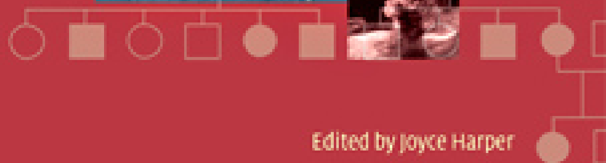
Second Edition

Preimplantation Genetic Diagnosis



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Edited by

Joyce C. Harper

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Preface

This book has been written by the leaders in the field of preimplantation genetic diagnosis (PGD) for everyone who has an interest in the field, including embryologists, reproductive specialists, cytogeneticists, molecular biolologists, obstetricians, gynecologists, genetic counselors, and nurses. Since the first PGD cases were performed in the late 1980s, PGD is now performed worldwide. This book brings together all the disciplines involved in PGD. The introduction summarizes all the disciplines and includes a history of PGD. The first section covers the background and includes chapters on in vitro fertilization (IVF), genetic disease, genetic counseling, prenatal diagnosis, preimplantation development, and preimplantation genetics. The second section covers the techniques used in PGD, including clinical practice, polar body biopsy, cleavage-stage biopsy, blastocyst biopsy, fluorescent *in situ* hybridization (FISH) for chromosome abnormalities, sexing and aneuploidy screening, the use of polymerase chain reaction (PCR) in PGD, and quality assurance and good practice. The last section covers ethical issues and future developments.

Since the book encompasses all aspects of PGD, readers from any background will be able to understand the entire field of PGD. Each chapter contains a list of key points summarizing the chapter. Readers can read the book from cover to cover or dip into the chapters that interest them. Since the field of PGD is at the cutting edge of IVF, molecular, and cytogenetic technology, it is continuously evolving and so this new edition has many updates to the first edition. This includes new chapters on polar body, cleavage-stage and blastocyst biopsy, PGD for sexing, chromosome abnormalities, and aneuploidy screening. This book is a must for anyone interested in PGD.

Section 1 Chapter 1

Background

Introduction to preimplantation genetic diagnosis

Joyce C. Harper

Key points

- Preimplantation genetic diagnosis (PGD) was first applied in 1988 using a polymerase chain reaction (PCR) protocol to amplify a sequence on the Y chromosome for embryo sexing for patients carrying X-linked disease.
- Patients have to go through in vitro fertilization (IVF) so that their embryos may be generated in vitro. Cells are removed from oocytes or embryos and used for the genetic diagnosis. Unaffected embryos are transferred to the patient.
- The most common biopsy procedure is cleavage-stage biopsy, but biopsy of polar bodies and trophectoderm cells is performed clinically.
- The indications for PGD are: monogenic disorders, chromosome abnormalities, sexing, or specific diagnosis of X-linked disease.
- PGD technology has been used to try and improve the pregnancy rate for infertile patients by screening for aneuploidies. Indications include advanced maternal age, repeated implantation failure, and repeated miscarriages (preimplantation genetic screening; PGS).
- Fluorescent *in situ* hybridization (FISH) is the technique used to analyze chromosomes in the biopsied cells, and is the method of choice for embryo sexing. It is also used for chromosome abnormalities and aneuploidy screening.
- PCR is the technique used to detect monogenic disorders but it has been hampered by problems with contamination and allele dropout.
- PGD has stimulated much ethical debate. Many countries have legislation controlling PGD and in some countries cleavage-stage and blastocyst biopsy are illegal. Social sexing is illegal in Europe and other countries.
- The first 20 years has shown major advances in the field of PGD. The next 20 years may include the use of arrays for examining all the chromosomes, multiple genes and gene expression. PGD may be used for all IVF patients to select the genetically "best" embryo.
- The European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium has collected nine years of data on PGD and PGS. Five working groups have been set up to look at PGS, accreditation, the database, guidelines, and misdiagnosis. Additionally a pediatric follow-up and external quality assessment for FISH and PCR have been developed.

Introduction

Preimplantation genetic diagnosis (PGD) was developed out of a need to provide an alternative to prenatal diagnosis for couples at risk of transmitting a genetic disease to their children. The options for such couples are: to remain childless; not to undergo genetic testing (reproductive roulette); or to go through prenatal diagnosis, PGD, gamete donation, or adoption. These are all difficult reproductive options. The majority of couples will opt for prenatal diagnosis by chorionic villus sampling (CVS) or amniocentesis (see Chapter 5). The procedures themselves take a few minutes, and for recessive disorders the couple have only a 25 percent chance of an affected pregnancy; with a dominant disorder this rises to 50 percent. But if the pregnancy is affected the couple have to decide if they wish to continue or consider termination. Neither is an easy option. Another advantage of prenatal diagnosis is

Table 1.1 The three methods of embryo biopsy used in preimplantation genetic diagnosis (PGD)

	Day performed	Types of cells removed	Indications	Zona drilling	Cell removal	Limitations
Polar body	First PB day 0 Second PB day 1 Or simultaneously on day 1	First and second polar bodies	PGS Monogenics carried by mother	Laser Mechanical Beveled pipette	Aspiration	Only maternal chromosomes/ genes
Cleavage-stage	Day 3	Blastomeres	PGS Monogenics Sexing Chromosome abnormalities	Laser Mechanical Acid Tyrodes	Aspiration Displacement	Postzygotic mosaicism
Blastocyst	Day 5	Trophectoderm	PGS Monogenics Sexing Chromosome abnormalities	Laser Mechanical Acid Tyrodes	Herniation	Postzygotic mosaicism Some embryos will arrest prior to biopsy Short time for diagnosis

Table 1.2 Methods used for preimiplantation genetic diagnosis (PGD)

	Indications	Cell preparation	Protocol	Limitations
FISH	Sexing Chromosome abnormalities PGS	Spreading cells using methanol:acetic acid or Tween HCl	Fix Denature Hybridization Wash off unbound probe Visualize	Cumulus contamination Mosaicism Overlapping signals Failure of probes to bind
PCR	Sexing Monogenic disorders	Tubing cells into lysis buffer	Lyse cell Cycles of denaturing, annealing, elongation, Detect products	Cumulus contamination Sperm contamination (use ICSI) Other contamination Amplification failure Allele dropout
Metaphase CGH	Sexing Chromosome abnormalities PGS	Tubing cells into lysis buffer	Lyse cell, whole genome amplification Co-hybridization with control sample on to metaphase spread Analysis of each chromosome using CGH software	Contamination Mosaicism Procedure takes several days and so currently embryos are frozen Requires many skills, PCR, and cytogenetics

FISH, fluorescent in situ hybridization; PGS, preimplantation genetic selection; PCR, polymerase chain reaction; ICSI, intracytoplasmic sperm injection; CGH, comparative genomic hybridization.

that in most countries this will be paid for by the health service. If the couple decide to go through PGD they have to undergo IVF procedures to produce embryos in vitro even though they are often fertile. IVF is a highly invasive procedure with a relatively low chance of success, and adding on PGD does not improve the chances of delivering a baby. Cells need to be removed from the embryo to allow single-cell genetic testing. These may be removed from the oocyte/zygote (first and second polar body biopsy), blastomeres may

be taken from cleavage-stage embryos, or trophectoderm cells taken from blastocysts (Table 1.1) The polymerase chain reaction (PCR) is used for the diagnosis of monogenic disorders, and fluorescent *in situ* hybridization (FISH) is used for chromosome analysis (Table 1.2).

PGD is a complicated procedure. As well as involving the IVF team, it requires a diagnostic team who are experts in single-cell diagnosis. Besides the technical difficulties, internationally PGD is a controversial procedure as there are ethical and moral concerns about genetic testing of the early embryo.

In this book every aspect involved in PGD is considered, from IVF, prenatal diagnosis, and genetic counseling to quality assurance and ethical considerations. This chapter offers the reader a history of PGD, an outline of each chapter, and a report on the ESHRE PGD Consortium.

History of PGD

Animal studies and preclinical work

The first biopsies on embryos were performed by removing one cell from two-cell embryos by Seidel (1952) and Tarkowski and Wróblewska (1967), working on rabbits and mice, respectively. The first PGD was performed by Gardner and Edwards (1968), who biopsied a small portion of the trophectoderm from rabbit blastocysts, sexed the embryos by identifying sex chromatin (which identifies females), and replaced them into recipient females. For a rabbit blastocyst to implant it needs to be expanded with an intact zona and so Richard Gardner made a very neat slit in the zona, sucked out a small amount of trophectoderm, pinched it off, and hoped that the remaining trophectoderm would block the hole in the zona. The offspring were found to be of the predicted sex (Edwards & Gardner, 1967; Gardner & Edwards, 1968). This technique was later tried on human blastocysts without success (Steptoe *et al*., 1971).

In 1985, at a Ciba Foundation meeting in London, scientists were discussing the possibility of diagnosing genetic disease in a human preimplantation embryo. It was generally agreed that there were no single-cell diagnostic techniques available, and that the biopsied cell(s) would have to be cultured to obtain sufficient cells for the diagnosis. The revolutionary PCR procedure had just been developed (Saiki *et al*., 1985) but it was not envisaged that PCR could work on a single cell.

Subsequently, the idea of performing PGD was reviewed by a number of people. Penketh and McLaren (1987) wrote a review on "Prospects for prenatal diagnosis during preimplantation human development" and Edwards and Hollands (1988) wrote a review on "New advances in human embryology; implications for preimplantation diagnosis of genetic disease" (Edwards & Hollands, 1988). Edwards and Hollands (1988) suggested that sexing sperm would be easier than sexing embryos but they said the advantage of typing embryos would be that the cells would be "fully representative of the embryonic genome." These authors suggested that noninvasive techniques in which the medium was examined would be the simplest; either secretion or uptake of substances from the culture medium would be possible. It is only now that noninvasive methods seem a possibility (Seli *et al*., 2007; Vergouw *et al*., 2008). Edwards and Holland (1988) suggested that if invasive methods were used they would involve dissolving the zona, disaggregating the embryo, separating the cells and culturing them for diagnosis, and putting the embryo back in an artificial zona for transfer. They further suggested performing this technique on two-cell embryos.

Several different approaches to embryo biopsy were being investigated in the late 1980s. In Australia, Leeanda Wilton was developing methods of removing cells from mouse embryos (Wilton & Trounson, 1986; Kola & Wilton, 1991) (Figure 1.1); André Van Steirteghem was exploring removing one cell from two-cell embryos (Nijs & Van Steirteghem, 1987); and Marilyn Monk and Alan Handyside were investigating taking one or two cells from an eight-cell embryo for diagnosis of hypoxanthine phosphoribosyl-transferase (HPRT) deficiency (Monk *et al*., 1987). Audrey Muggleton-Harris and Marilyn Monk demonstrated that PGD in a mouse model for Lesch–Nyhan disease could also be done by biopsy and analysis of a few trophectoderm cells extruded through the zona pellucida, a technique perfected by Audrey Muggleton-Harris in David Whittingham's unit (Monk *et al*., 1988). Trophectoderm biopsy was also tested by Dokras *et al*. (1990) and Summers *et al*. (1988). Another approach to obtaining blastocysts was to perform uterine lavage where embryos would be flushed on day five of development (Buster *et al*., 1985). The diagnosis and transfer of blastocysts would avoid the low implantation rate of in vitro fertilized cleavage-stage embryos, which was only 15 percent at that time. Bruno Brambati suggested that uterine lavage would be an efficient, practical, and

Figure 1.1 Leeanda Wilton doing embryo biopsy in Melbourne, Australia in 1986.

safe method to obtain blastocysts for PGD (Brambati & Tului, 1990). However, the problem with using lavage was that it would be impossible to be sure that all of the blastocysts had been flushed, allowing the possibility that undiagnosed embryos could implant. Whatever method was used, it was predicted that the biopsy technique would almost certainly affect implantation (Edwards & Hollands, 1988).

The challenge of the introduction of molecular biology for PGD was the move from working with millions of cells to the very few cells of the embryo. Edwards and Hollands (1988) suggested that the most reliable method for single-cell diagnosis would be "to use DNA probes for identifying the genotype of the human embryo" and they predicted that high levels of chromosome abnormalities (Plachot *et al.*, 1987) would "lead to complications in the interpretation of some diagnostic tests." Monk, working in Anne McLaren's MRC Mammalian Development Unit at University College London in the 1970s, had already developed an array of single-cell-sensitive molecular procedures for the study of gene expression and its regulation in early mouse development, most notably for the study of X chromosome inactivation in female embryonic development. In the late 1980s Alan Handyside collaborated with Marilyn Monk to carry out mouse embryo biopsies of single blastomeres, single-cell diagnosis, and embryo transfers to show that Monk's single-cell molecular diagnoses were correct. Handyside had been working on mouse embryo biopsies at Cambridge University and joined Robert Winston at the Hammersmith Hospital. The first single-cell diagnoses were performed on embryos from the first genetically engineered mouse carrying a defect in the HPRT gene, the mouse model for Lesch–Nyhan syndrome in the human. The mouse was created by mutation of the HPRT gene in embryonic stem cells in culture, transferring some of these mutated cells to a host blastocyst, and returning that blastocyst to the uterus of a foster mother to produce a chimeric male offspring carrying the mutated gene in his sperm (Hooper *et al.*, 1987). Thus, some of his daughters were heterozygous for the HPRT mutation. Monk was able to use biopsied cells from embryos from this heterozygous female mouse to diagnose the mutant embryos (half the males) carrying the mutation on their single X chromosome. This was the first demonstration that preimplantation diagnosis by biopsy and analysis of a single blastomere for a single gene defect was a feasible proposition (Monk *et al.*, 1987) and many key early papers followed (Monk *et al.*, 1988; Benson & Monk, 1988; Monk, 1988; Monk & Handyside, 1988; Holding & Monk, 1989; Monk & Holding, 1990; Monk, 1990a, 1990b, 1990c; Monk, 1991a, 1991b, 1991c). Work with human embryos also began at this time; in collaboration with Braude and Johnson at Cambridge

Figure 1.2 Marilyn Monk and Cathy Holding at Anne McLaren's MRC Mammalian Development Unit in the Galton Laboratory, University College London, 1998/1999.

University, Monk assayed HPRT gene activity in single blastomeres biopsied from human preimplantation embryos (Braude *et al*., 1989), although, in the human, the maternally inherited enzyme at the eight-cell stage would obscure the diagnosis of Lesch–Nyhan syndrome by this method.

In many of the first papers the procedure was called "preimplantation diagnosis" (PID), as an extension of prenatal diagnosis (PND). However, the name was changed to "preimplantation genetic diagnosis" (PGD) by people entering the field later on to avoid confusing the acronym PID with that for pelvic inflammatory disease.

Marilyn Monk and Cathy Holding set out to create further single-cell enzyme assays for common inherited genetic diseases as well as maintaining their interest in single-cell assays for X-linked genes to further their studies on the regulation of X chromosome inactivation in development (Figure 1.2). One of these was adenosine deaminase (a deficiency in this enzyme is the basis of severe combined immunodeficiency disease (SCID)) (Benson & Monk, 1988). In Brussels, too, that same line of research led Karen Sermon, in André Van Steirteghem's team, to evaluate the possibility of diagnosing Tay–Sachs disease through measuring the enzyme beta-N-acetylhexosaminidase activities in single blastomeres (Sermon *et al*., 1991). They could show that it would work in the mouse, but, unfortunately, not in the human. Later, the same group (Van Blerk *et al*., 1991) showed the same for β-glucuronidase, the lysosomal enzyme deficient in mucopolysaccharidosis type VII.

Holding and Monk, in collaboration with Cathy Abbott, were moving tubes from water bath to water bath to try to develop the procedures of PCR and testing out the new PCR machine that was being developed by Martin Evans and BioCam in Cambridge. They wanted to look directly at the actual mutation in the DNA of a specific gene in a single cell. It was an immense struggle to find the way to make PCR work at the single-cell level but their hard work and perseverance led to eventual success using a mouse model for β thalassemia (Holding & Monk, 1989). They used nested primers, first amplifying the larger sequence and then, in a new reaction, amplifying an inner sequence with the inner primers. This vastly increased the specificity and sensitivity of the reaction, and they were able to analyze single cells and publish the first nested PCR on a single cell detected by a simple agarose gel assay (Holding & Monk, 1989) as well as establishing PGD for β thalassemia in a mouse model system.

In 1990, Holding and Monk extended their single-cell PCR analyses to the human to develop single-cell detection of the sickle cell mutation in the betaglobin gene in human oocytes. In collaboration with Peter Braude, then at the Rosie Maternity Unit, Addenbrookes Hospital, in Cambridge, they were the first to show that it was possible to diagnose genetic disease by analysis of the polar body of a human unfertilized egg, thus avoiding working on the human embryos themselves (Monk & Holding, 1990).

Development of human embryo biopsy

In the late 1980s many teams worldwide were attempting clinical PGD, including the Hammersmith team in London, Jacques Cohen's team in New York, and Yury Verlinksy's team in Chicago. The first two groups were attempting cleavage-stage biopsy and the Verlinsky team was working on polar body biopsy.

The Hammersmith Hospital team, led by Handyside and Winston, tried day two and day three human embryo biopsy. Alan Handyside, with the help of Kate Hardy, applied his mouse cleavage-stage biopsy techniques to day three human embryos using acid Tyrodes to drill a hole in the zona and aspirating one or two cells from eight-cell embryos, and allowed the embryos to grow on to day five of development. Hardy used differential staining to count the number of trophectoderm and inner-cell mass cells of the control (32 embryos) and biopsied (45 embryos) to determine if the biopsy technique affected blastocyst development and measured the uptake of pyruvate and glucose (Hardy *et al*., 1990). Since this study showed little effect on the ratio of the inner-cell mass and trophectoderm

Figure 1.3 Elena Kontogianni, PhD viva. From left to right: Murdo Elder, Charles Rodeck, John West, Elena Kontogianni, Alan Handyside, and Robert Winston, Department of Obstetrics and Gynaecology, Hammersmith Hospital, 1993.

cells, or on metabolism, it gave the green light to human cleavage-stage biopsy. Today the same basic biopsy technique (of day three cleavage-stage biopsy) is used (Harper *et al.*, 2008a). The zona is breached and single blastomeres are aspirated. Studies on day two biopsy did not show such favorable results as day three biopsy (Tarin *et al.*, 1992).

The first clinical cases

Elena Kontogianni was studying for her PhD at the Hammersmith Hospital, on single-cell PCR for sexing, which she did by amplifying a repeated region of the Y chromosome (Figure 1.3). It was this approach that was used for the world's first PGD cases (Handyside *et al.*, 1990). Female embryos were selectively transferred in five couples at risk of X-linked disease, resulting in two twins and one singleton pregnancy. Because the Y chromosome region Kontogianni was amplifying contained many repeats, it gave fewer problems than trying to amplify a unique region. A band on the PCR gel indicated that the embryo was male and the absence of a band indicated that the embryo was female. However, failure to tube the cell, an anucleate blastomere, or failure of the PCR also resulted in absence of a band on the PCR gel. A total of 21 cycles were performed in two series and one misdiagnosis occurred. To reduce the risk of misdiagnosis, Kontogianni went on to co-amplify sequences on the X and Y (Kontogianni *et al.*, 1991). At that time nothing was known about allele dropout, cumulus cell contamination, or amplification failure from single cells.

During the 1980s, human IVF embryos were exclusively transferred on day two of development as the culture medium used was incapable of reliably growing embryos past this stage. Since the biopsy was to be performed on day three, the first diagnoses were all performed in one day, with transfer of the embryos late on day three. A comparison of day two and day three transfers indicated that this would not adversely affect pregnancy rates (Dawson *et al.*, 1995). The worry of embryos arresting was so high that some transfers took place in the early hours of day four so that the embryos were removed from culture as soon as possible. There were many evenings at the Hammersmith when a transfer was performed at 1 a.m. on day four and researchers returned to the laboratory at 7 a.m. to start the next case. Winston helped deliver most of the first PGD babies.

Development of FISH

During the same period that single-cell enzyme activity and gene mutation detection were being developed in the UK, others were analyzing whole chromosomes. Kola and Wilton (1991) biopsied single cells from embryos from mice that were carrying a Robertsonian translocation. These single cells were karyotyped and normal embryos transferred. Analysis of the fetuses showed that the PGD was 100 percent accurate. This was the first PGD of aneuploidy. In the late 1980s Wilton moved to London to work at the Institute of Zoology, and began to collaborate with Handyside at the Hammersmith Hospital.

Jones *et al.* (1987) were the first to report the use of highly specific DNA probes to detect the human Y chromosome which could successfully be used on chromosomes from single cells of human embryos.

Figure 1.4 Members of the Galton Laboratory, University College London, in 1990. From left to right: Sioban SenGupta, Rajai Al Jehani, Joy Delhanty, Darren Griffin, Kiran Gulati, and Sarah Leigh.

Joy Delhanty was working with Richard Penketh at the Galton Laboratory, University College London and they thought about sexing embryos using radiolabeled probes, but detection of hybridization by autoradiography took several days and was not reliable enough at the single-cell level. They reported on the rapid sexing of human embryos by use of biotinylated probes in 1989 (Penketh *et al*., 1989).

The Hammersmith team was aware that its PCR sexing protocol was flawed, so Delhanty contacted them to say that she had taken on a PhD student (Darren Griffin) to set up the new fluorescence *in situ* hybridization (FISH) technology that she thought would be ideal for PGD (Figures 1.4, 1.5, and 1.6). Griffin started his PhD with Delhanty in 1988 and his first job was to get FISH working, which involved learning the radioactive and enzymatic *in situ* hybridization (ISH) approaches then adapting them to a fluorescent approach (i.e. FISH). In those days there were no commercial FISH probes and everything had to be prepared in-house; this led to some stressful times when things stopped working. The first set of experiments using single-color FISH with a Y probe were relatively successful; about 50 percent of the cells had a single signal as expected. Blastomeres for research were hard to come by and these single cells were initially spread by Penketh (Griffin *et al*., 1991). But, for PGD, both X and Y probes were required. Delhanty and Griffin thought their salvation would come with the newly available Oncor X probe. Handyside spread the cells this time and some were from whole embryos as well as single cells. It was here they got the first inkling of how

Figure 1.5 Robert Winston and Darren Griffin in Prague, 1990.

chromosomally abnormal human embryos were going to be, with some cells having two, three, four, five, or more X chromosomes (Griffin *et al*., 1991). Two things happened to make dual FISH work in human embryos. After a trip to Leiden Griffin learned the dual FISH technique and Leeanda Wilton joined the team, being very productive in spreading embryos. Wilton was working with the Hammersmith team trying to karyotype human blastomeres but was struggling to obtain reliably spread chromosomes. Handyside suggested that Wilton retrieve the fixed nuclei from the bin and allow Griffin and Delhanty to have a go at “FISHing” them, and to everyone’s amazement the FISH worked first time. The team was still aware of a tiny flaw in the plan as, at that time, FISH took 24 hours to complete. On February 11, 1991 (his 24th birthday) Griffin finally cracked the means by which FISH could be done

Figure 1.6 Darren Griffin in the Galton Laboratory, University College London, 1993.

in seven hours. Things then moved very quickly, with Wilton now spreading the cells, and the following week they were doing a case (Delhanty *et al.*, 1993; Griffin *et al.*, 1993; Griffin *et al.*, 1994). The problematic PCR sexing protocol was abandoned in favor of the FISH technique, which could clearly identify a male embryo, a female embryo, and an embryo with a single X chromosome but no Y (Turner syndrome). Many people were involved in these early cases: Handyside doing the biopsy; Wilton spreading the cells; and Griffin and Delhanty the FISH. These were the world's first PGD cases using FISH (Griffin *et al.*, 1993; Griffin *et al.*, 1994).

IVF was not quite as organized as it is today. In one of the first PGD cycles using FISH, the patient forgot to attend for her egg collection, which went ahead 12 hours later. Winston famously took 10 of his staff skiing every year (Figure 1.7(a) and (b)), and the skiing party was due to leave the day after the case. This meant an evening biopsy, spreading just before midnight, and FISH through the night. At 7 a.m. Griffin faxed the results off to the Hammersmith Hospital, picked up his skis, and got on a plane to Switzerland with the rest of the team.

The first clinical cases of PGD coincided, perhaps not accidentally, with the years of debate leading up to the passage of the Human Fertilisation and Embryology Bill through the UK Parliament in 1990. The hard work by Winston, Monk, Handyside, Wilton, and Delhanty was a positive influence on the Bill. Anne McLaren played a key role in public debate and media coverage, as well as liaising with politicians during the debate in

(a)

(b)

Figure 1.7 Robert Winston's skiing trip, Murren, Switzerland: (a) 1993, John Mansfield, Robert Winston, Pierre Ray, Joyce Harper, Vivienne Hall, Fiona Robinson, Kate Hardy, Debbie Taylor, Ben Winston, and Joe Conaghan; (b) 1994, Asangla Ao, Joyce Harper, Kate Hardy, Antony Lighton, Thanos Paraschos, Pierre Ray, Debbie Taylor, and Joe Conaghan.

(a)

(b)

Figure 1.8 (a) Meeting of the International Working Group at European Society for Human Reproduction and Embryology (ESHRE), Thessaloniki, Greece, 1993, including: Alan Handyside, Marilyn Monk, Leeanda Wilton, Elena Kontogianni, Yury Verlinsky, Michelle Plachot, Audrey Muggleton-Harris, Sandra Carson, Anver Kuliev, Paul De Sutter, Carles Gimenez, Nikica Zaninovic, Charles Strom, Peter Braude, Joe Leigh Simpson, Edith Coonen, Inge Liaebers, Math Pieters and others; (b) The second international symposium on "Preimplantation Genetics" held in Chicago, 1997. From left to right: Santiago Munné, Debbie Taylor, Dagan Wells, Stuart Lavery, Paul Kendrick, Patrizia Ciotti, Joyce Harper, Andre Duyker, Mason Wilton (baby), Leeanda Wilton, Pierre Ray, and Pia Cau.

Parliament. The passage of the Bill through Parliament, which was to permit embryo research under license in the UK, was greatly influenced by this early pioneering work demonstrating the clinical relevance of embryo research for PGD, which featured at this time as a significant medical breakthrough.

In the USA

Several groups were also developing PGD in the USA. Yury Verlinsky took into account the ethical concerns associated with the biopsy of cleavage-stage embryos and, with the help of Jacques Cohen, who taught him mechanical polar body biopsy, applied PGD to polar bodies, and called the procedure "preconception diagnosis" as originally only the first polar body was used. Verlinsky worked on his first cases in 1988/1989 and sent a paper to *Nature*, which was rejected but was accepted by *Human Reproduction* (Verlinsky *et al.*, 1990). He used the first polar body to detect a maternally transmitted alpha 1 antitrypsin deletion in one patient. Eight eggs were collected, seven polar bodies were aspirated, six embryos fertilized, and PCR was successful in five cases. Two embryos were transferred but the patient did not get pregnant. In the same year the Verlinksy team reported on preconception diagnosis for cystic fibrosis (Strom *et al.*, 1990). In 1990 Verlinsky set up the First International Symposium on Preimplantation Genetics in Chicago, and at this meeting held the first meeting of the International Working Group on Preimplantation Genetics. The aims of the working group were to collect and distribute information on the progress of centers involved in PGD, and to coordinate their activities, organize annual working group meetings, ensure coordination with other relevant research, and organize conferences on PGD. The international working group met during various congresses in Washington (1991), Thessaloniki, Greece (1993) (Figure 1.8(a)), New York (1994), Hamburg (1995), Rio de Janero (1996), Chicago (1997), Los Angeles (1998), Sydney (1999), and Bologna (2000) (Verlinsky *et al.*, 1994a). Verlinsky organized several symposia on preimplantation genetics. The second was held in Chicago in 1997 (Figure 1.8(b)).

In 1988/1989 Jamie Grifo was doing a fellowship in reproductive endocrinology with Alan Decherney at Yale, and he was interested in trying to set up PGD. He sent one of his medical students to Atlanta to work with Henry Malter to develop embryo biopsy. They returned to Yale, where they taught Grifo the procedure of partial zona dissection on four- to eight-cell mouse embryos using calcium and magnesium-free media, and they applied FISH to the biopsied cells with probes for chromosomes X and Y (Grifo *et al.*, 1990). In this paper they also performed FISH on human blastomeres. In 1990, while still at Yale, Grifo and his technician, Ysui Tang, were working on FISH for sexing mouse and human embryos and sperm, and they continued this work at Cornell (Grifo *et al.*, 1992a). Cohen and colleagues, first at Reproductive Biology Associates (RBA) and later at Cornell, had developed and improved many

Figure 1.9 Cohen and Munnés team, 1994 including Jacque Cohen, Mina Alicani, Santiago Munné, and others.

micromanipulation techniques and Grifo joined the Cornell team (Cohen, Malter, Talanski, Rosenwaks, and Berkley) (Figure 1.9). The Cornell team performed its first PGD cases by sexing single-cell blastomeres using co-amplification of DNA on the X and Y chromosome (Grifo *et al*., 1992b). Santiago Munné had studied male infertility and cytogenetics of mouse embryos with Anna Estop and Josep Egozcue, a pioneer in the study of cytogenetics of gametes and embryos. He joined the Cornell team in 1991, bringing fixation skills with him, and developed the FISH technique. In 1992, in collaboration with Ulli Weier, he was the first to apply FISH with directly labeled probes (Munné *et al*., 1993a).

Development of PCR for monogenic disorders

Several groups were now working on using PCR for the detection of specific gene mutations for PGD (Li *et al*., 1988; Holding & Monk, 1989; Monk & Holding, 1990; Bradbury *et al*., 1990; Coutelle *et al*., 1989; Gomez *et al*., 1990; Navidi & Arnheim, 1991; Sermon *et al*., 1991; Sermon *et al*., 1992). Mark Hughes came to the Hammersmith from the USA to develop single-cell PCR for cystic fibrosis (Figure 10(a), (b), and (c)). Along with Pierre Ray, who was studying for his PhD, Hughes developed nested PCR to amplify the ΔF508 region followed by heteroduplex formation for rapid detection of the deletion (Lesko *et al*., 1991; Handyside *et al*., 1992; Liu *et al*., 1992). It is amazing that the cystic fibrosis gene was only described in 1989 (Riordan *et al*., 1989), and by 1992 the first diagnosis of cystic fibrosis in a single cell was possible. The first report was on just three couples, all carrying the ΔF508 mutation, of which one woman became pregnant (Handyside *et al*., 1992). At the same time, the Brussels team developed its own protocol for cystic fibrosis (Liu *et al*., 1992; Liu *et al*., 1993) and later was the first team to perform PGD for Duchenne muscular dystrophy (Liu *et al*., 1995).

At the Genetics & IVF Institute (GIVF) in Virginia, USA, Gary Harton was in the process of developing PGD in 1992, and he performed the Institute's first clinical case in 1993 (Levinson *et al*., 1992). Work focused on monogenic disease diagnosis, including tests for cystic fibrosis, Huntington's disease (non-disclosing), Fragile X, and the first birth of an unaffected child following PGD for spinal muscular atrophy (SMA) (Fallon *et al*., 1999), as well as the first clinical PGD test for an autosomal dominant disease, Marfan syndrome (Harton *et al*., 1996). GIVF also pioneered the separation of X and Y sperm using MicroSort® (Levinson *et al*., 1992; Schulman & Karabinus, 2005).

Marilyn Monk's team developed mouse PGD for Lesch–Nyhan syndrome, SCID, thalassemia, and sickle cell disease, X-linked Duchenne muscular dystrophy, Fragile X, myotonic dystrophy, and Kennedy disease (Daniels *et al*., 1995; Monk *et al*., 1995). Monk published the first quality control experiments to verify sensitivity, efficiency, and accuracy to lay down the standards for this sensitive work and to convince the field that single-cell PCR was indeed possible (Monk *et al*., 1993). Monk's group was already aware of the problem of carryover contamination (millions of copies of product were being produced). Cathy Holding separated the sites of loading samples into the PCR tubes (which were carried out in the Galton Laboratory car garage) and the PCR procedure in the laboratory. Later, Monk and colleagues began developing single-cell technology for the triplet repeat diseases – Fragile X and myotonic dystrophy (Daniels *et al*., 1995; Daniels *et al*., 1996) and imprinted genes (Daniels *et al*., 1995; Daniels *et al*., 1996; Daniels *et al*., 1997; Huntriss *et al*., 1998; Salpekar *et al*., 2001). Monk's team also developed a method they called "cell recycling," in which a single cell could be analyzed by PCR for a specific gene defect (Duchenne muscular dystrophy) as well as the same single cells being studied for sex by *in situ* hybridization (Thornhill *et al*., 1994; Thornhill & Monk, 1996) (Figure 1.11).

The Cornell group published one of the first papers on whole-genome amplification using primer extension preamplification (PEP) (Xu *et al*., 1993). The group developed three PEP protocols on single blastomeres from arrested embryos. Three aliquots of each PEP product were used as templates for exon 10 of the cystic fibrosis gene, or the human X chromosome.

(a)

(b)

(c)

Figure 1.10 IVF meeting, Israel 1994: (a) From left to right: Alan Handyside, Joyce Harper, Robert Winston, and Mark Hughes; (b) Mark Hughes, Alan Handyside, Joyce Harper, and Elena Kongtogianni; (c) Robert Winston and Alan Handyside.

Figure 1.11 Dinner at the European Society for Human Reproduction and Embryology (ESHRE), Bologna, 2000. From left to right: Alpesh Doshi, Giles Palmer, Pierre Ray, Joyce Harper, Alan Thornhill, Marilyn Monk, and Joep Geraedts.

In 1992 Dagan Wells started his PhD in Joy Delhanty's laboratory, working on cancer genetics, and joined Darren Griffin, who was doing the clinical PGD FISH cases (Figure 1.12(a) and (b)). Wells then moved on to PGD for monogenic disorders and, along with Asangla Ao, who had graduated with her PhD at Monk's laboratory, and had been working at the Hammersmith Hospital with myself, Delhanty, Handyside, Kontogianni, and Ray, did the first PGD case for a cancer predisposition syndrome, the APC gene, in 1996. This case was notable for two other reasons: it represented the first use of whole-genome amplification in conjunction with PGD; and it combined direct mutation detection and analysis of informative linked markers, a key strategy for increasing the accuracy of PGD for dominant disorders (Ao *et al*., 1998).

(a)

(b)

Figure 1.12 (a) Dagan Wells and Darren Griffin's publicity shot, Galton Laboratory, University College London, 1992; (b) Dagan Wells and Joyce Harper in the Galton Laboratory, University College London, 1996.

ESHRE campus workshop on PGD, 1993

After working as a clinical embryologist since 1987, I joined the Hammersmith team in 1992 and was thrown into the deep end, assisting in biopsies done by Selmo Geber, a clinician from Brazil, during my first week (Figure 1.13). I spent some time trying and failing to perfect freezing of biopsied embryos. In 1993 Winston, Handyside, Griffin, and myself organized a European Society for Human Reproduction and Embryology (ESHRE) campus workshop on "Preimplantion Genetic Diagnosis of Inherited Disease" at the Hammersmith Hospital. We typed out the abstracts from the faxes we received. Invited speakers included Audrey Muggleton-Harris, talking about blastocyst biopsy, Joy Delhanty, talking on FISH, Mark Hughes, talking on DNA amplification, and Eugene Pergament, talking on preimplantation genetics. I spoke on our new two-hour FISH (Harper *et al*., 1994) and other speakers included Anna Veiga, Ian Findlay, Jiaen Lui, and Sue Pickering. We went on a Thames boat cruise and were very worried that if the boat sank PGD would stop as all the key people were on board. Since that time I have organized annual PGD workshops, initially at the Hammersmith Hospital and later at University College London (Figure 1.14).

New method of spreading blastomeres and embryos

In 1993 University College London was contacted by Edith Coonen and Joep Geraedts from the Netherlands, who had developed a novel method of spreading blastomeres from mouse embryos (Coonen *et al*., 1994). Leeanda Wilton had been using traditional methanol: acetic acid for spreading but owing to the high amount of cytoplasm present in human blastomeres, it was often difficult to control the spreading procedure. With Tween/HCl the cell membrane lysed and the nucleus could be clearly observed during the whole spreading procedure. The cytoplasm could be easily cleaned away from the nucleus, giving a very high FISH efficiency. Since the reagents are nontoxic the cells may be spread in the IVF laboratory. Edith Coonen and I applied this to human embryos with great success (Harper *et al*., 1994) also significantly reducing the time of the FISH procedure by using probes that were directly labeled with the fluorochromes (Harper *et al*., 1994). Another advantage of using Tween/HCl is that, for the first time, whole embryos could be spread and "FISHed." Previously, since methanol: acetic acid could not be used to spread

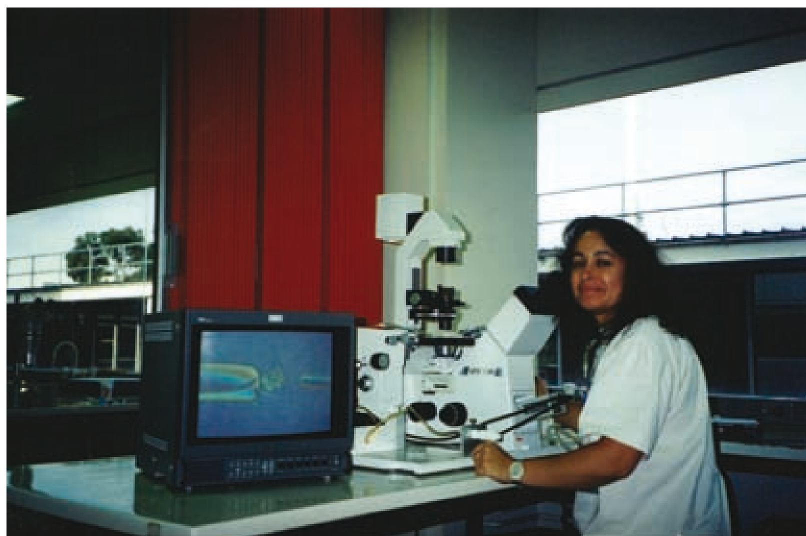

Figure 1.13 Joyce Harper demonstrating embryo biopsy during a micromanipulation workshop organized by Leeanda Wilton in Melbourne, Australia, 1993.

Figure 1.14 First hands-on preimplantation genetic diagnosis (PGD) workshop at the Hammersmith Hosptial, 1996: Alan Handyside, Asangla Ao, Pierre Ray, Debbie Taylor, Joyce Harper, Theo Atkoulis, Marianne Bergere, Dorthe Cruger, Marianne Schwartz, Francine Lossos, Chaque Khatchadourian, Clare Conn, Jane Meintjes, Elisabeth Larsen, Geraldine Viot Szoboszlai, Lianna Schulman, Dagan Wells, Patrizia Cotti, Usanee Jetsawangsri, Sirpa Makinen, Annette Bonhoff, Mark Lelorc'h, Erik Iwarsson, Lars Ahrlund Richter, Stephane Viville, Ralf Bohm, and Jean Cozzi.

whole embryos, the diagnosis results had to be confirmed on single cells, which was very labor intensive. It was while spreading whole embryos that I realized how essential it was to remove all the cumulus cells from the embryo as, when spread, the cumulus nuclei are indistinguishable from embryonic nuclei (we had found eight- cell embryos giving 20 or more nuclei and male embryos with numerous female cells, all caused by cumulus contamination). We then realized that it was vital that all cumulus was removed prior to the biopsy procedure to avoid maternal contamination for both FISH and PCR diagnosis, something that we had not been doing previously (Wilton *et al.*, 2008).

Mosaicism

The early cases of embryo-sexing using FISH quickly demonstrated the potential of the technique in not only providing information on copy number of chromosomes but also the detection of aneuploidy, polyploidy, and mosaicism (Delhanty *et al.*, 1993). When I started doing FISH I was surprised at the frequent highly abnormal nuclei I was finding. I knew the karyotyping

studies (Angell, 1989; Plachot *et al*., 1989; Zenzes & Casper, 1992; Zenzes *et al*., 1992) had indicated that high levels of chromosome abnormalities were present in human embryos, but I found many embryos where all the nuclei had differing chromosome abnormalities. I had wondered if I was doing the FISH incorrectly, but Joy Delhanty and I decided that this was a true phenomenon, and since I was reading the Chaos theory book, we decided to define these embryos as "chaotic" (Harper *et al*., 1995; Harper & Delhanty, 1996) (Figure 1.15). After publication of these findings I discussed my results with Michelle Plachot and Maria Zenzes, and both said that they had seen such nuclei when they were performing karyotypes but they had not reported them. We later showed that the frequent occurrence of chaotic embryos was a patient-related phenomenon (Delhanty *et al*., 1997). At Cornell, and later at Saint Barnabas, the Munné–Cohen team did extensive studies on chromosome abnormalities at the embryo level, and together with the data coming from our group, revealed a high rate of mosaicism at cleavage stages (Munné *et al*., 1994; Harper *et al*., 1994; Harper et *al*., 1995). The work of the Munné team obtained them the first-prize paper of the American Fertility Society in 1994. Santiago Munné and coworkers have spent considerable effort in differentiating mosaicism and error rate, developing scoring criteria, and searching for ways to reduce that error rate (i.e. Munné *et al*., 1997; Magli *et al*., 2001; Colls *et al*., 2007).

Paternal contamination

Sperm become embedded in the zona during normal IVF and can become dislodged during zona drilling. It is possible that these stray sperm may contaminate the diagnosis. This has been less of a problem for FISH, as to FISH sperm, steps need to be taken to decondense the sperm nucleus to make it accessible to the FISH probes. But for PCR paternal contamination is a problem. In the early 1990s the Brussels group was performing subzonal insemination (SUZI) for male infertility. During one case the researchers accidentally pierced the oolema and the sperm was deposited in the ooplasm. Since this was the only embryo that developed for this patient it was transferred and resulted in a live birth. This was the start of intracytoplasmic sperm injection (ICSI), which is now used throughout the world for the treatment of male infertility (Palermo *et al*., 1992; Liebaers *et al*., 1992). The use of ICSI increased at an incredible rate and soon we realized that this should be used for PGD cases where PCR is used to prevent paternal contamination.

Allele dropout

Pierre Ray and Alan Handyside noticed another problem with PCR, apart from contamination. When Ray was testing some heterozygous cystic fibrosis single lymphocytes while doing a PGD workup, he occasionally found that one of the alleles did not amplify (allele dropout, ADO). He analyzed single blastomeres from

Figure 1.15 Joy Delhanty and Joyce Harper in a hotel room in New York at the Serono Symposium on "The Genetics of Gametes and Embryos," June 1994.

untransferred embryos after PGD and detected an ADO rate of 18 percent (Ray *et al.*, 1996). From further analysis of single lymphocytes Ray reported that increasing the denaturation temperature reduced the rate of ADO without affecting amplification efficiency, and different lysis protocols also affected the ADO rate (Ray *et al.*, 1996; Ray & Handyside, 1996). As the PGD community at this time was small, discussions at various meetings led to the realization that others had also experienced this phenomenon.

Preimplantation genetic screening

The Cornell team published the first cases of preimplantation genetic screening (PGS) using five probes (X, Y, 18, 13, and 21) in 1993 (Munné *et al.*, 1993b). The team examined 157 blastomeres from 30 human embryos. Successful FISH was achieved in 93 percent of the blastomeres and aberrations were found in 70 percent (14/20) of abnormally developing monospermic embryos and 70 percent (7/10) of normally developing embryos. In an American Society of Reproductive Medicine (ASRM) meeting in 1994, Munné and Verlinsky agreed that PGS should be applied to polar bodies. Both teams independently published work on the FISH analysis of polar bodies in 1995 (Munné *et al.*, 1995; Verlinksy *et al.*, 1995), starting a prolific, friendly but competitive race between both teams. While the Cornell team focused on embryo diagnosis, the Chicago team focused on polar body analysis.

In Italy in 1993, Santiago Munné and Luca Gianaroli collaborated to learn more about the chromosomal status of embryos in relation to different conditions such as temperature and stimulation (Figure 1.16(a) and (b)). They performed the first PGS cases in Italy in 1996, which led to some of the most important papers on PGS (Gianaroli *et al.*, 1999; Munné *et al.*, 1999; Magli *et al.*, 2001; Magli *et al.*, 2007). Some of these studies determined that the chromosome abnormalities detected in spontaneous abortions were different than those found at the embryo level (Munné *et al.*, 2004), helping to define the PGS standard tests used today with FISH. This concept was later applied to other patients with recurrent miscarriage (Munné *et al.*, 2005; Garrisi *et al.*, 2008). The collaboration between Munné and Gianaroli refined the study of indications for PGS, such as repeated implantation failure, advanced maternal age, repeated miscarriage, previous trisomic conceptions, male factor, and so on. But the use of PGS today is very controversial (see later).

PGD for translocations

In 1994 Clare Conn joined the Delhanty team at the Galton Laboratory, UCL to do her PhD and began to approach the problem of carrying out PGD for couples who were carriers of chromosomal rearrangements, initially Robertsonian and reciprocal translocations. At that time, the only commercially available probes were those to detect repetitive sequences, mainly alpha satellites specific to certain chromosomes, whereas to detect unbalanced products of translocations locus-specific probes are required. We had to obtain YAC probes from the Medical Research Council (MRC)

(a) (b)

Figure 1.16 (a) Luca Gianaroli 1985; (b) Cristina Magli and Santiago Munné in a restaurant in Bologna, 1995.

Human Gene Mapping Resource Centre to grow and test; most were either useless for interphase work or turned out to map to the wrong region. So this was a highly labor-intensive process, but Conn persisted and PGD was successfully offered to several couples with translocations and to one couple with gonadal mosaicism for trisomy 21 (Conn *et al.*, 1995; Conn *et al.*, 1998; Conn *et al.*, 1999; Cozzi *et al.*, 1999). These were the world's first PGD cases for inherited chromosomal abnormalities. At Cornell, the Munné team also reported PGD for translocations using polar body biopsy (Munné *et al.*, 1998a) and showed that PGD decreases the rate of miscarriage in these patients (Munne *et al.*, 1998b).

Worldwide PGD

By 1994 there were eight centers worldwide, with four in the USA. Alan Handyside and I wrote a paper on the current status of PGD (Harper & Handyside, 1994) and reported on 83 cycles of sex-selection for patients carrying X-linked disease using PCR or FISH and 51 cycles of PGD for monogenic disorders, including cystic fibrosis. Three centers dominated the field: the Hammersmith/UCL team; the team at Cornell University Medical College in New York run by Cohen and Munné; and the Reproductive Genetics Institute in Chicago run by Verlinsky. The other centers were: the University Hospital, Ontario; the Academic Hospital, Brussels; the Jones Institute, Norfolk; the Genetics & IVF Institute, Fairfax; and GIEPH, Barcelona. All centers except the Reproductive Genetics Institute in Chicago were performing cleavage-stage biopsy with rapid diagnosis being done in one day and transfer the evening of the biopsy. Our paper reported on three misdiagnoses; one of sexing by PCR and two of cystic fibrosis out of 34 pregnancies. We said that no attempt was made to do blastocyst biopsy as developmental rates in vitro remained low.

The first world data paper was a success and so I repeated the process two years later (Harper, 1996). Now there were 14 centers worldwide that had performed 197 sexing cycles and 65 for monogenic disorders. Fifty pregnancies were reported (pregnancy rate 25 percent per cycle and 29 percent per embryo transfer), resulting in 28 deliveries and 34 babies born. We said "analysis of multiple nuclei from individual embryos has revealed a surprisingly high incidence (about 16 percent per embryo) of mosaicism and aneuploidy for the sex chromosomes of human embryos" (Delhanty *et al.*, 1993; Harper *et al.*, 1994; Munné *et al.*, 1994). I wish it was really that low today; the use of multiple chromosomal probes now detects up to 60 percent of embryos as mosaics. DNA amplification methods mostly involved nested PCR and we suggested that development of whole-genome amplification by PEP was the way forward.

The ESHRE PGD Consortium and PGDIS

In 1997 at various meetings, including ESHRE in Edinburgh (Figure 1.17) and the Second International Symposium on Preimplantation Genetics in Chicago (see earlier Figure 1.8(b)), some of the key players in PGD agreed that data collection was important but also that other aspects of PGD would benefit from discussion and communication in the PGD community. Involved in this discussion were Alan Handyside, Leeanda Wilton, Inge Liebaers, Joep Geraedts, Karen Sermon, and myself. Handyside decided this group should be called a "consortium," which was a good move as this best describes the activities. After much discussion of how it should be done, it was agreed that the consortium should be set up under the umbrella of the Reproductive Genetics special interest group in ESHRE, and so the ESHRE PGD Consortium was formed (see later). The Consortium had its first meeting at the ESHRE Central Office at the end of 1997 (Figure 1.18) and has had many other successful meetings (Figure 1.19).

Joe Leigh Simpson had a long-term interest in PGD and was instrumental in setting up the PGD International Society (PGDIS), which was established in 2003. This society took over the main role of the international working group, which had been to organize meetings on PGD and preimplantation genetics. The first PGDIS meeting was held in 2005 in London (Figure 1.20) followed by meetings in Australia and Spain.

1998–2008: The last 10 years

John Dumoulin performed an important study in 1998, reporting on the use of Ca^{2+}/Mg^{2+}-free medium for biopsy of human embryos at the 4- to 10-cell stage on the third day of development (Dumoulin *et al.*, 1998). These workers showed that use of this medium gave a lower rate of cell lysis as well as requiring a shorter time to perform the procedure. Subsequent embryo development to the blastocyst stage was not affected by the choice of biopsy medium. This medium is now in standard use in embryo biopsy. Another advance in embryo biopsy has been the gradual increase in the use of the

Figure 1.17 In a pub at the European Society for Human Reproduction and Embryology (ESHRE) Congress in Edinburgh, 1997. From left to right: Dagan Wells, Jon Sherlock, Leeanda Wilton, Elena Kontogianni, Joyce Harper, Marianne Bergere, and Stephane Viville.

Figure 1.18 The first ESHRE PGD Consortium Steering Committee Meeting, 1997, European Society for Human Reproduction and Embryology (ESHRE) Central Office, Belgium. From left to right: Catherine Staessens, Joep Geraedts, Karen Sermon, Joyce Harper, Stephane Viville, Inge Liebaers, and Alan Handyside.

laser for zona drilling, which gives greater control over hole size and makes biopsy quicker (Boada *et al*., 1998; Joris *et al*., 2003; Harper *et al*., 2008a).

Polar body biopsy has become popular in countries where legal restraints prevent cleavage-stage biopsy, such as Italy and Germany. Another important development for embryo biopsy is the introduction of blastocyst biopsy (de Boer *et al*., 2004; McArthur *et al*., 2005; McArthur *et al*., 2008), which gives more cells for diagnosis but in some cases blastocysts have to be frozen to allow time for the diagnosis. It will be interesting to see how embryo biopsy develops over the next 10 years.

Freezing of biopsied embryos using conventional methods proved unsuccessful (Joris *et al*., 1999; Magli *et al*., 1999; Ciotti *et al*., 2000), but various modifications to the cleavage-stage protocol appear to improve post-thaw survival of biopsied embryos (Lee & Munné, 2000; Voullaire *et al*., 2002; Jericho *et al*., 2003; Magli *et al*., 2006). The recent introduction of vitrification into human embryo freezing may be the ideal method to freeze biopsied embryos, although more experience

Figure 1.19 ESHRE PGD Consortium Steering Committee meeting held at University College London, 2007 including Sioban SenGupta, Joyce Harper, Karen Sermon, JanTraeger-Synodinos, Celine Moutou; and back row: Alan Thornhill, Veerle Goossens, Sjoerd Repping, Paul Scriven, Katerina Vesela, and Christine de Die-Smulders.

Figure 1.20 Jacques Cohen, Robert Edwards, and Yury Verlinsky at a meeting of the organizing committee for the PGDIS meeting in London, 2005 (at University College London).

and published results are necessary (Zheng *et al.*, 2005; Kuwayama, 2007).

In 2000 Dagan Wells, whilst still in Joy Delhanty's laboratory at UCL, and Leeanda Wilton, working in Melbourne, both published papers using comparative genomic hybridization (CGH) on human embryos, which is a method that can be used for assessing all of the chromosomes in single cells. Both studies analyzed 12 embryos and reported embryos with chaotic nuclei and high levels of mosaicism (Wells & Delhanty, 2000; Voullaire *et al.*, 2000). The Wilton group successfully applied the method in 28 clinical cases (22 for repeated implantation failure and six for advanced maternal age or repeated miscarriage), freezing the biopsied embryos to allow time to perform and analyze the CGH (Voullaire *et al.*, 2002; Voullaire *et al.*, 2007; Wilton *et al.*, 2001; Wilton *et al.*, 2003). Wells joined the Saint Barnabas team in 2000 and published the first clinical case of CGH on polar bodies (Wells *et al.*, 2002).

The Verlinsky group was the first to report on PGD for human leukocyte antigen- (HLA-) matching (Verlinksy *et al.*, 2001). The couple, both carriers of the IVS 4 + 4 A≥T mutation in the FANCC gene with an affected child required an HLA-compatible donor for cord blood transplantation. Thirty embryos were tested in four IVF attempts and six were homozygous affected and 24 were unaffected, and five of these were also HLA-compatible. Three embryo transfer procedures were performed and the patients delivered a child after the third treatment cycle. HLA typing has now become an important indication for PGD (Harper *et al.*, 2008a).

One important change in PGD for monogenic disorders is the diagnosis of late-onset disorders where prenatal diagnosis is not normally indicated, such as predisposition to inherited cancer. Some of the most frequent requests for PGD are now for the inherited cancer predisposition syndromes, such as retinoblastoma, Li Fraumeni syndrome, neurofibromatosis I and II, and familial adenomatous polyposis coli (Harper *et al.*, 2008a). Couples who are reluctant to terminate an otherwise normal pregnancy appear to be more willing to consider selection at the preimplantation stage.

Fluorescent PCR, as introduced by the Brussels group (Sermon *et al.*, 1998), has enabled multiplexing and a more sensitive PCR reaction, and the use of linked or unlinked markers has became routine (Sermon, 2002; Sermon *et al.*, 2004) (Figure 1.21). The most exciting prospect for the future is that both FISH and PCR are replaced by array technology, which would permit the amount of information obtained from a single cell to be increased way beyond current technology.

The quest for a comprehensive chromosome count has involved techniques which were never taken up; spectral karyotyping (SKY) (Márquez *et al.*, 1998) and cell conversion (Willadsen *et al.*, 1999; Verlinksy *et al.*, 1994b). Márquez *et al.* (1998) karyotyped 60 unfertilized oocytes and two fresh polar bodies, and it was concluded that this method could be used as an alternative to karyotyping second meiotic metaphase chromosomes from human oocytes and polar bodies. In cell conversion, Dyban *et al.* (1992) treated second polar bodies with okadaic acid so the chromosomes were converted to metaphases and could be karyotyped

Figure 1.21 Karen Sermon, Yury Verlinsky, Leeanda Wilton, and Joyce Harper at the International Society of Prenatal Diagnosis meeting in Kyoto, Japan, 2006.

Figure 1.22 Jacque Cohen and Santiago Munné in Taiwan, 2002.

(Verlinsky *et al.*, 1994b) and Willadsen *et al.* (1999) attempted this in blastomeres using bovine eggs.

Transport PGD

Most PGD Centres were originally set up as an add-on to an already existing IVF unit. Now the field has matured from research to mainstream genetic testing and so specialist PGD Centres have been established which work with many IVF units, such as Reprogenetics (Figure 1.22), Genoma, Genesis Genetics and our own centre, UCL Centre for PGD. IVF units perform the biopsy and prepare the cells (tubing for PCR and spreading for FISH) which are then transported to the PGD Centre for the diagnosis. This is how we did the first FISH cases; the Hammersmith Hospital did the biopsy and UCL did the diagnosis. It is essential that patients are adequately counselled and that there is excellent communication between the IVF unit and PGD Centre.

Background

IVF

Internationally a growing number of patients are using assisted reproductive technologies to conceive. One of the main reasons for this is that the average age of conceiving a first child has been steadily increasing, and so many couples experience age-related infertility. It is well documented that chromosomal abnormalities in oocytes increase in frequency as a woman

ages, and these have a significant effect in women aged over 35 (Hassold, 1986). These meiotic chromosome abnormalities lead to aneuploidy in the oocytes, which can lead to unbalanced chromosome arrangements in the embryo. The majority of these abnormal embryos either will not implant or will miscarry.

The most common causes of female infertility include polycystic ovarian syndrome, unexplained infertility, endometriosis, tubal disease, and premature menopause. The most common causes of male infertility are azoospermia (nonobstructive and obstructive), hormonal and genetic.

Infertility treatments include timed intercourse, ovulation induction, intrauterine insemination (IUI), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) (see Chapter 2).

For patients going through IVF and ICSI, a number of tests are performed prior to the onset of treatment. The male's semen is analyzed, the woman's ovarian reserve is assessed to predict if she will respond well to fertility drugs and to determine which dose of drugs to use, the uterus is checked to ensure that there are no abnormalities, and a dummy embryo transfer is performed to ensure that the actual embryo transfer procedure will run smoothly. The basic superovulation regimen involves downregulation of the pituitary and then administration of follicle stimulating hormone (FSH) to stimulate development of the oocytes. Timing the administration of these drugs may be varied for patients who have a suboptimal response. Stimulation is monitored by serial ultrasound and estrogen levels. If suitable follicle development occurs, human chorionic gonadotrophin (hCG) (to mimic the luteinizing hormone surge) is given 36 hours before the scheduled egg collection to allow final maturation of the oocytes. The oocytes are collected by ultrasound-guided follicle aspiration under light sedation and the collected oocytes are stored in appropriate culture conditions.

The IVF laboratory is the hub of the fertility clinic and a high-quality laboratory is essential for good success rates. There is no point setting up PGD in an IVF clinic that has poor success rates. Oocytes and embryos are cultured here until transfer or freezing. Insemination may be performed by standard IVF or by ICSI, and is done on the day of the oocyte retrieval. In both cases the sperm is prepared to remove the seminal plasma; usually by use of a density gradient. Standard IVF involves adding some prepared sperm to the dish where the oocytes are being cultured. ICSI is used in cases where sperm quality or quantity is very low. It involves selection of a particular sperm and injection of this sperm directly into the cytoplasm of the oocyte. It is essential that ICSI is used for all PGD cases where PCR is performed to reduce the risk of paternal contamination (see misdiagnosis below). A fertilization check is performed around 18–20 hours after insemination to look for the appearance of two pronuclei and two polar bodies. Embryos are cultured for a further one to four days, depending on the policy of the center. In standard IVF embryos may be transferred on days two, three, or five of development. The IVF laboratory will also be involved in oocyte, sperm, and embryo freezing. Vitrification is an ultra-rapid freezing protocol which has shown great success for oocyte and embryo freezing (Kuwayama *et al*., 2005; Zheng *et al*., 2005). If a pregnancy results, patients are given progesterone to support the pregnancy.

Internationally there has been an increase in the success rates of IVF, and many centers report a delivery rate of between 40 percent and 50 percent (Data from the Human Fertilisation and Embryology Authority, HFEA).

Genetic disease

Inherited disease may be caused at the level of the gene or the chromosome. In principle, single-gene mutations may lead to genetic diseases, which can be distinguished on the basis of five simple modes of inheritance: autosomal dominant, autosomal recessive, X-linked recessive, X-linked dominant, and Y-linked. In practice, only the first three are relevant. At the chromosome level a distinction may be made between numerical and structural chromosomal abnormalities. The first usually result from meiotic nondisjunction and are sporadic in the vast majority of cases. Chromosomal rearrangements are carried by a parent and may lead to the production of chromosomally unbalanced gametes. There is also mitochondrial inheritance, which shows completely maternal inheritance.

Mutation simply means change. Change in gene function may be brought about by loss of the whole or part of the coding sequence of the gene (deletion) or by alteration in the bases that make up the DNA molecule (substitution). More rarely, gene duplication can cause disease. Inherited disorders are divided into autosomal-recessive, autosomal-dominant, and X-linked. In an autosomal-recessive disease, two abnormal copies of the gene are required for the disease to occur. Therefore parents may be carriers and will have no

symptoms of the disease. Any of their children will be at a 25 percent risk of being affected with the disease. If the parents already know they are carriers they can plan their family with this knowledge, but many couples are first aware that they are carriers when they have an affected child. This includes diseases such as cystic fibrosis, β thalassemia, and many inborn errors of metabolism.

Autosomal-dominant conditions only require one copy of the gene to be abnormal and they will affect 50 percent of the offspring. Generally, at birth dominant disorders are not as severe or life-threatening as recessive disorders. Therefore the parent carrying the affected gene will have the disease, but since dominant conditions are often late-onset, the disease may not yet have manifested itself, for example Huntington's disease, inherited cancer predisposition, and Marfan syndrome.

X-linked disorders (caused by mutation in genes that are carried on the X chromosome) may be either recessively or dominantly inherited. The vast majority are recessive and are carried by females who are themselves unaffected, or only mildly so, because of the normal copy of the gene on their second X chromosome. Common X-linked recessive diseases include Duchenne muscular dystrophy and hemophilia.

Numerical chromosome abnormalities are abnormalities that deviate in number from the normal karyotype, which has $2n = 46$ chromosomes; that is, two haploid sets. They may be divided into aneuploidy (an extra (trisomy) or missing (monosomy) chromosome of a pair) and ploidy (the number of haploid sets of chromosomes is abnormal) abnormalities. Aneuploidy is not normally inherited. Typically, the imbalance arises at meiosis I in the mother when two homologous chromosomes pass to the same pole of the spindle at anaphase. The most common numerical chromosome abnormalities are trisomies and monosomies, arising *de novo* as a result of meiotic nondisjunction during gametogenesis in parents with a normal karyotype. For all chromosomes, except the largest, the nondisjunction rate increases with maternal age. An increase in maternal age results in an increased embryonic aneuploidy rate as well as an increased frequency of spontaneous abortion. This is reflected in a higher miscarriage rate and a lower pregnancy rate. The most frequent abnormality in spontaneous abortions is trisomy 16, representing about 30 percent of all such trisomies, but in cleavage-stage embryos trisomies for chromosomes 13, 21, and 22 are the most common.

Structural chromosomal rearrangements are common in the human population. They are the result of simultaneous chromosome breakage, either within the same chromosome or in different chromosomes, and subsequent rejoining in an abnormal fashion. Providing that the breakage in the chromosomes has not affected gene transcription, the individual carrying the rearrangement will develop normally. The problems occur in oogenesis or spermatogenesis when the rearranged chromosomes have to pair with their normal homologs and abnormal products of segregation result.

The most common type of chromosome rearrangement is a translocation, which is the movement of a segment of chromosome from its normal position to a new site. Reciprocal translocations involve breaks along the arms of two chromosomes and exchange of material, with reunion creating two abnormal derivatives. Robertsonian translocations involve breakage and reunion around the centromere of the "acrocentric" chromosomes; the minute short arms of these chromosomes are normally lost in the process so that the chromosome number per cell is reduced to 45; again, with no phenotypic effect.

Much rarer types of translocation occur, known as "insertions." These involve three simultaneous breaks, either within one chromosome or involving two different chromosomes. The segment freed by two of the breaks within one chromosome is then inserted into a new position within the same chromosome, or into a second one. Inversions occur when two breaks occur within one chromosome and the free segment may rotate through 180 degrees before rejoining. This produces an inversion of genetic material between the breakpoints, which usually has no phenotypic effect in the heterozygous form when only one of a pair of chromosomes is involved. Ring chromosomes are formed when genetic material is lost from both ends of a chromosome, creating an unstable situation that is resolved by the fusion of the broken ends. It is unusual to find ring chromosomes in a normal adult, but they do occasionally occur as ring X-chromosomes or affecting an autosome if only the telomeres themselves are lost .

Genetic counseling

Couples found to be at risk of transmitting a genetic or chromosomal disorder to their children need to have nondirective genetic counseling. They usually require

genetic counseling as they have a child or pregnancy diagnosed with a genetic condition, have a child or pregnancy with dysmorphic features or physical or developmental abnormalities, the couple or another family member carries a genetic condition or they have experienced recurrent miscarriages. Genetic counseling should include medical facts about the disorder and perception of their genetic risk and impact of the genetic disorder on the family. It is important that the counseling is nondirective as the patients should be able to make up their own mind about how they manage their future treatment. Their reproductive options are to remain childless, to take a chance (reproductive roulette), or to consider prenatal diagnosis, PGD, gamete donation, or adoption.

If the couple are considering PGD they will need a consultation with a PGD specialist who will outline the advantages and disadvantages of PGD. An outline should be given of the IVF and PGD procedures, with written information for the patients to read at home. Issues requiring special attention include discussion about the impact PGD may have on the couple and their family, for example when there is an affected child at home, their perception of PGD and likely success, perception of their fertility, the welfare of the child in relation to genetic conditions that may cause ill health or early death in a parent (e.g. cancer predisposition), and the impact of failure of treatment. The patients have to be fully informed about the misdiagnosis risks, the impact of multiple pregnancies, and the option of confirmatory prenatal testing.

PGD for late-onset disorders, such as Huntington's disease, creates additional counseling issues. Patients may know they are a mutation carrier or may not wish to know their own genetic status. Since the person carrying the mutation will develop the disease, the future care of the child and the care of the affected partner must be discussed.

Adequate counseling may be problematic in cases of transport PGD, especially for monogenic or chromosomal abnormalities, as the IVF unit undertaking the treatment may not have a PGD specialist who can give the patients the necessary information (see transport PGD below).

Prenatal diagnosis

Prenatal diagnosis of congenital birth defects or genetic disorders is based on screening a low-risk population using noninvasive tests. In the past, standard screening policies to detect Down syndrome (trisomy 21) relied on offering fetal karyotyping to pregnant women over the age of 35 years. However, this failed to significantly reduce the incidence of Down syndrome babies that were born, as the majority of affected babies are born to women under the age of 35 as this age group delivers the most babies. This stimulated the development of screening tests to identify high-risk pregnancies with increasing sensitivity. The various strategies available include screening by maternal age, genetic and obstetric history, maternal serum, ultrasound, and magnetic resonance imaging. For those whose screen results indicate they are at a high risk of aneuploidy, an invasive diagnostic test for fetal karyotyping may be considered, with the knowledge that invasive testing carries a 1 percent risk of miscarriage. Using screening before applying an invasive diagnostic test limits the number of invasive prenatal diagnostic tests that are offered.

First- and second-trimester ultrasound may detect some markers which indicate the possibility of aneuploidy. First-trimester sonographic assessment of fetal anatomy has become an important component of prenatal diagnosis and includes measurement of nuchal translucency (NT). NT is the maximum thickness of the subcutaneous translucency between the skin and the soft tissue overlying the cervical spine of the fetus (normal NT measurement <3 mm). Other markers for aneuploidy may also be examined in the first trimester, including absence or hypoplasia of the nasal bone, tricuspid regurgitation, and raised pulsatility index in the ductus venosus.

The routine second-trimester "20 week" anomaly ultrasound scan is usually performed between 18 and 22 weeks of gestation, and includes a series of predefined images to obtain standardized measurements. An evaluation is made of the key structures, including the head shape and internal structures, the spine, abdominal shape and contents, the heart in the four-chamber view, and the extremities. Studies show that this detects 60–80 percent of major, and 35 percent of minor, congenital malformations (Chitty *et al.*, 1991; Luck, 1992), and in clinical practice up to 50 percent of significant abnormalities may be detected (Boyd *et al.*, 1998).

The analysis of specific biochemical markers in maternal serum is a noninvasive technique to identify pregnancies at high risk of certain birth defects. Second-trimester serum screening includes analysis of free β-human chorionic gonadotropin (hCG), alpha-feto-protein (AFP), unconjugated estriol, and inhibin. First-trimester screening for Down syndrome

is more recent and includes analysis of pregnancy-associated plasma protein-A (PAPP-A) and hCG, in particular the free β-subunit.

Tests that combine serum and ultrasound markers have the highest detection rate. In the combined test (Wald & Hackshaw, 1997) NT measurement is combined with serum levels of PAPP-A and hCG in the first trimester (11–13 + 6 weeks of gestation). The integrated test was found to have detection rates of 93 percent and 95 percent at a 5 percent false-positive rate (FPR), or 85 percent for a 1 percent FPR, in studies in Europe (Serum, Urine, and Ultrasound Screening Study, SURUSS) (Wald *et al*., 2003) and in the USA (First- and Second-Trimester Evaluation of Risk trial, FASTER) (Malone, 2005).

Invasive tests in pregnancy are used to obtain fetal cells, fluids, or tissues that are used for prenatal diagnosis of aneuploidy, fetal congenital disease, and fetal infection. The choice of invasive test is determined by the indication and fetal gestation balanced against the safety of the procedure. Patients who have diagnostic invasive procedures are screened-positive or at increased risk owing to a known genetic abnormality.

Amniocentesis is performed from 15 weeks' gestation but not before. It is used to determine the fetal karyotype, obtain fetal DNA, for investigation of fetal infection or to detect inborn errors of metabolism by biochemical assay. Using an aseptic technique and under constant ultrasound visualization, a fine needle is passed directly into the amniotic sac and 18–20 ml of fluid is aspirated. Amniotic fluid contains a variety of fetal cells that require one to three weeks' culture to provide sufficient dividing cells (metaphase nuclei) for karyotype analysis.

Fetal blood sampling (FBS) was performed for many indications such as hemoglobinopathies, coagulopathies, and immunodeficiencies, and to diagnose fetal infections. These are now mostly amenable to chorionic villus sampling (CVS) and DNA-based diagnosis. FBS is still used to investigate fetal nonimmune hydrops, and to confirm the level of fetal anemia or platelets prior to fetal transfusion.

The indications for CVS are similar to those of amniocentesis. It is particularly useful in the prenatal diagnosis of genetic diseases because it provides a larger amount of fetal DNA when compared with amniocentesis. It may be performed via the abdomen (transabdominal) or through the cervix (transcervical) (Jauniaux *et al*., 2000). Chorionic villi from CVS consist of an inner mesenchymal core and an outer cytotrophoblast, the latter in particular containing dividing cells. The cytotrophoblast will yield metaphases suitable for direct analysis and short-term culture (48 hours) and results can be backed up by long-term culture of the mesenchymal core.

The gold standard for analysis of prenatal diagnosis samples to examine chromosomes is a karyotype. However, since a karyotype requires the cells to be cultured, the karyotype results take several days or weeks to obtain. Quantitative fluorescence (QF) PCR has been introduced to allow rapid detection (one or two days) of the common aneuploidies. In this technique, highly polymorphic short tandem repeats (STRs) on chromosomes 13, 18, 21, X, and Y are amplified by use of fluorescent primers and PCR in a multiplex assay. The fluorescence intensity of the alleles is detected using an automated genetic analyzer and generates reliable results even with very small samples (Cirigliano *et al*., 2004). The limitation of QF PCR is that it does not look at all of the chromosomes and most structural rearrangements would be missed. Therefore a full karyotype is usually still performed in addition to QF PCR.

Cell-free fetal DNA was first detected in the maternal circulation a decade ago, and it has already proved its usefulness in prenatal diagnosis. The original report first described the identification of the SRY gene in the plasma of pregnant women carrying a male fetus (Lo *et al*., 1997) and it is being used in some clinics for gender selection for X-linked diseases. It is also used to type the Rh group of the fetus in pregnant women who are Rh-negative and at risk of allo-immunization (Lo, 1998; Finning, 2002). Blood is taken from the Rh-negative pregnant woman early in the first trimester (eight to nine weeks of gestation), and using a sensitive real-time PCR method, the *RHD* gene is amplified in a highly accurate and specific reaction. Cell-free fetal DNA detection has been used in the prenatal diagnosis of several autosomal-dominant monogenic disorders such as myotonic dystrophy (Amicucci *et al*., 2000) and achondroplasia (Saito *et al*., 2000) when the gene has been inherited from the father.

Preimplantation embryo development

Gametogensis is the development of the sperm and the oocyte. Spermatogenesis is a continual process that may be divided into three stages: proliferation (mitosis), reduction division (meiosis), and differentiation. In the male, germ cells start to proliferate by mitosis at puberty. This is followed by meiotic division and a

gradual re-organization of cellular components, characterized by a loss of cytoplasm. The four early spermatids produced then undergo a process of terminal differentiation and structural remodeling known as "spermiogenesis." Spermatozoa leaving the testis are not normally capable of fertilizing oocytes. They gain this ability while passing down the epididymis, a process known as "epididymal maturation." When they enter the female reproductive tract, the sperm will swim through the cervical os, up the uterus, and down the Fallopian tubes. If they encounter an oocyte, they will swim through the cumulus cells and, upon meeting the zona pellucida, they undergo primary binding, lose their acrosomal cap, and then undergo secondary binding, which enables the sperm to enter the ooplasm of the oocyte.

The mitotic phase of germ-cell proliferation in the human female terminates before birth, and, by the fifth month of fetal life, all oogonia have entered their first meiotic division to become primary oocytes. Around week nine of gestation, the oogonia are in the first meiotic prophase. The oocyte nucleus (germinal vesicle) is arrested in diplotene (the last stage of prophase I), after chromatid exchange and crossing-over have taken place. The primordial follicles remain quiescent, in meiotic arrest, for many years, and resume their growth and development to produce mature oocytes capable of fertilization only after puberty. Over 99 percent of follicles are destined to die rather than ovulate. Following puberty, when a primordial follicle is recruited into a developmental pathway that will lead to ovulation, the oocyte enters a growth phase, which involves a significant increase in size. When the oocyte has completed its growth phase, the follicle is "pre-antral," and is able to undergo rapid development in response to follicle stimulating hormone (FSH) secreted from the pituitary. The fully expanded antral follicle is now responsive to luteinizing hormone (LH) secreted from the pituitary, which is released rapidly (LH surge) in response to feedback signals from the ovary. Under natural conditions, the endogenous LH surge will induce final nuclear maturation of the oocyte within the next 24–36 hours. In this final maturation stage of oogenesis the nuclear membrane breaks down (germinal vesicle breakdown, GVBD), meiosis is re-initiated, and the first polar body is extruded. The oocyte is now in the stage of meiosis II (M-II).

If a sperm is able to fertilize an oocyte, the oocyte becomes activated and resumes meiosis and extrudes the second polar body. Approximately 18–20 hours after meeting the sperm the oocyte forms into a zygote. The two pronuclei (one from the sperm and one from the oocyte) may be seen in the center of the zygote. Over the next few days the early embryo undergoes a series of key events, including cleavage, activation of the embryonic genome, changes in its metabolic requirements, compaction, cavitation, and differentiation, to form the blastocyst (around day five of development). The last stage of preimplantation development occurs when the blastocyst hatches from the zona pellucida and becomes embedded in the uterus.

Preimplantation genetics

An array of research and clinical studies has confirmed a high frequency of cytogenetic abnormalities in human oocytes and embryos. Methods used to study the chromosomes of gametes and embryos include karyotyping, FISH, metaphase-CGH and array-CGH.

Although the incidence of constitutional chromosome abnormalities is about tenfold higher in the infertile male population than in normal men, 95 percent of infertile men have a normal karyotype (Egozqcue *et al.*, 2005). Males with constitutional chromosomal rearrangements or other anomalies are clearly at increased risk of producing aneuploid sperm, but extensive investigation by several groups over a number of years has shown that men with a normal somatic karyotype but with various sperm pathologies are also at increased risk.

Chromosomal imbalance in oocytes may result from losses or gains of individual chromatids or whole chromosomes. As the chromosomes in the primordial follicles are arrested in meiosis I (M-I), oocytes are vulnerable to nondisjunction, which may include predivision of chromatids (Angell, 1989). The overall rate of chromosome and chromatid imbalance in human oocytes detected by karyotyping is about 11 percent for women of maternal age 32–34 years (Mahmood *et al.*, 2000; Pellestor *et al.*, 2002; Cupisti *et al.*, 2003), but more recent studies using metaphase CGH put this figure at over 40 percent (Gutiérrez-Mateo *et al.*, 2004a; Gutiérrez-Mateo *et al.*, 2004b; Fragouli *et al.*, 2006a; Fragouli *et al.*, 2006b; Fragouli *et al.*, 2006c).

Chromosomes in human embryos show high levels of chromosomal abnormalities affecting all chromosomes (Delhanty *et al.*, 1993; Delhanty *et al.*, 1997; Harper *et al.*, 1995; Munné *et al.*, 1993a; Munné *et al.*,1998a). Naturally, embryos inherit meiotic errors from the oocyte and sperm from which they are derived but additionally embryos exhibit postzygotic

Table 1.3 Chromosome groups found in human preimplantation embryos

Definition	
Normal	All chromosomes normal
Uniformly abnormal	Uniform abnormality
Mosaic – aneuploid or polyploidy	At least two cell lines present
Chaotic	Every cell has a different chromosome complement

errors. Chromosomal abnormalities may be caused by mitotic nondisjunction or anaphase lag (Daphnis *et al*., 2005). Chromosome abnormalities in embryos can be separated into three groups; uniformly abnormal, mosaic, and chaotic (Table 1.3) (Delhanty *et al*., 1997). Mosaicism is seen in over 50 percent of human embryos and has caused many problems in PGD as the cell biopsied from such embryos is not representative of the rest of the embryo. Evidence from detailed FISH analysis of early spontaneous abortions suggests that preimplantation embryos containing lethal chromosome anomalies in more than half of their cells or with chaotic mosaicism have a minimal chance of survival beyond implantation or early pregnancy. Those that have few abnormal cells may implant successfully, since a low number of cells will be involved in forming the inner cell mass from which the embryo proper is derived.

The predominant type of mosaicism affecting most human preimplantation embryos from routine IVF patients is that of the diploid/aneuploid type, where the embryo begins life as normal diploid and an aneuploid line develops during the cleavage stage. However, in many of these embryos other cell lines are also present, including polyploid, haploid, and "chaotic types" with multiple anomalies. Several cell-cycle checkpoint genes, essential for maintaining accurate chromosome segregation, show extremely low levels of expression during the first two cell divisions of the embryo.

The definition of a "chaotic" embryo is that the chromosome constitution of individual cells varies widely, affecting several chromosomes, and that the mechanisms involved cannot be determined. There are two types, *fully chaotic* where no diploid cells are present, and *partially chaotic* with a core of diploid cells, indicating that the embryo began life with a balanced complement of chromosomes. A possible mechanism leading to the fully chaotic type would be a multipolar spindle at the first mitotic division. This could arise because of centrosome anomalies; interestingly, in humans it is the sperm that contributes the centrosome.

Procedures used in PGD

Clinical aspects of PGD

Couples requiring PGD are usually fertile but have to go through IVF so that PGD may be performed. When PGD was first developed it was thought that these fertile patients would achieve a higher pregnancy rate compared with infertile patients but this has been shown not to be the case (Harper *et al*., 2008a). A PGD cycle is more likely to be successful if at least nine oocytes are generated (Vandervorst *et al*., 1998). This increases the likelihood that after the biopsy and diagnosis some genetically good-quality embryos would be available for transfer. If the patient is carrying a chromosome abnormality or dominant disorder, and less than eight embryos are produced, there is a high chance that there would be no normal embryos for transfer.

In PGD cycles the embryos are manipulated during the biopsy procedure and embryos are selected for transfer based on their genetic status rather than their developmental and morphological status. In every indication for PGD, there are always cycles where no suitable embryos are available for transfer (Harper *et al*., 2008a). In cycles where genetically normal embryos are identified, it is not always possible to choose an embryo of good morphology. As a result, in most PGD clinics the PGD pregnancy and delivery rates will be less than those for infertile couples.

PGS was developed to try and aid embryo selection for infertile couples who are going through IVF (see Chapter 13 and below). Determining the genetic status of the embryo may help increase their pregnancy rates. But for this to occur, the advantage of examining the chromosomes has to compensate for the disadvantage of embryo biopsy.

For a successful PGD center, close collaboration between clinical genetic departments and IVF centers is necessary (Geraedts *et al*., 2001). The IVF center is responsible for evaluation of the suitability of the couple for IVF, for the IVF/ICSI treatment, that is, ovarian stimulation, ovum pick-up, embryo transfer, and for

the embryo biopsy. Clinical geneticists or counselors, or both, and the PGD center are responsible for evaluation of the genetic indications for PGD, pretest counseling, and genetic testing of the embryo.

Patients referred for PGD show a number of different histories, but basically they are referred because they are carrying an inherited disorder which they could pass on to their future child. Many couples have already had an affected child and are bringing up a child with a disorder, have suffered the tragedy of losing a child, or have experienced recurrent miscarriages of genetic origin or (repeated) terminations of pregnancy. Others, like carriers of Huntington disease, are in the presymptomatic phase of their diseases. Moral or ethical objections to prenatal diagnosis and abortion may be another reason for PGD.

PGD is technically possible for most monogenic disorders in which a gene defect is known. Controversial indications for PGD remain the relative "mild" disorders, such as (late-onset) inherited deafness, blindness, and skin diseases or disorders with incomplete penetrance. PGD is used for social sexing, that is, sex-selection for nonmedical reasons. PGD is also used for HLA typing in order to create a sibling without the disorder who is HLA-identical to his or her affected sibling, and who may serve as a stem cell donor for the affected sibling (Verlinsky *et al.*, 2001).

The patient file should include: a genetic counseling report, copies of the original chromosomal or DNA testing reports, or other specific testing of affected child, future parent(s), or other family members. Also, a full pedigree and family history (at least three generations) should be present, as well as data on health problems of female and male partners, and specialist consultations which may affect genetic diagnosis or IVF success and pregnancy. Furthermore, a female reproductive history, gynecologic and fertility status, and a male reproductive history, andrological history, and results of sperm analysis are needed (Thornhill *et al.*, 2004).

The intake procedure involves taking a complete history from or about the affected individual and the future parents, including their general profile and their motivation for and expectations of PGD. A team discussion of the acceptability of new referrals, especially with new indications, and of the acceptability of individual cases with several complicating medical, gynecologic, or genetic factors is necessary and may prevent couples from undergoing "useless," disappointing, or potentially harmful PGD cycles.

All indications for PGD rely on the condition being confirmed by the PGD laboratory. In appropriate cases, chromosome analysis or DNA studies, or both, should be carried out in the patient, the prospective parents, and other family members. Basic gynecologic evaluation does not differ significantly from that in routine IVF/ICSI. The genetic laboratory needs to work up the specific diagnosis for each family. In many countries patients are referred for PGD by clinical genetics centers, which means that they have been counseled with respect to the genetic condition relevant to that particular family and their reproductive options (Geraedts *et al.*, 2001). Once admitted to the PGD center, couples receive further extensive information on IVF and PGD: brochures or written information may be sent to them before their appointment. We should check the awareness of couples regarding the nature and the severity of the genetic disorder, its recurrence risk, and their reproductive options and alternatives to PGD. The benefits and limitations of PGD compared to the alternative options, such as prenatal diagnosis, should be explained. Suitable counseling about PGD may be very limited in centers operating transport PGD as it is unlikely that staff at the IVF center will be suitably trained to offer detailed counseling (see Chapter 4).

Embryo biopsy

There are three methods of embryo biopsy (see Table 1.1 earlier). Until recently, almost all centers used cleavage-stage biopsy, but in recent years polar body biopsy and blastocyst biopsy have grown in popularity. There are advantages and disadvantages to each procedure.

Polar body biopsy

Some of the initial PGD cycles performed in Chicago by the Verlinsky team were performed on the first polar body only and were termed "preconception diagnosis." However, it soon became apparent that, for both chromosome analysis and analysis of monogenic disorders, the second polar body was vital. Since the second polar body is only extruded during fertilization, removal of the first and second polar bodies could not be defined as preconception diagnosis.

The polar bodies may be removed sequentially or simultaneously (see Chapter 9). Since the first polar body degenerates quite rapidly, sequential biopsy will ensure that the DNA in the first polar body is still viable, but this procedure results in three manipulations of

the oocyte; first polar body removal, ICSI and second polar body removal. The polar bodies are removed by use of a beveled pipette which pierces the zona (Montag *et al*., 2006), a laser to drill a small opening (Montag *et al*., 2002), or by using three-dimensional dissection (Cieslak *et al*., 1999). Acid Tyrodes should not be used for zona drilling as it has been shown to affect spindle development and so will affect the resumption of meiosis (Gordon & Talansky, 1987).

Biopsy of the first polar body alone has limited applicability for PGD for a number of reasons. The procedure only allows the detection of maternal genetic defects and crossing over of homologous chromosomes leads to a reduction in the number of embryos available for transfer (Dreesen *et al*., 1995). There is only the possibility of a single cell for analysis, leading to lower overall reliability (in contrast to cleavage-stage biopsy, in which two cells may be taken for independent analysis). It is thought that more unacceptable errors result from polar body analyses when compared to blastomere analysis (Navidi & Arnheim, 1991). To overcome these disadvantages Verlinsky *et al*. (1990) proposed and undertook to remove both first and second polar bodies for analysis. After assessing the safety of removing the second polar body in mice (Kaplan *et al*., 1995) this approach has met with success and has been applied to PGD for the detection of numerous monogenic diseases (Strom *et al*., 1998; Verlinsky *et al*., 1999), chromosomal aneuploidies (Verlinsky *et al*., 1996) and maternal chromosome translocations (Munné *et al*., 1998b).

Despite the large number of cycles reported with this approach, only a few centers worldwide have used it. This may be because of a number of factors, including: it only looks at maternal chromosomes; it is very time-consuming as not all oocytes will achieve fertilization but all oocytes have to be biopsied; biopsy of both the first and second polar bodies is required for optimal diagnostic efficiency and although this can be achieved by either sequential (Strom *et al*., 1998) or simultaneous (Verlinsky *et al*., 1998) biopsy with successful results, it is very labor-intensive. Many centers may have established cleavage-stage biopsy as the tool for PGD as a result of the large number of referrals for sexing (see Chapter 14).

Laws in Germany and Switzerland make cleavage- and blastocyst-stage biopsy illegal, but do not seem to prohibit first polar body biopsy (and maybe also second polar body biopsy). Also recent changes in Italian law mean that all embryos have to be transferred. Genetic diagnosis is not appropriate as any abnormal embryos would still have to be replaced. The decision about which oocytes to inseminate is required prior to insemination.

Cleavage-stage embryo biopsy

Cleavage-stage biopsy is the most common method used in PGD and PGS (Harper *et al*., 2008a). The procedure is performed on day three of development. There are two stages to cleavage-stage biopsy: zona drilling and blastomere removal. Three methods of zona drilling have been reported but, until recently, the original method using acid Tyrodes to drill the zona (Hardy *et al*., 1990) had been the main technique used (Harper *et al*., 2008a). Now the most common method of zona drilling is achieved by use of a laser (Boada *et al*., 1998). The technique is much quicker than acid Tyrodes drilling, it allows a more accurate hole to be made, and it reduces blastomere lysis. The third method is mechanical opening of the zona. This method has the advantage that it leaves a flap of zona that can be put back in place after blastomere removal and no chemicals or lasers are used which may affect embryo development (Cieslak *et al*., 1999; Selva, 2000).

Removal of the blastomeres is mostly performed using simple aspiration. The biopsy pipette is introduced into the perivitelline space through the hole in the zona in order to reach a blastomere. Close location of the blastomere respective to the opening allows limited penetration of the microtool. One or two blastomeres are removed by gentle aspiration. Other methods have been described but they are rarely used. These include extrusion, flow displacement, single-needle biopsy, stitch and pull, and puncture and aspiration. In extrusion the blastomere(s) is extruded through the aperture by pushing against the zona at another site (usually at 90 degrees to the aperture) using a blunt pipette (Levinson *et al*., 1992). Flow displacement involves creating a second puncture site through which medium is injected to dislodge the blastomere through the first puncture site (Pierce *et al*., 1997). However, the routine clinical application of this technique is limited.

The biggest problem with cleavage-stage biopsy is that the embryos are starting to undergo compaction at this stage of development and tight junctions form between blastomeres which may make removal of one or two cells very difficult. In the early days of PGD, this resulted in a relatively high level of blastomere lysis and some biopsies could take a long time to perform. Ca^{2+}/Mg^{2+}-free medium has been used to loosen the

membrane adhesions between blastomeres (Santaló *et al*., 1996; Dumoulin *et al*., 1998), which permits an easier removal of cells, and results in less blastomere lysis and a shorter procedure time. Use of this medium has made cleavage-stage biopsy much quicker and simpler. The effects are reversible, so that when the embryos are returned to normal medium the tight junctions are reformed, but more than a few minutes in Ca^{2+}/Mg^{2+} -free medium may cause irreversible results (Doshi, unpublished observation). Ca^{2+}/Mg^{2+} -free medium is now used routinely in cleavage-stage biopsy.

In PGD cycles the morphologically best embryos may not be replaced. The main criteria for embryo selection will be the results of the genetic test. On this basis alone, PGD pregnancy rates would be expected to be below IVF pregnancy rates. Added to the equation is the fact that the embryos are also manipulated and cells removed. It is obvious that embryo biopsy must have some detrimental effect on the implantation potential of the embryo. The best situation for the individual embryo would be not to be biopsied. Therefore any PGD cycle has to be performed for a defined indication where the damage caused by biopsy is outweighed by the indication (see Chapter 8). A similar argument may be made for embryo freezing where the pregnancy rates of most clinics in a frozen–thawed cycle will be lower than the fresh IVF cycle. Embryo freezing is often compared to cleavage-stage biopsy since, after thawing, most embryos will have lost a few cells (Cohen *et al*., 2007).

One of the ongoing discussions in PGD is how many cells to biopsy. Continuing the argument above, removing one cell will be less harmful than removing two (and will also be technically much simpler). Goossens *et al*. (2008) studied the effects of biopsy of one or two cells for various PGD and PGS indications. Their results show that removing two cells reduces the developmental potential of the embryo in vitro but does not have any significant effect on implantation potential or delivery rates. This study only compared one- and two-cell biopsy. An interesting study would be to compare no biopsy, one-cell, and two-cell biopsies. For PGS, where the aim is to get the patient pregnant, it is still recommended to remove only one cell (see Chapter 13), but for some cases of monogenic disorders and chromosomal abnormalities, it is important to analyze two cells. The usual rule is that from embryos containing six cells or more, two cells may be removed, and from embryos with five cells just one cell may be removed. It is unlikely that a two- or three-cell embryo would be biopsied, and whether one cell should be biopsied from a four-cell embryo is debatable, but if embryos are not biopsied then they cannot be considered for transfer. In the adverse situation where there are few day three embryos, four- or five-cell embryos may have to be considered for biopsy.

One of the fundamental problems with cleavage-stage biopsy is that, at this stage, high levels of mosaicism are seen. When PGD was developed it was thought that taking one cell from the embryo would be representative of the rest of the embryo but we now know that this is not the case (see misdiagnosis below).

Blastocyst biopsy

Blastocyst biopsy would seem to be the most logical procedure as it is thought that only the most viable embryos make it to the blastocyst stage but also more cells may be removed and they are from the cells that go on to make the placenta rather than cells that will form the embryo proper. However, a high proportion of embryos will not make it to the blastocyst stage, which may have implanted if they had been transferred earlier. Since a good number of embryos are needed for a successful PGD cycle (Vandervorst *et al*., 1998) but only approximately 50 percent of embryos will make it to the blastocyst stage, this method of biopsy will result in the fewest embryos for PGD. It is also the method with the shortest time for the diagnosis as the embryos will need to be replaced within 24 hours or frozen.

Blastocyst biopsy has only recently been incorporated into clinical practice (de Boer *et al*., 2004; Kokkali *et al*., 2005; McArthur *et al*., 2005; McArthur *et al*., 2008) and has been closely linked to the use of laser technology (Veiga *et al*., 1997). The main benefits of using a laser in the process are speed, accuracy, and the non-acidification of the medium, thus facilitating the biopsy procedure (Boada *et al*., 1998). Furthermore, its use during blastocyst biopsy is essential in order to achieve a rapid, easy, and efficient detachment of the cells to be biopsied.

Two approaches with respect to timing have successfully been used in clinical practice. In the first approach, the hole is made on the third day of development (or occasionally on the fourth day, at the morula stage) (McArthur *et al*., 2005). The aim of making the hole at this stage is to minimize the risk of damaging the cells adjacent to it, as at the blastocyst stage the perivitelline space is almost nonexistent. In this approach,

the zona pellucida (ZP) of all embryos available on day three is drilled. The embryos are then kept in culture until they reach the blastocyst stage. At this point the cells closest to the hole tend to extrude and the herniated cells are then biopsied (it is hoped, trophectoderm [TE] cells). Only those embryos which reach the blastocyst stage are biopsied. The other possibility consists of making the hole at the blastocyst stage. The area chosen for ZP drilling must be directly opposite the inner cell mass (ICM). After a few hours of culture (4–10 h) and the subsequent herniation of a few trophectoderm cells, the biopsy may be performed.

In both cases, the biopsies are obtained by securing the blastocyst with a holding pipette (internal diameter, 20–30 μm), with the herniated cells located at the three o'clock position. The TE cells (two to nine cells) are gently aspirated with a biopsy pipette (internal diameter 30 μm) and three to five laser pulses are applied in order to detach them from the blastocyst. The cells obtained are finally ejected from the biopsy pipette and are ready to be processed.

The time available for analysis in cases of PGD based on blastocyst biopsy is reduced to a maximum of 24 hours or the embryos have to be frozen. The fact that the results of genetic analysis must be obtained in so limited a time means that high levels of coordination are required between all parties involved in the process. Although most diagnostic protocols can be carried out in this timeframe, it nevertheless restricts the possibility of re-analysis or rehybridization, when necessary, and limits the advantages associated with PGD in blastocysts.

The TE cells used as a source of biopsy material give rise to extra-embryonic tissues while the fetus originates from the ICM. Therefore, and as it occurs with polar-body PGD, the biopsy does not lead to a reduction in total embryo mass, the ICM remaining intact.

Diagnosis

The two main techniques used for the diagnosis in PGD are PCR and FISH (see Table 1.2 earlier). For monogenic disorders, PCR is used. There have been many advances in PCR since the first cycles of PGD amplifying only a sequence on the Y chromosome (Handyside *et al.*, 1990) but the problems of contamination, allele dropout, and amplification still persist. Most centers now make use of multiplex fluorescent PCR or whole-genome amplification (WGA). The method used to look at chromosomes is FISH, which has progressed little since the first cycles of PGD for sexing using FISH (Griffin *et al.*, 1994; Munné *et al.*, 1994). The same types of probes are used but now multiple probes are used in several rounds of hybridization. FISH is used for sexing for X-linked disease, PGD for inherited chromosome abnormalities and aneuploidy screening. The diagnostic tests used are still far from ideal, and misdiagnosis continues to be reported (Harper *et al.*, 2008a, Goossen *et al.*, 2008b) (see misdiagnosis below).

FISH

The ideal way to examine chromosomes from any tissue is to perform a karyotype so that all of the chromosomes may be examined. But to do this from a single embryonic cell has proved very difficult and not efficient enough for PGD. Therefore PGD cycles where chromosome analysis is required are done by FISH. FISH uses fluorescently tagged DNA probes which bind to their complementary sequence and may be visualized under a fluorescent microscope.

FISH can be done in multiple rounds but the efficiency will decrease with each round as the test DNA becomes weaker. The most efficient way to perform FISH would be to use just one or two probes. As more probes are added the technique becomes less efficient (Ruangvutilert *et al.*, 2000), but for PGS as many chromosomes as possible need to be examined and so, often, five probes are used per round and as many as three rounds may be performed (Munné *et al.*, 2006).

There are two basic methods of preparing embryonic nuclei for FISH. The traditional way of spreading any type of cells for cytogenetic analysis uses 3:1 methanol:acetic acid (Tarkowski method), and this has been applied to embryonic nuclei (see Chapter 13). However, this is a difficult technique to learn and the nuclei are not visible during the spreading procedure. Nuclei can become very large and diffuse. Also, it is difficult to spread a whole embryo by this method as it is difficult to clean the cytoplasm away from multiple cells. The second method was developed in mouse embryos specifically for embryonic nuclei and uses an acidified detergent solution of Tween and HCl (Coonen *et al.*, 1994). The technique was successfully applied to human embryonic cells in 1994 (Harper *et al.*, 1994). This method may be used to spread single blastomeres, whole cleavage-stage embryos, and blastocysts. This technique is relatively easy to learn but does require practice to become efficient. A huge advantage is that it may be used in the embryo biopsy room, whereas methanol:acetic acid cannot as it is both

toxic and volatile. Both methods are equally efficient for PGD and PGS (Handyside, personal communication), but people usually prefer the method they are used to (see Chapter 13). One study compared the two methods from a laboratory where the methanol:acetic acid method had been used for many years. It was not surprising that the results showed that the methanol:acetic acid method was more efficient, as this was the technique that they had more experience with (Velilla *et al.*, 2002). In my view both techniques are equally efficient.

FISH is a very simple procedure. After spreading the cells, a pepsin digestion is performed to remove any cytoplasm from the nuclei and to make the nuclei accessible to the probes. The nuclei are refixed to ensure that they are stuck to the slides. Probe and test DNA may be denatured simultaneously or separately, depending on the probe combinations used to make the DNA single-stranded, and the probes and samples are hybridized for between one and six hours to allow the probes to anneal to their complementary sequence. Any unbound probes are washed off and the slides are observed under the fluorescent microscope. FISH may be a technically simple procedure to perform but analysis of FISH signals is not as easy as counting dots (see Chapter 12). Ideally, two independent, suitably trained people should view each FISH result.

Metaphase CGH has had a limited application in PGD (see the future below).

PGD for chromosome rearrangements

Couples carrying a chromosome abnormality may have experienced repeated miscarriages, the birth of an affected child, or infertility. Many of these couples do not reach the stage of prenatal diagnosis as they miscarry before such procedures can be performed. If these couples keep trying naturally they have a high chance of eventually conceiving a normal child, but for some the risk of another miscarriage is not acceptable and so PGD is the reproductive choice for them. An experienced cytogeneticist needs to establish which probe combination should be used for the PGD. Some misdiagnoses have been caused as incorrect probe combinations were used (Scriven, 2003; Mackie Ogilvie & Scriven, 2002). Chromosome abnormalities cause problems as during meiosis crossing over becomes confused. This leads to unbalanced chromosomes in the gametes which go on to give chromosome imbalance in the embryos.

The main class of chromosome abnormalities are the translocations. Robertsonian translocations involve the acrocentric chromosomes where the satellite is lost and the two chromosomes become stuck together. Therefore someone carrying a Robertsonian translocation will only have 45 chromosomes. PGD is relatively easy to do as efficient probes are available commercially for the acrocentric chromosomes. Two probes may be used for the chromosome that could lead to a viable fetus (e.g. chromosome 21 in a translocation involving chromosome 21).

Reciprocal translocations may be difficult as every patient comes with different chromosomes and break points so an individual FISH protocol needs to be designed. But, with the increasing numbers of commercial FISH probes available, the majority of reciprocal translocations can be done by FISH. It is vital that a qualified cytogeneticist decides which probe combination is used and the efficiency of the protocol needs to be established in interphase nuclei such as lymphocytes. Testing the protocol on blastomere nuclei may be performed but it is not essential.

Other types of chromosome abnormalities may be diagnosed by PGD such as inversions (Escudero *et al.*, 2001), insertions (Melotte *et al.*, 2004), and ring chromosomes (our own group, unpublished).

PGD for infertility (PGS)

Embryo biopsy and single-cell analysis methods have also been used to try and help infertile couples achieve a pregnancy. Since chromosome abnormalities increase with advanced maternal age, it seems logical that checking embryos from these patients for chromosome abnormalities may increase their chance of having a chromosomally normal embryo transferred. At the time PGS was started (late 1990s) the current practice of prenatal screening was in the early stages and so at that time most women of advanced maternal age were offered an invasive procedure (CVS or amniocentesis – see Chapter 5). Therefore PGS was suggested as a means of eliminating the need for prenatal diagnosis in these women since the embryos could be checked for the most common abnormalities at the preimplantation stage. However, around the same time it was realized that human preimplantation embryos exhibit a high level of chromosome abnormalities, especially mosaicism, so that the one or two cells that are biopsied may not be representative of the rest of the embryo (Harper *et al.*, 1995).

With advances in prenatal screening methods to detect chromosome abnormalities in the fetus, and the realization that taking one cell from an embryo is

Table 1.4 Differences between preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)

	PGD	PGS
Primary aim	Identify normal embryos	Get the patient pregnant
Indication	Monogenic disorder, X-linked disease, known chromosome abnormality	Advanced maternal age Repeated implantation failure Repeated miscarriage Severe male factor infertility
Fertility	Often fertile	Infertile or subfertile
Biopsy	Usually day 3	Usually day 3
Number of cells for analysis	Two cells	One cell
Diagnosis	FISH for chromosome abnormalities and sexing. PCR for monogenic disorders	FISH with as many probes as possible
Undiagnosed or inconclusive results	Never transfer these embryos	Can transfer these embryos
Prenatal diagnosis	Indicated	Not indicated

FISH, fluorescent *in situ* hybridization; PCR, polymerase chain reaction.

not representative of the rest of the embryo, the use of PGS as an alternative to prenatal diagnosis was no longer suggested. Instead, it was suggested that PGS may improve implantation and delivery rates in "difficult" IVF patients such as those of advanced maternal age (AMA), with repeated implantation failure (RIF), repeated miscarriage (RM) with a normal karyotype in the couple, and severe male factor (SMF) infertility. The procedure has been called many things, including PGD for infertility, PGD-AS (PGD for aneuploidy screening), and PGS.

What is the difference between PGD and PGS?

PGD is the diagnosis of specific genetic and chromosomal abnormalities in couples who are at high risk of transmitting these abnormalities to their children. Most of the couples are fertile and the primary aim is to identify which embryos are free from the disorder the parents carry and the secondary aim is to get pregnant. Most of the patients could explore the other reproductive options available to them (prenatal diagnosis, gamete donation, and so on). To go through PGD they have to go through IVF. Aneuploidy screening (PGS) is the analysis of as many chromosomes as possible in embryos from patients with subfertility with the aim of increasing their chances of a normal pregnancy. Most of the patients are going through IVF anyway and the PGS is added on, with the primary aim being to get the patient pregnant. The main differences between PGD and PGS are summarized in Table 1.4.

Randomized controlled trials for PGS

To date there have been hundreds of studies on PGS but only two randomized controlled trials. The basis of a well-designed clinical trial is that it is properly randomized into a treatment and control group, and for PGS the end-point should be delivery of a normal baby. To detect a significant difference in the control and treatment groups approximately 200 patients would be needed in each arm. Following this criterion only two studies have been conducted. The first, by Staessens *et al*. (2004), was an excellent study that showed that PGS did not improve the delivery rate in patients with AMA. The control and PGS group both achieved relatively high pregnancy rates. The only criticism of this study was that since the center performs many cycles of PGD where, usually, two cells are removed, the researchers also removed two cells for PGS. There is no conclusive evidence of whether biopsy of one or two cells has a different effect on the implantation potential of the embryo, but, logically, the more an embryo is manipulated the more adverse the effect. Therefore, for PGS, the majority of clinics only remove one cell, but then mosaicism can never be detected.

The second randomized controlled trial on PGS was published in 2007, by Mastenbroeck *et al*., and it caused a huge stir in the IVF community. Their data on over 400 patients showed that PGS had a detrimental effect on delivery rates since the control group achieved a higher delivery rate. However, the paper has been severely criticized (Harper *et al*., 2008b; Munné *et al*., 2007; see also Chapter 13). The paper showed

inappropriate patient selection (PGS for AMA will give best results in older patients, such as those aged over 40), poor embryo selection, 20 percent of embryos were undiagnosed after FISH, probes for 15 and 22 were not included in the FISH panel, the control pregnancy rate was very low, and when undiagnosed embryos were transferred the pregnancy rate was only 7 percent.

The current status of PGS is controversial. To date there has been no randomized controlled trial that has shown an improvement in delivery rates using PGS for AMA, but no randomized controlled trials have been conducted on PGS for RM or RIF. A randomized controlled trial on PGS for RM would be almost impossible as RM patients often have no problem achieving a pregnancy and so they could not be randomized into an IVF or PGS treatment group. Therefore the debate about PGS is ongoing and ESHRE PGD Consortium data will show if the uptake of PGS decreases or continues to grow. A number of large randomized controlled trials are under way, including one organized by ESHRE.

Sexing

The first PGD cycles used PCR to amplify a sequence on the Y chromosome but even with improvements in PCR, FISH should be the preferred method of embryo-sexing. FISH with probes for the X and Y chromosomes can differentiate XX from XO and other sex chromosome abnormalities but PCR cannot. Also additional probes may be used for the chromosomes common in aneuploidy, such as 13, 16, 18, and 21. ESHRE PGD Consortium data have shown that there have been three misdiagnoses of sex by use of FISH. These could have been caused by cumulus cell contamination, resulting in a female result instead of a male; the transfer of the wrong embryo; unprotected sex; or failure of the FISH. It is essential that when performing sexing the X and Y probes should be present in the first round of FISH with few additional probes to obtain the maximum efficiency of the procedure. The more FISH probes used in each round, the less efficient the FISH becomes (Ruangvutilert *et al.*, 2000). Therefore three-probe FISH will be more efficient than five-probe FISH, and the first round will be more efficient than the second round.

In addition to establishing the sex of the embryo, FISH will also identify abnormalities of sex chromosome copy number (for instance, a single X chromosome, which is associated with Turner syndrome, and XXY, which is associated with Klinefelter syndrome), as well as embryos with aneuploidy (monosomy or trisomy) for the autosome. The presence of an autosome probe indicates the ploidy of the embryo, which allows discrimination between a single X chromosome and haploidy (one copy of every chromosome), XXX or XXY and triploidy (three copies of every chromosome), and XXXX or XXYY and tetraploidy (four copies of every chromosome).

The ideal way to perform PGD for X-linked disease is to do a specific diagnosis for the mutated gene using PCR. This will become the most common method in the future but currently it takes time to work up a diagnosis for each new X-linked disorder.

PCR for monogenic disorders

PCR is a technically demanding procedure and single-cell PCR should only be attempted by experienced molecular biologists. PCR is a continually developing field and the methods currently used are very different to those used in the first PGD cycles for sexing embryos. The biggest problems in single-cell PCR have been contamination and allele dropout. In PGD, contamination may be maternal (cumulus cells), paternal (sperm embedded in the zona), the personnel doing the biopsy, tubing, or PCR, or the media and other reagents used. To avoid maternal contamination, all the cumulus cells must be removed before biopsy. To avoid paternal contamination, ICSI should be used to prevent multiple sperm becoming embedded in the zona. However, ESHRE PGD Consortium data show that centers continue to use IVF when performing PGD for monogenic disorders (Harper *et al.*, 2008a). To avoid other types of contamination, extreme care must be taken with all stages of PCR, including the use of a positive pressure room to set up the PCR reagents, testing of all culture and biopsy media, an accurate work-up using a larger number of single cells, and the use of blanks during the PGD case. Currently, PGD for monogenic disorders is usually performed using multiplex fluorescent PCR. This has the advantage that linked or unlinked markers may be used, which permits detection of contamination.

Allele dropout (ADO) is a phenomenon specific to single-cell PCR, and it is caused by the preferential amplification of one allele. It was first identified in known heterozygous single cells that were being examined for the development of PGD protocols (Ray & Handyside, 1996). For a recessive disorder where both partners are carrying the same mutation, ADO in a homozygous affected or unaffected cell would not lead to a misdiagnosis, but in a heterozygous carrier the embryo could be diagnosed as normal or affected, depending on which allele dropped out. This was the case in a twin pregnancy, resulting after PGD of cystic

fibrosis where it was thought that normal embryos were transferred and the children were carriers (Harper *et al.*, 2008a). Such misdiagnoses are considered acceptable as they do not result in the birth of an affected child. However, if the couple are carrying different mutations, both mutations need to be identified as otherwise allele dropout could lead to the transfer of an affected embryo. The same applies for dominant disorders. ADO of the affected allele in a heterozygous cell will give a normal result when the embryo is affected. The best way to deal with ADO is to include informative markers which are linked to the gene being diagnosed, but couples are not always fully informative for linked markers.

When PGD was first developed, the diseases being diagnosed were those which had an early onset and were considered to be life-threatening. These were diseases for which prenatal diagnosis would routinely be offered. More recently there has been a trend to offer PGD in cases where prenatal diagnosis is not routinely offered, such as late-onset disorders, including the cancer predispositions. PGD has already been performed for polyposis coli (Ao *et al.*, 1998), BRCA1, and other cancer predispositions (Harper *et al.*, 2008a). PGD may be the treatment of choice for those diseases that are not thought to be serious enough for PND, but where the couple do not want to transmit the disease to their children.

Another controversial use of PGD is for HLA typing. This involves HLA matching the embryos to an already existing ill child. In some cases, such as β thalassemia and Fanconi anemia, this is done in tandem with PGD for the disorder. In this situation the chance of obtaining an embryo that is free from the monogenic disorder and HLA-matched is slim, but this has been successfully applied (Verlinsky *et al.*, 2001). HLA matching may also be done without PGD when there is no genetic link, such as when the sick child has leukemia. Couples embarking on this must be sure that they have explored every other possibility as it will take time to commence PGD and they will need to wait until the delivery of the PGD baby before they can take the stem cells from the cord blood, which may then be used to treat the sick child. This use of PGD has caused much debate and the family have to be considered as a whole. It might be thought that it is of no direct benefit to the savior sibling to have their embryo undergo PGD, but since family dynamics are severely affected when a child dies, there will be an overall benefit to the family if the sick child survives.

PCR techniques have advanced greatly over the last 10 years. The latest method to be used for PGD is whole-genome amplification (WGA) and DNA haplotyping (Renwick *et al.*, 2006). Many methods of WGA have been tried, but the one that seems to have the most success has been multiple displacement amplification (MDA). Once the DNA is amplified, multiple tests can be done, including using multiple markers to trace the normal and affected chromosomes. The future of single-cell diagnosis probably lies in the use of array technology to be able to identify a much larger amount of information about the biopsied cell (see Chapter 18).

Quality assurance and good practice

All PGD laboratories should be accredited and should follow ISO 15189. There are some key aspects of accreditation that are mandatory for PGD laboratories. These include center organization, personnel, facilities and safety, staff training and continued professional development (CPD), equipment servicing and maintenance, process control, documentation, producing appropriate reports, adverse events, assessments, services, horizontal and vertical audits, and satisfaction and process improvements (see Chapter 16). Standard operating procedures should be written for every step of the PGD process and they should be adhered to.

One of the most important issues in PGD is that the procedures are carried out by suitably qualified personnel. In some units the medical director might wish to perform the biopsy and the clinical embryologist may learn FISH or PCR. This is not the way it should be done. All personnel should undertake the task they are fully trained to perform. Biopsy should be performed by a clinical embryologist who is performing embryology on a day to day basis. FISH should be performed by a cytogeneticist and PCR by a molecular biologist; both require appropriate training in single cell diagnosis. In the UK scientists performing clinical diagnostic services have a recognized training program which leads to state registration as a clinical scientist. Specialized training in PGD is not available, but state registration may be obtained through a six-year in-house training program and submission of a portfolio to the Association of Clinical Scientists. PGD centers should have a clearly defined training program for molecular and cytogenetic personnel as well as provision for CPD. All stages of training and CPD need to be recorded and assessed annually during the staff appraisal process. The person responsible for signing the results report should be a state-registered scientist and/or a member of an appropriate professional body (in the UK this is the Royal College of Pathologists).

To participate in an accreditation scheme it is essential to have an external quality assessment (EQA) program. The PGD Consortium has set up schemes for EQA of FISH and PCR (see Consortium).

PGD is a multidisciplinary procedure that requires excellent organization and communication. During a treatment cycle, accurate communication is required to ensure that the PGD team knows the status of the patient and that the correct result is reported to the IVF unit.

Ethics and the future

There are many ethical and legal issues that need to be considered with PGD. This is clearly illustrated as embryo biopsy and PGD is illegal in some countries which do not allow the manipulation of the early embryo (Italy, Germany, and Switzerland) as it is felt there that the embryo should be respected as a human person, and that the embryo has an absolute right to life. In Germany, Italy, and Switzerland it is possible to perform prenatal diagnosis and selective abortion of a fetus, but not possible to perform PGD on a cleavage-stage embryo. This argument has always been inconsistent (Gesetz, 1990). The same rules do not apply to the analysis of oocytes and so first polar body (and sometimes also second polar body) biopsy is permissible in most countries. However, once fertilization is complete, ethical considerations come into play.

One ongoing issue in PGD is what should we be allowed to diagnose, and there is always substantial disagreement on this point. In the UK all PGD applications have to be approved by the HFEA. When inquiries began about offering PGD for breast cancer, the HFEA set up a consultation document and offered its first license to perform PGD for breast cancer to our center in 2007. When the Consortium first started data-reporting, PGD cycles for social sexing were submitted and only after some very heated debates was it agreed that these data should be included in Consortium reports. Many key figures in PGD still do not feel these data should be reported. This issue is going to become even more difficult when arrays are used that can diagnose many disorders from a single cell, such as a single nucleotide polymorphism (SNP) array.

Another question is: who decides which embryos are replaced? As less-severe diseases are diagnosed a situation may arise where the couple decide to transfer an affected embryo. The majority of PGD clinics state in their consent forms that they will only transfer unaffected embryos. This has been raised repeatly with respect to mutations causing inherited deafness as already couples who want to actively select a child who will be deaf have come forward. Another scenario concerns the couple who come for IVF as they are infertile but one partner is also carrying a late-onset disorder. The couple therefore decide to check for the late-onset disorder being carried but after diagnosis it is discovered that all of the embryos are affected. What if they wish to transfer an affected embryo – not because this is their first choice but since no other embryo is available; the desire for a child is stronger than that to wipe out the disease from their family?

Future developments

The biopsy technique has remained relatively unchanged over the last 20 years. Ca^{2+}/Mg^{2+} -free medium and the laser for zona drilling have speeded up the procedure and made it more efficient, but the majority of clinics still use the same basic technique as was first reported (Handyside *et al.*, 1990). But in the future optical tweezers may be used for biopsy as has been applied to polar body biopsy (Clement-Sengewald *et al.*, 2002). The FISH technique is also the same basic technique that was applied in 1991 (Griffin *et al.*, 1991) except now the probes are directly labeled with the fluorochromes, more probes may be used in multiple rounds, and there have been improvements in embryo spreading (Harper *et al.*, 1994). However, PCR technology has changed since the first sexing cases. Multiplex fluorescent PCR (F-PCR) has enabled multiple sites to be analyzed so that the mutation and linked informative markers can be used for contamination detection, and the technique is much more sensitive. Various methods of whole-genome amplification have been developed (Renwick *et al.*, 2006), which enables more information to be gained from a single cell. But the future of PGD most probably lies in the development of array technology that will replace both PCR and FISH.

There are many types of array platforms and many methods of analyzing the arrays but a common method used is array CGH (a-CGH). Metaphase CGH has been applied to embryonic nuclei (Wells & Delhanty, 2000; Voullaire *et al.*, 2000; Wilton *et al.*, 2001), but the technical challenges of metaphase CGH have meant that it has not been routinely introduced into the PGD arena. Array CGH has been applied to single cells (Fiegler *et al.*, 2006; Le Caignec *et al.*, 2006) and very recently has been reported in PGD (Hellani *et al.*, 2008). The presence of copy number variants has meant that understanding the information obtained from the arrays is not that

Table 1.5 Aims of the ESHRE PGD Consortium

- To survey the availability of PGD
- To collect, prospectively and retrospectively, data on the accuracy, reliability, and effectiveness of PGD
- To initiate follow-up studies of pregnancies and children born
- To produce guidelines and recommended PGD protocols
- To formulate a consensus on the use of PGD

simple. Once a whole chromosome array becomes used in PGD it will be closely followed by arrays for the diagnosis of monogenic disorders, perhaps by haplotyping or the use of S typing. It may be that one array can test for common monogenic disorders and aneuploidy, which will greatly increase the information that we can obtain from a single cell. But with this will arrive the possibility of diagnosing multiple genes, which will lead to PGD for characteristics.

In many areas of biology the "lab on a chip" concept is developing. Microfluidic PCR chips are fast and use small reaction volumes, and they have been used to amplify multiple genes from a single bacterium (Ottesen *et al.*, 2006).

Noninvasive methods of PGD may be possible in the future. This could be performed by use of four-dimensional confocal fluorescence, which has been used to observe spindle and chromosome dynamics in live mouse embryos (Schuh & Ellenberg, 2007). Metabolic profiling of biomarkers may be performed by high-performance liquid chromatography (HPLC) to analyze amino acids, or near infra-red (NIR) spectroscopy to obtain a metabolic profile (Seli *et al.*, 2007; Vergouw *et al.*, 2008). This field has been termed "metabolomics." Preliminary studies using HPLC have shown that different profiles are obtained from chromosomally normal and abnormal human embryos, which might mean that noninvasive PGS is possible.

Another important advance in the field of PGD has been the introduction of vitrification for freezing biopsied embryos. Traditional slow freezing methods give poor survival of biopsied embryos, probably due to the zona breach. Vitrification is a rapid freezing method which works well whether the zona is present or absent, and so may easily be applied to biopsied embryos (Kuwayama *et al.*, 2005; Zheng *et al.*, 2005). Freezing biopsied embryos will open up the field to allow blastocyst biopsy and a number of diagnostic techniques that may take several days, such as metaphase or array CGH. Suitable embryos can be transferred in a future cycle.

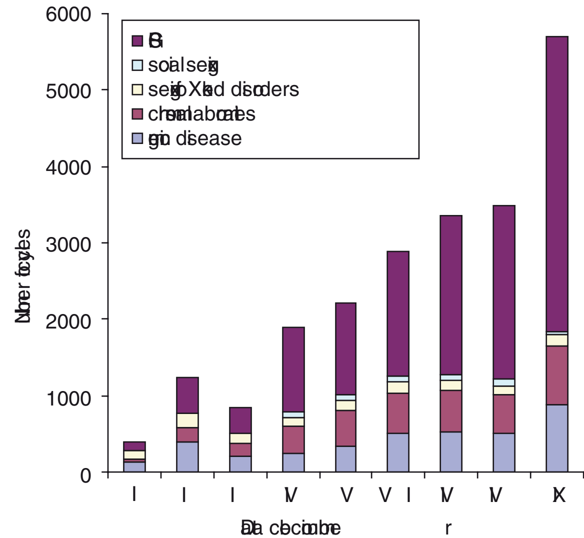

Figure 1.23 Evolution of PGD and PGS data over the first nine data collections.

ESHRE PGD Consortium

The ESHRE PGD Consortium is an international consortium dealing with all aspects of PGD. The aims of the Consortium are summarized in Table 1.5.

There are over 90 active centers that are members of the Consortium, from countries including the majority of European centers and members from Argentina, Australia, Brazil, Egypt, India, Israel, Japan, Korea, Saudi Arabia, Singapore, South Africa, Thailand, Taiwan, United Arab Emirates, and the USA.

Data collection

Over the last 11 years the main role of the Consortium has been collection of world data (ESHRE PGD Consortium Steering Committee, 1999, 2000, 2002; Sermon *et al.*, 2005; Harper *et al.*, 2006; Sermon *et al.*, 2007; Harper *et al.*, 2008a; Goosens *et al.*, 2008). Data VIII reported on a total of 15 885 cycles. These publications have shown the evolution of PGD over the last 10 years (Figure 1.23). The Consortium collects data on every cycle of PGD and reports data under the headings of PGD for chromosome abnormalities, sexing for X-linked disease, monogenic disorders (Table 1.6), PGS, and sexing for social reasons (Table 1.7). Details of the pregnancies and deliveries are also reported. One of the initial aims was to collect data on canceled cycles, but since most centers only reported on cycles that reached oocyte retrieval, there was gross underrepresentation

Table 1.6 Top 10 monogenic disorders reported to the Consortium

Recessive
Cystic fibrosis
β-thalassemia
Spinal muscular atrophy
Sickle-cell disease
Dominant
Myotonic dystrophy
Huntington's disease
Charcot–Marie–Tooth disease
Sex-linked (specific diagnosis)
Duchenne muscular dystrophy
Fragile X syndrome
Hemophilia

Table 1.7 Summary of data collections I–VII

	Cycles to OR	No. of embryos biopsied	No. of embryos transferred	Embryo transfer procedures	Pregnancy rate (per OR and per ET) (%)
Monogenics	2090	12903	3514	1640	20/26
Chromosomes	2192	14717	2733	1460	15/23
Sexing X-linked	816	5113	1164	613	19/26
Aneuploidy	6878	38796	9378	4971	18/25
Social sexing	413	2586	661	298	22/30

OR, oocyte retrieval; ET, embryo transfer
Source: Harper *et al., 2008a.*

of canceled cycles. Therefore later publications only reported on cycles that reached oocyte retrieval.

The Consortium has found that pregnancy rates between centers vary widely (Figure 1.24).

The initial data were collected on paper and transferred to the Excel computer program by the University College London PGD team. After a few years of this prehistoric method of working, centers were sent Excel spreadsheets to complete themselves. In 2002 Celine Moutou designed a sophisticated filemaker Pro database with separate files for patient details, cycles, pregnancies, and babies. There is now a database working group looking at ways of further improving data collection.

The Consortium has set up several working groups; misdiagnosis, accreditation, data collection, PGS, and guidelines. It also has a pediatric follow-up study and has set up external quality assessments for FISH and PCR (see below).

Misdiagnosis

In Goosens *et al.* (2008) there were 24 misdiagnoses; 12 each for FISH and PCR (Table 1.8(a) and (b)).

There are several ways that misdiagnoses may occur in PGD (Table 1.9) (Harper *et al.*, 2008a; Wilton *et al.*, 2009). General ways include unprotected sex, confusion of the embryo and cell number by the laboratory, transfer of the wrong embryo, paternal or maternal contamination (the former can be overcome by using ICSI and the second by removing all cumulus cells prior to the biopsy). The Consortium has published a detailed paper on misdiagnosis (Wilton *et al.*, 2009).

The only time that embryonic cells may be tested to determine the accuracy of a diagnosis is when a couple come through PGD and affected embryos are generated. It is considered good practice, therefore, to confirm the initial diagnosis in any untransferred embryos. The Consortium is conducting a survey of centers that follow up untransferred embryos.

Accreditation and external quality assessment

All PGD laboratories should be accredited and should follow ISO 15189. The Consortium is preparing a

Table 1.8(a) Misdiagnoses reported after FISH

Sexing for X-linked disease	PND-Postnatal	Outcome
45, XO, Hemophilia A	PND	TOP
46, XY Hemophilia A	Postnatal	Born
Translocations		
T13 after 45,XY,der(13;14)(q10;q10)	Mis	Mis
47,XX,+der(22)t(11;22)(q23.3;q11.2)	PND	TOP
46,XY,der(15)t(13;15)(q25.1;q26.3)pat	PND	TOP
PGS		
T16 after 1st PB biopsy only	Mis	Mis
T16 after 1st PB biopsy only	Mis	Mis
Trisomy 16	Mis	Mis
Trisomy 16	Mis	Mis
Trisomy 21	Post	Born
47,XXX	PND	Lost to follow-up
Social sexing		
Requested male but female fetus	PND	TOP

Source: Goossens *et al.*, 2008.

(b) Misdiagnoses reported after PCR

Monogenics	PND-Postnatal	Outcome
Myotonic dystrophy type 1	PND	TOP
SMA	Post	Born
ß-thalassemia	PND	TOP
ß-thalassemia	PND	TOP
Familial amyloid polyneuropathy	PND	Born
Cystic fibrosis	PND	Born
Cystic fibrosis (1 of twins)	Post	Born
CMT1A	PND	Born
CMT1A (twins)	PND	TOP of both twins
Fragile X	PND	Born
Sexing for X-linked disease		
46 XY in retinitis pigmentosa	PND	Born
46 XY in Duchenne muscular dystrophy twin	PND	TOP of one twin

PND, prenatal diagnosis; TOP, termination of pregnancy; Mis, miscarried.
Source: Goossens *et al.*, 2008.

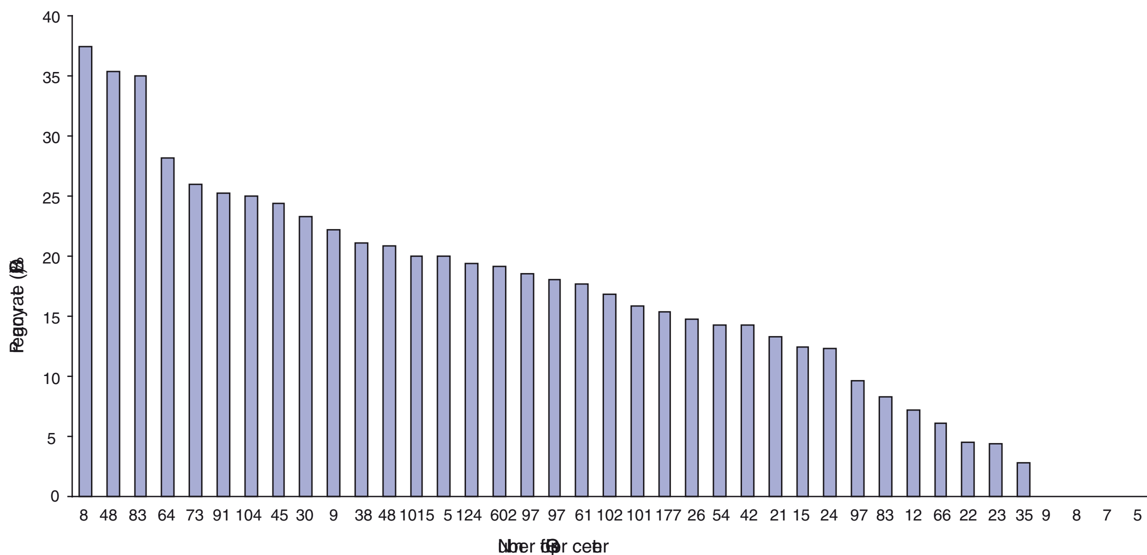

Figure 1.24 Pregnancy rates in relation to individual centers for data VIII. The average pregnancy rate was 20 per cent.
Source: Harper *et al.*, 2008a.

Table 1.9 Possible causes of misdiagnosis

Factor	Error type	PCR	FISH	Examples of preventative action
Unprotected sex	Human	√	√	Written instructions for patients to avoid unprotected sex during treatment Use of oral contraceptive pill
Mislabeled tube or slide	Human	√	√	Comprehensive, robust labeling system and SOP
Misidentified tube, slide, or embryo	Human	√	√	Appropriate witnessing procedures
Misinterpreted report	Human	√	√	Appropriate training, report, and counseling
Transfer of wrong embryo	Human	√	√	Appropriate training, report, witnessing, and counseling
Use of incorrect probes or primers	Human	√	√	Appropriate witnessing procedures
Haploid cells	Intrinsic (embryo)	√	√	Removal of second cell Use of informative linked STR/SNP markers
Chromosomal mosaicism	Intrinsic (embryo)	√	√	Removal of second cell Use of informative linked STR/SNP Test polar bodies Develop test to determine origin of aneuploidy (PCR only)
Probe or primer failure	Extrinsic (technical)	√	√	Adequate preclinical validation Use whole-genome amplification (WGA) to allow repeat sample testing (PCR only)
Maternal contamination	Extrinsic (technical)	√	√	Remove all cumulus cells prior to biopsy
Paternal contamination	Extrinsic (technical)	*		Use ICSI to introduce only a single sperm into the oocyte (PCR)
Operator contamination	Extrinsic (technical)	√		Wear appropriate protective clothing in a controlled environment
Carry-over contamination	Extrinsic (technical)	√		See above Use dedicated reagents and equipment in controlled environment
Allele dropout	Intrinsic (technical)	√		Remove more than one cell at biopsy Include up to three informative linked STR/SNP markers when performing analysis
Wrong segregation analysis	Human	√	√	Ensure validation is overseen by experienced or licensed molecular biologist (PCR) or cytogeneticist (FISH)
Uniparental disomy	Intrinsic (embryo)	√		Removal of more than one cell at biopsy Include up to three informative linked STR/SNP markers when performing PCR analysis

SOP, standard operating procedure.

beginner's guide to accreditating a PGD laboratory. In 2008 the Consortium set up external quality assessment systems for FISH and PCR. The FISH EQA is an online scheme developed by Ros Hastings at the UK National External Quality Assessment Scheme/Cytogenetics European Quality Assessment (UK NEQAS/CEQA). Participating centers have to assess online FISH images, make reports, and submit reports for previous cases they have carried out. The molecular EQA has been set up in collaboration with the UK NEQAS. In this scheme centers are sent DNA and single cells for particular disorders and they have to determine the genotype.

Pediatric follow-up

In 2007 the Consortium launched a retrospective pediatric follow-up scheme, organized by Alison Lashwood. This project involved 10 centers and follow-up of more than 1000 babies born after embryo biopsy and PGD.

Conclusion

This book covers all aspects of PGD written by experts in the field. As its editor I have mostly left the authors to express their personal opinions, which do not always agree with mine. PGD is a field where many people have

opposing views, such as in the use of PGD for social sexing, HLA typing, late-onset disorders, and PGS. The views of the authors are therefore not necessarily my own.

PGD has developed significantly over the last 20 years, and the next 20 years will be a very exciting time. We may see the development of noninvasive PGD and refinement of arrays to examine chromosomes and multiple genes. We may use expression arrays to determine the best IVF embryo. Maybe we will use PGD for all IVF cases so that we can select the most viable and genetically best embryo produced by that couple. Or we may be altering genes to enhance future generations.

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Chapter 2

Assisted reproductive technologies

Joyce C. Harper, Alpesh Doshi, and Paul Serhal

Key points

- In vitro fertilization (IVF) has been offered as a form of fertility treatment for more than 30 years. IVF is performed globally for the treatment of infertility and millions of babies have been born.
- Female infertility may be caused by blocked Fallopian tubes, endometriosis, abnormal uterine cavity, polycystic ovaries, and so on.
- Male infertility may be caused by poor sperm parameters attributed to obstructive, hormonal, genetic, or chromosomal abnormalities.
- Patients require preliminary investigations to determine the cause of their infertility. If they require assisted reproductive technology (ART) they will attend an ART clinic for a consultation to determine which treatment they require.
- Patients undergoing IVF or intracytoplasmic sperm injection (ICSI) require pretreatment tests, including an infection screen (chlamydia, HIV, and so on), assessment of uterine cavity (HyCoSy), dummy embryo transfer, semen assessment, genetic screen for severe male factor infertility, and assessment of ovarian reserve.
- Superovulation is achieved using a gonadotrophin releasing hormone (GnRH) agonist to downregulate the pituitary and then gonadotrophins (follicle stimulating hormone, FSH) to stimulate the ovaries. A single dose of human chorionic gonadotrophin (hCG) is given to mimic the luteinizing hormone (LH) surge. Oocytes are collected 36 hours later.
- Oocytes are cultured in the laboratory and mixed with a prepared sample of sperm for traditional IVF or a single sperm is injected into the oocyte (ICSI). Embryo development is monitored over the following days. Embryos can be transferred at the cleavage stage (days two to three) or blastocyst stage (days five to six).
- Cryopreservation of surplus embryos is possible at any stage of the treatment to allow embryos for future treatment.

Introduction

In vitro fertilization (IVF) was initially developed to treat patients with damaged Fallopian tubes (Steptoe & Edwards, 1978). IVF treatment has since been extended far beyond tubal infertility to treat a whole host of indications, including unexplained infertility, endometriosis, and male infertility. The introduction of intracytoplasmic sperm injection (ICSI) was a landmark in the treatment of male infertility (Palermo *et al.*, 1992). IVF is also a prerequisite for preimplantation genetic diagnosis (PGD). The average female patient age in most IVF clinics is mid- to late thirties, which reflects the trend to delay having a family until later in life. This causes added problems as fertility is greatly affected by the age of the woman, with fertility rapidly decreasing at around 36 years of age. The IVF laboratory is the hub of the IVF clinic where semen is prepared, oocytes collected, embryos identified and cultured, and oocytes, sperm, and embryos frozen. In the last 30 years many advances have been made in IVF which have resulted in a gradual increase in the delivery rates worldwide. As a result many clinics now opt for single embryo transfer.

Female infertility

Female infertility may be related to a number of causes; the most common include polycystic ovarian

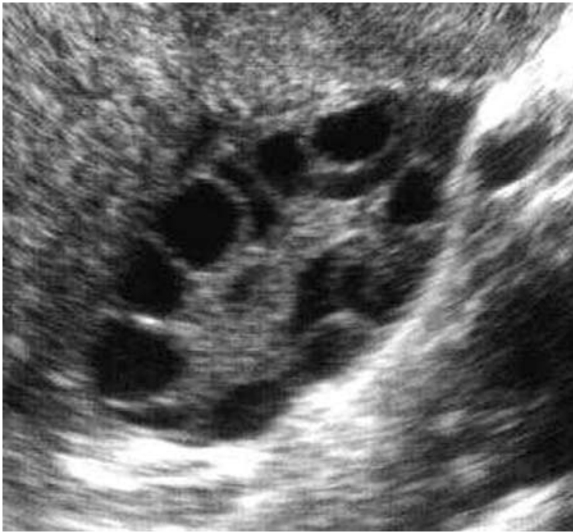

Figure 2.1 Polycystic ovaries.

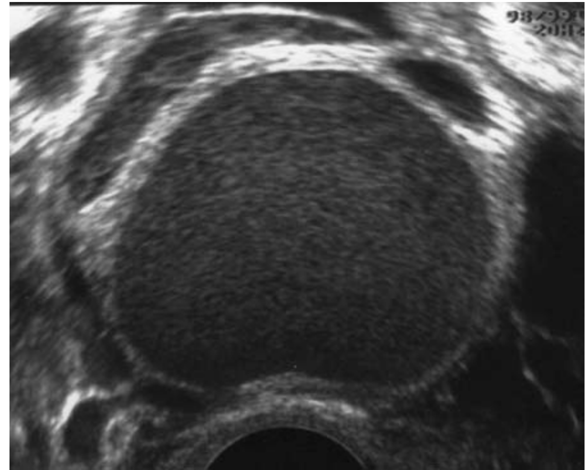

Figure 2.2 Ovarian endometrioma.

syndrome, unexplained infertility, endometriosis, and tubal disease.

Polycystic ovarian syndrome

Polycystic ovary syndrome is the most common cause of anovulatory infertility in women, and may affect up to 80 percent of women with anovulatory infertility (Hull, 1987) (Figure 2.1). Correction of anovulation using medicated ovulation induction regimens is a highly successful approach to establishing monofollicular ovulatory cycles and achieving pregnancy (Adams & Franks, 1985). However, a proportion of women will either fail to ovulate, achieve a multifollicular response, or not achieve pregnancy with this approach, and this group of women may be considered for IVF. Ovarian stimulation in women with polycystic ovaries is approached with caution owing to the propensity of the polycystic ovary to have an uncontrolled overresponse to stimulation.

Unexplained infertility

Unexplained infertility is a diagnosis of exclusion, which is reported to occur in up to 25 percent of couples. Couples with unexplained infertility have a cumulative three-year live-birth rate of 33.3 percent without treatment (Collins *et al.*,1995). However, if a couple have been attempting to conceive for over three years and are over 30 years of age, their chances of conceiving spontaneously in the next year is only 14.3 percent. Consequently, to maximize their chance of conception superovulation combined with intrauterine insemination (IUI) is performed (Serhal *et al.*, 1988). The aim of this treatment is to generate two follicles by ovarian stimulation, trigger ovulation by human chorionic gonadotrophin (hCG), and perform IUI 36 hours later with washed prepared sperm.

Endometriosis

Endometriosis is caused when endometrial tissues appear in extrauterine sites, mainly in the pelvis (Figure 2.2). The causes of endometriosis have been a matter of continuous debate. Early endometrial implants typically occur on the surface of the pelvic organs. Endometriosis may trigger localized inflammatory responses leading to scar formation and adhesions. Active endometriotic lesions result in invasive nodules or surface plaques. Deep implants in the ovaries result in the formation of endometriomas. In patients with severe disease, the endometriosis distorts the pelvic anatomy and causes infertility.

There is evidence that in couples with otherwise unexplained infertility, the fecundity of women with minimal and mild endometriosis is improved by laparoscopic ablation of endometriosis (Marcoux *et al.*,1997). IVF is an effective treatment for patients with severe pelvic endometriosis. Before embarking on IVF, any endometriomas should be drained by laparoscopy and the cyst wall should be stripped or ablated (Donnez *et al.*, 2001). The ovarian response to gonadotropin stimulation, fertilization, and embryonic development is normal except in severe cases. In women

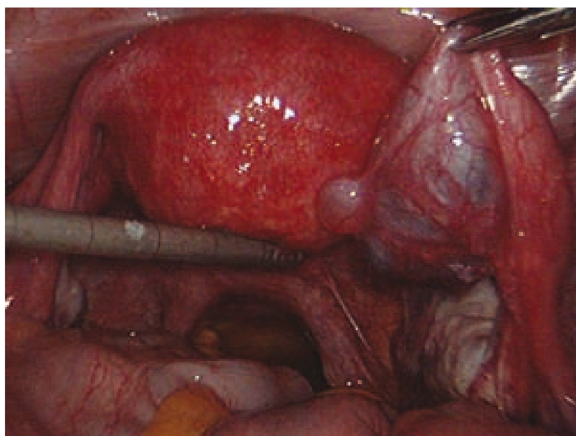

Figure 2.3 Laparoscopy and chromopertubation (lap and dye).

with Grade VI endometriosis the implantation rate is reduced (Oehninger *et al.*, 1989).

Tubal disease

Blocked tubes may be caused by chlamydia and other pelvic infections and also by endometriosis. To determine whether the Fallopian tubes are blocked hysterosalpingography and/or laparoscopy and dye are undertaken (Figure 2.3).

Surgery may be performed to repair a damaged Fallopian tube. However, after such surgery, the chances of a normal pregnancy are small and there is an increased risk of ectopic pregnancy. Consequently, surgery is not often recommended. IVF is recommended for most couples.

Premature ovarian failure

It is estimated that approximately 1 percent of women experience premature menopause, in which case the only option for them would be oocyte donation. Patients with Fragile X syndrome are at risk of developing early menopause. They have a 10-fold increase in the incidence of premature ovarian failure (Conway *et al.*, 1998). Consequently, patients with Fragile X syndrome who are considering PGD must undergo an assessment of their ovarian reserve (see below) and appropriate counseling. Some couples may opt for oocyte donation.

Male infertility

Male infertility may be caused by poor sperm parameters attributed to obstructive, hormonal, genetic, or chromosomal abnormalities.

Azoospermia is complete absence of sperm in the ejaculate. Azoospermia may be divided into *obstructive* (ductal) or *nonobstructive* (secretory) azoospermia. Up to 70 percent of men with congenital bilateral absence of the vas deferens (CBAVD) may be carriers of the cystic fibrosis gene. Nonobstructive azoospermia may be caused by testicular failure, such as Sertoli cell-only syndrome or Klinefelter syndrome (XXY).

A hormone evaluation should be performed to measure follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, and testosterone. A karyotype should be performed to ensure that the male is not carrying a translocation or other chromosome abnormality. Patients with CBAVD should undergo a cystic fibrosis screen and those with nonobstructive azoospermia should have a Y gene microdeletion analysis. If azoospermia is present the sperm will need to be aspirated either from the epididymis or the testes.

Percutaneous epididymal sperm aspiration (PESA) involves sperm being retrieved directly from the epididymis using a needle. Testicular sperm aspiration (TESA) involves sperm being retrieved directly from the testes using a needle, and testicular sperm extraction (TESE) involves sperm being retrieved from a biopsy of testicular tissue. Severe male factor infertility is treated by ICSI.

Infertility treatments

Infertility treatments include timed intercourse, ovulation induction, IUI, gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), IVF, and ICSI.

Timed intercourse involves follicle-tracking or ovarian stimulation, or both, to ensure the couple have intercourse at the right time. Ovulation kits may be used to measure the LH surge and so give the couple a better idea of when ovulation is occurring.

Ovulation induction is the controlled administration of fertility drugs that stimulate the ovary to produce mature eggs. This is usually reserved for women who have very irregular menstrual cycles and in whom investigations have shown that they do not ovulate on a regular basis. Ultrasound scans and blood tests are undertaken to ensure that the treatment is effective and that a mature follicle is produced so that intercourse may be timed optimally to achieve a pregnancy. If the patient is not scanned and has treatment, this could result in a multiple pregnancy. Follicular stimulation is usually achieved with clomiphene

citrate (an anti-estrogen that works by stimulating the pituitary gland to release more FSH), which is normally given in a short course at the beginning of the menstrual cycle for five days. Under normal circumstances women ovulate naturally in response to clomiphene citrate. Occasionally an added injection of hCG is given when the follicle is mature to ensure that it is released. Injections of FSH may also be used, which can be given on a daily basis (usually in a low dose) to try and stimulate the development of a single mature follicle, which is then primed to release the egg by use of an hCG injection when the follicle has reached an appropriate size.

For IUI, GIFT, and ZIFT the patient needs patent Fallopian tubes. In IUI the patient may be given mild ovarian stimulation, and development of follicles can be tracked by ultrasound. At the time of ovulation a semen sample is prepared (see below) and injected into the uterine cavity. Care has to be taken to ensure that there are not multiple follicles, which could lead to a multiple pregnancy. GIFT involves a stronger amount of stimulation so that multiple follicles are produced. Oocytes are collected by laparoscopic methods and immediately mixed with a prepared semen sample. One or two oocytes or sperm may be transferred directly into the Fallopian tube and the remaining oocytes are retained for in-vitro culture.

ZIFT is now rarely used. The eggs are collected and fertilized in the laboratory and at the zygote stage they are transferred to the Fallopian tubes. A general anesthetic is required for zygote transfer.

For IVF, patients are superovulated (see below) and oocytes are usually collected by vaginal ultrasound-guided aspiration under light sedation. All the oocytes are cultured in the laboratory and mixed with a prepared semen sample. Embryo development is monitored over several days and the transfer of one or two embryos may be done on days two, three, five, or six. ICSI is identical to IVF except that, instead of sperm being mixed with the oocytes, a single sperm is isolated and injected directly into the egg.

Surplus embryos from GIFT, IVF, and ICSI may be cryopreserved for future treatment.

Organization of IVF treatment

A successful IVF program will depend on carefully coordinated teamwork involving clinicians, embryologists, and nurses. For PGD to be successful it needs to be undertaken in a successful assisted reproductive technology (ART) clinic.

The infertility consultation

All patients are seen for a full consultation to assess their suitability for treatment. During this consultation the following occur: gynecologic and medical history is taken and ART treatments are explained as are the realistic chances of success and risks of the treatment. The patient's previous investigations are also reviewed. Patients should be given details of their options and their chances of success. Information sheets and consent forms should be given to them. In the UK, all IVF patients have to be offered counseling, especially if they are using donor gametes.

A general and pelvic examination is made, including chlamydia screening and updating of cervical cytology. The body mass index (BMI) of the woman should be calculated (weight divided by the square of the height) and weight loss advised for any woman with a BMI >30. Women are advised to stop cigarette smoking and reduce alcohol intake, not only in pregnancy but also because they reduce fertility. Folate supplementation is recommended before conception and during the first 12 weeks of a pregnancy (400 μg folic acid per day or if there is a history of neural tube defect, or the woman is taking medication for epilepsy, 4 mg daily). Her BMI, sickle cell, β thalassemia, Tay–Sachs, HIV, Hepatitis B, and Hepatitis C status are checked. Rubella immunity should be checked and immunization offered if the woman is susceptible. The male partner should be asked to produce a semen sample for analysis.

IVF clinicians are used to dealing with infertile couples who have experience of fertility tests and treatment. PGD patients often have little background knowledge of IVF, which is a prerequisite for PGD, and it is important that treatment is explained from basic principles and backed up by written information. Careful explanation should include a realistic assessment of success. Side effects and potential risks of treatment are discussed and consent forms are issued with sufficient time to allow patients to make an informed decision whether to proceed or to have a natural conception with prenatal diagnosis. Patients may feel that the stress of undergoing IVF is more than the psychological stress of undergoing a natural conception and prenatal diagnosis.

PGD patients will require an additional consultation with the PGD specialist (see Chapter 8).

For PGD it is important to obtain about 10 oocytes and good-quality embryos so that after biopsy and diagnosis, at least two good-quality embryos, free

Table 2.1 Summary of ovarian reserve tests

Type of test	What is measured	Reduced ovarian reserve
Static tests		
Estradiol	Day three estradiol	>80 pg/ml
FSH	Day three FSH	>10–15 IU/l
Measurement of follicles	Antral follicle count	Low number of antral follicles
Inhibin B	Day 5 inhibin	<45 pg/ml
AMH	Day 3 AMH	<7 pm/L
Dynamic tests		
Clomiphene citrate challenge	Days five to nine FSH	20 IU/l rise in FSH
GnRH agonist stimulation	Estradiol and FSH	Estradiol <180 pmol/L FSH >9.5 IU/l

FSH, follicle stimulating hormone.

from disease, are available for transfer. For all indications, there are cycles which result in all embryos being abnormal (Harper *et al.*, 2008). A Brussels group reported that it only admits couples to the PGD program if at least seven good-sized follicles are present (Vandervorst *et al.*, 1998).

Assessing ovarian reserve

Ovarian reserve assessment refers to a group of tests that aim to predict the response of an individual patient to gonadotrophin stimulation (Table 2.1). The assessment of ovarian reserve not only aims to identify those patients who are likely to respond well to gonadotrophin stimulation, and therefore have a low chance of cycle cancelation, but, more importantly, aims to identify those women in whom response is likely to be suboptimal, with a higher chance of cycle cancelation due to poor response. The latter group of women is likely to benefit from a modified stimulation protocol that takes into account the reduction of available primordial follicles and, additionally, identifies a further subgroup of women who are unlikely to respond to gonadotrophin stimulation and facilitates counseling at an early stage for alternatives such as ovum donation.

Advancing maternal age is associated with a reduction in fertility potential, and beyond the age of 36 this rate of reduction accelerates (Magarelli *et al.*, 1996). Several studies have been done to assess age-only as an ovarian reserve test, and although overall there is concordance that older women are less likely to succeed with IVF, the cut-off age used varies from 35 to 40 years. This reflects the biological variation in decline in fertility between individuals and thus is of poor specificity; therefore, age alone cannot be used but should be a consideration when assessing patients (Bukman & Heineman, 2001).

The assays of serum estradiol and FSH in the early follicular phase of the menstrual cycle have been the mainstay of ovarian reserve testing for over a decade. These tests rely on the fact that a diminishing number of follicles in the ovaries is followed by a rise in FSH, as the ovary requires more pituitary stimulation to recruit follicles. Similarly, women with reduced ovarian reserve have a shortened follicular phase with more advanced follicular recruitment and a higher basal estradiol. The test is simple and inexpensive, and is, overall, considered to be reliable (Toner *et al.*, 1991; Muasher *et al.*, 1988). However, the cut-off above which FSH may be considered to be predictive of poor outcome is highly variable between studies, although overall a value above 10–15 IU/l is generally used. Above this level there is a significant reduction in the number of mature oocytes retrieved and in ongoing pregnancy rates (Scott *et al.*, 1989; Sharif *et al.*, 1998; Bancsi *et al.*, 2000). Similarly, the definition of elevated serum estradiol level varies between studies. Smotrich *et al.*(1995) and Licciardi *et al.* (1995) have shown that above an estradiol level of 80 pg/ml and 60 pg/ml on day three of the cycle the chances of pregnancy are dramatically reduced. In contrast, Scott *et al.* (1989) and Lee *et al.* (1988) have shown that, in normally menstruating women, serum estradiol has no prognostic value. In a systematic review of ovarian reserve tests that predict the outcome of IVF treatment, Broekmans *et al.* (2006) assessed a total of 37 studies which investigated the predictive performance of basal FSH and 10 studies investigating basal estradiol. However, this meta-analysis was significantly limited by highly

heterogeneous study populations, study design, cut-off point for definition of poor FSH and elevated estradiol, and end-point of study, and, as a result, a single-point predictive sensitivity and specificity could not be calculated. However, the present authors do conclude that from the data available, unless grossly abnormal, neither basal estradiol nor basal FSH is predictive of response to stimulation and chances of pregnancy, but both serve as a screening step for further tests.

The increased resolution and availability of transvaginal ultrasound for the assessment of women considering IVF has enabled assessment of ovarian reserve by use of nonbiochemical markers. Notably, a cohort of follicles measuring between 2 mm and 5 mm is available at the beginning of each menstrual cycle, is clearly visible, and is considered to be reflective of ovarian reserve (Scheffer *et al.*, 1999; Kline *et al.*, 2005). Ultrasound studies have shown that antral follicle counts decline with advancing age, and a decline in antral follicle count may precede a detectable decline in serum markers for ovarian reserve. Additionally, antral follicle count has been shown to be a better predictor of ovarian response, number of oocytes retrieved, and the incidence of ovarian hyperstimulation syndrome (Chang *et al.*, 1998; Fratarelli *et al.*, 2000; Ng *et al.*, 2000). Hendriks and colleagues (2005) performed a meta-analysis of 11 studies using antral follical count (AFC) and 32 studies using basal FSH in the assessment of ovarian reserve and have confirmed that women with suboptimal AFC respond less well to ovarian stimulation and have lower rates of ovarian hyperstimulation. However, there was no correlation with chances of pregnancy. The finding that basal FSH was less effective as an ovarian reserve test was not entirely surprising as basal FSH levels are under the influence of a host of factors and are not therefore a direct assessment of ovarian reserve. In contrast, AFC reflects the available pool of follicles and is therefore a more direct, biophysical measure. The inability of either basal FSH or AFC to predict pregnancy is, again, not entirely surprising as these tests are a quantitative rather than qualitative reflection of ovarian reserve. Whether ovarian reserve testing may be employed for pregnancy outcome has not been assessed and requires further research.

Dynamic ovarian reserve tests provide additional information in women with normal or borderline abnormal static ovarian reserve tests. These tests evaluate the change in absolute levels of either FSH or estradiol in response to an exogenous stimulus. The clomiphene citrate challenge test (CCT) is the most basic of these and involves administering 100 mg of clomiphene citrate from cycle days five to nine. If there is a 20 IU/l rise in FSH, this is considered to reflect poor ovarian reserve (Scott *et al.*, 1993; Fahri *et al.*, 1997). An abnormal clomiphene citrate test (CCT) is seen in older fertile women who have lower spontaneous conception rates (Scott *et al.*, 1993). In the context of IVF, a suboptimal CCT is predictive of poor response to ovarian stimulation, cycle cancelation, and chances of pregnancy (Tanbo *et al.*, 1992; Scott *et al.*, 1993; Scott & Hoffman, 1995). Gülekli *et al.* (1999) have additionally shown that follicle count in ovarian biopsy specimens has a significant correlation with CCT. A further refinement of dynamic tests is the GnRH agonist stimulation test. Administration of a gonadotropin releasing hormone (GnRH) agonist on cycle days two and three induces an initial surge of FSH, LH, and estradiol. The estradiol response to stimulation reflects the functional integrity of ovarian follicles and a low E2 response may be regarded as a consequence of dwindling cohorts of secretory follicles. Ranieri *et al.* (1998) have shown that the assessment of both FSH and estradiol in response to GnRH agonist administration has the best predictive value of all ovarian reserve tests, and, using receiver operating characteristic (ROC) curves, they determined that an FSH <9.5 IU/l and estradiol rise of >180 pmol/l were most likely to be associated with a good response to gonadotropin stimulation. In a subsequent study, Ranieri *et al.* (2001) used these FSH and estradiol levels in response to GnRH agonist stimulation and found that in women with poor ovarian reserve stopping GnRH administration at the time of commencing gonadotropins resulted in an ovarian response and pregnancy rates comparable with those in women with normal ovarian reserve.

More recently, two novel hormones, anti-Müllerian hormone (AMH) and Inhibin B, have been investigated as potential markers of ovarian reserve. Both are produced by the granulosa cells of the ovarian follicles. AMH production from the granulosa cells is not gonadotropin-dependent, whereas inhibin B production is.

Inhibin B levels rise progressively through the menstrual cycle and peak at days five to six, and the level then falls gradually until ovulation, when there is a sharp rise again, followed by a steep decline to undetectable levels in the luteal phase. The rise of inhibin B in the early follicular phase indicates that small antral follicles secrete it in response to rising endogenous FSH and then, as a dominant follicle emerges, the

levels fall as those small antral follicles not recruited undergo atresia. Static tests of inhibin B show a great deal of variability and depend on which day in the early follicular phase the assay is performed. AMH is produced by small, growing pre-antral follicles and is not dependent on FSH, and so, therefore, does not display variation through the menstrual cycle. Treatment of IVF patients with a single, high dose of GnRH agonist results in a rise of endogenous FSH, estradiol, and Inhibin B, but does not affect AMH serum levels (van Rooij *et al.*, 2002). Similarly, in conditions where FSH levels are suppressed, such as pregnancy, AMH levels remain constant (La Marca *et al.*, 2005). Thus, AMH is not influenced by the gonadotropic status and reflects only the follicle population. Several studies have shown that AMH is a good marker for ovarian responsiveness to IVF protocols, and levels in the early follicular phase are lower in women who are likely to respond less well (Seifer *et al.*, 2002; van Rooij *et al.*, 2002).

There are several potentially useful markers of ovarian reserve; however, as data accumulate on their use it becomes clear that each marker is specific for a certain aspect of reproductive performance. Muttukrishna *et al.* (2005) investigated the relationship between AMH, Δinhibin B, Δ estradiol, and AFC with ovarian response in women having dynamic ovarian reserve testing with 300 IU/l exogenous FSH. No single test had a sensitivity <87 percent for prediction of poor response; however, the specificity varied considerably, with AFC having the lowest, 39 percent, and AMH the highest, at 64 percent. Basal FSH on day three, Δinhibin and AMH all had a significant correlation with the number of eggs collected, with inhibin B being the best predictor. Interestingly, the study confirmed previous findings of AFC association with number of eggs collected, but also showed that AFC was significantly associated with chances of a clinical pregnancy. However, combining all these significant parameters into a single cumulative score gave the best overall predictive ability for poor response, with 87 percent sensitivity and 80 percent specificity.

Optimizing response to ovarian stimulation protocols

Standard stimulation protocols require pituitary desensitization with a GnRH agonist, and this strategy has become almost universal practise in assisted conception clinics with the induced hypogonadotropic hypogonadism enabling almost complete control over follicular development. The standard long protocol starts on day 21 of the preceding cycle with the administration of GnRH agonist. When the period starts, a pelvic scan is performed to measure endometrial thickness, which should be <5 mm. Gonadotropins are started daily from days three or four, usually at a dose of 225 IU (Figure 2.4). Scans and E2 are done on days 7, 9, and 11. If two or three leading follicles are 18 mm in diameter or more, hCG (Profasi®), 10 000 IU is given. The oocytes are retrieved 36–37 hours post-hCG and progesterone 400 mg twice a day (Cyclogest®) is given from the day of the oocyte collection.

The standard "long" protocol with pituitary downregulation started in the preceding cycle has been credited with a higher pregnancy rate, greater numbers of oocytes collected, and lower cycle cancelation rates (Hugues *et al.*, 1991). However, it also has a higher requirement for exogenous gonadotropin and higher incidence of ovarian hyperstimulation syndrome (Hugues *et al.*, 1991).

Identification of women who are likely to respond poorly to ovarian stimulation leads to a further dilemma of what, if anything, may be done to improve response. In women who already have poor ovarian reserve, pituitary downregulation will not only predictably increase the requirement for gonadotropins but also results in significantly fewer numbers of oocytes collected (Hazout *et al.*, 1993).

The logical treatment of the poor responder would be to increase the dose of gonadotropin and thereby the intensity of the ovarian stimulation so that the greatest numbers of follicles are recruited. Several authors have reported on this strategy; however, when giving high doses throughout (Karande *et al.*, 1990) or increasing the dose sequentially (Van Hooff *et al.*, 1993; Cedrin-Durnerin *et al.*, 2000) there does not seem to be an improvement in either numbers of oocytes collected or pregnancy rates, but there is a higher cycle cancelation rate for poor response. In contrast, studies using a high initial dose of FSH (450 IU/day) do report a significantly higher number of oocytes retrieved and better pregnancy rates (Crosignani *et al.*, 1989; Hofmann *et al.*, 1989). This is in keeping with follicular physiology as follicle recruitment occurs in the very late luteal and early follicular phases, beyond which time the window for gonadotropin-mediated follicle recruitment has passed.

In an attempt to reduce the negative effects of GnRH agonist-induced ovarian suppression, several alternative protocols have been developed which involve

Figure 2.4 Gonadotropin releasing hormone (GnRH) agonist and gonadotropins.

giving the GnRH agonist in the early follicular phase along with the gonadotropin – the "flare" protocols. This then would not be hampered by excessive ovarian suppression and, additionally, the endogenous surge – or flare – of gonadotropins induced by the agonist would enhance the exogenous gonadotropin. A further modification proposed involves administration of several smaller or "micro" doses to achieve some control over ovarian function, but reduce the negative effects of full suppression. This micro-dose could be given repeatedly through the follicular phase to achieve suppression of LH, androgen, and progesterone secretion seen in standard flare-up protocols (Scott *et al.*, 1993; Deaton *et al.*, 1996). Overall, there is a consensus in the published literature that the standard flare protocols do achieve a lower cycle cancelation rate, a better yield of oocytes, and improved pregnancy rates (Howles *et al.*, 1987; Padilla *et al.*, 1996; Toth *et al.*, 1996). The results from micro-dose flare-up protocols are less consistent. Although initial results seemed to suggest an improvement, more recent data indicate that there is a higher cycle cancelation rate, a lower yield of oocytes, and lower pregnancy rates (Schoolcraft *et al.*, 1997; Leondires *et al.*, 1999). None of the variations of the flare-up protocol have been assessed in a randomized controlled trial.

An alternative approach to reducing ovarian suppression is to initiate the GnRH agonist in the mid-luteal phase of the preceding cycle and stop once menses has commenced. This strategy has been assessed in two randomized controlled trials, neither of which have demonstrated an improvement in pregnancy rates. Dirnfeld *et al.* (1999) initiated GnRH agonist on day 21 of the preceding cycle and stopped on the day of biochemically confirmed ovarian suppression (estradiol <140 pmol/l). The maximum dose of gondotropin was 375 IU/day and no increase in oocyte yield or pregnancy rate was shown. In contrast, the randomized controlled study by Garcia-Velasco *et al.* (2000), which used a higher maximum dose of gonadotropin (450 IU/day), did show a significant improvement in oocyte yield but no statistically significant improvement in either cycle cancelation or pregnancy; however, the trend for the latter two parameters was toward benefit.

Several other nonrandomized trials have shown a significant improvement in response to stimulation, pregnancy rates, and cancelation rates. Consistent with these trials is that the maximum dose of gonadotropin used is higher, indicating the increased level of stimulation required for women with poor reserve despite minimal suppression (Faber *et al.*, 1998; Karande & Gleicher, 1999; Schachter *et al.*, 2001). Ranieri *et al.* (2001) reported the response of women with poor ovarian reserve, determined by a combination of both static and dynamic tests, and found that cycle cancelation rates were lower, pregnancy rates higher, and oocyte yield higher; of particular interest with this study is that the same group previously reported a

similar strategy but in this study they used a higher starting dose of gonadotropins. In their previous study the cancelation rate was 27 percent, as opposed to 6 percent in the follow-up study. Additionally, there were no cancelations attributed to premature LH surge, in keeping with previous data (Ranieri *et al.*, 1998; Karande & Gleicher, 1999).

Finally, complete avoidance of ovarian suppression by the use of GnRH antagonists has been proposed. The obvious advantage would be the simplicity and ability to control ovarian response with no suppression and no risk of premature LH surge. Craft *et al.* (1999) used gonadotropins combined with clomiphene citrate and started GnRH antagonist on day six of stimulation. In comparison with previous cycles in the same group of women, there were fewer cycle cancelations due to poor response, and improved oocyte yield and pregnancy rates. Similarly, Akman *et al.* (2000) reported improvement in these parameters; however, in both studies, although there was a clear and consistent trend toward improvement, statistical significance was not reached. The main drawback of GnRH antagonist regimens is the cost, and improvements in response (when compared to GnRH agonist flare protocols) do not show any differences in cycle cancelation or pregnancy rates but do show a poorer yield of oocytes in the antagonist group (Akman *et al.*, 2001).

Treatment of coexisting gynecologic pathology

Hydrosalpinges

Tubal factor infertility remains a major indication for IVF. Studies have consistently shown that the presence of a hydrosalpinx will halve pregnancy rates, reduce live birth rates, and increase risk of spontaneous miscarriage (Camus *et al.*, 1999) (Figure 2.5). The mechanism of reduction of pregnancy rates remains unclear; however, several theories have been proposed and include a direct impact of tubal fluid on endometrial receptivity, a mechanical flushing effect, and a direct toxic effect on the embryo. Although the mechanism remains uncertain there are consistent data suggesting that the total amount of hydrosalpinx fluid present correlates with the reduction in chances of achieving a pregnancy. Therefore, in women with ultrasound-visible hydrosalpinges and women with bilateral hydrosalpinges, pregnancy rates are even lower.

Surgical removal of hydrosalpinx has been shown to significantly improve outcomes with IVF. In a prospective randomized controlled trial of salpingectomy prior to IVF, Strandell *et al.* (1999) randomized 204 women with hydrosalpinges to laparoscopic salpingectomy or no treatment prior to IVF. Clinical pregnancy rates were 36.6 percent in women who had salpingectomy compared with 24 percent in those who did not. This difference was not statistically significant but there was a significant difference in the live birth rates: 28.6 percent versus 16.3 percent. A subgroup analysis revealed significant improvement in implantation rates, clinical pregnancy rates, and delivery rates in women who had ultrasound-visible hydrosalpinges prior to treatment.

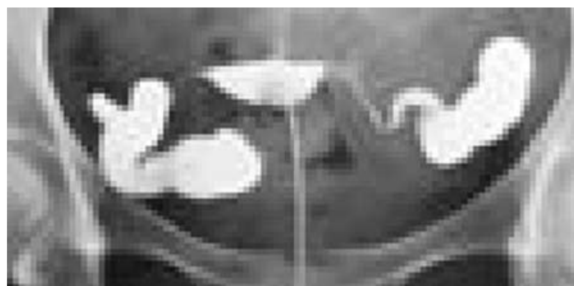

Figure 2.5 Hysterosalpingogram showing bilateral hydrosalpinges.

Although there remains little doubt that salpingectomy improves the outcome of IVF, the procedure itself remains controversial. This is primarily based on the effect of salpingectomy on ovarian function. In the study by Strandell *et al.* (1999), women who had had salpingectomy had an almost fivefold increased risk of cycle cancelation owing to failed stimulation or poor-quality embryos. It is unclear if it is the salpingectomy or initial pelvic inflammation that adversely affects ovarian function.

Evaluation of the endometrial cavity

The presence of endometrial polyps, submucous fibroids, and intrauterine adhesions may be associated with a reduction in pregnancy rates and an increase in risk of miscarriage with IVF. Advances in stimulation protocols and embryo culture conditions have seen a concomitant improvement in embryo quality. With good-quality embryos, the focus should be on the endometrium and the essential role this plays in determining success (Edwards, 2006). Therefore, an attempt should be made to exclude the presence of endometrial pathology which may adversely affect pregnancy rates.

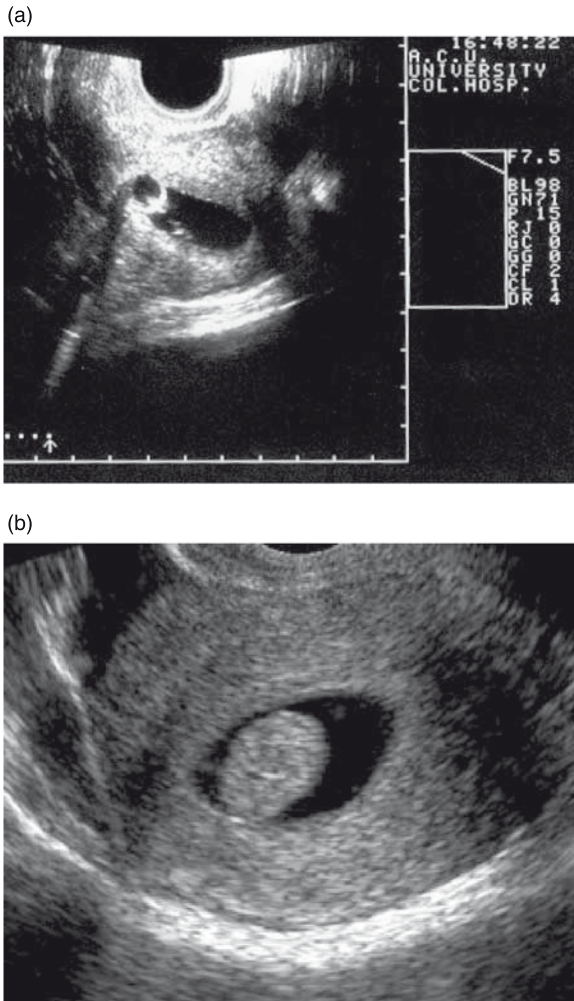

Figure 2.6 (a) Hysterosalpingo contrast sonography (HyCoSy) showing a normal uterine cavity; (b) HyCoSy showing an intrauterine polyp.

The increasing use of high-resolution transvaginal ultrasound has enabled the outpatient assessment of endometrial cavity lesions with increasing confidence. However, the sensitivity and specificity of unenhanced ultrasonography are limited and provide a high number of false-positive diagnoses; in order to effectively triage women who require surgical correction of intrauterine pathology instillation of saline into the uterine cavity to act as a contrast agent has been shown to be a highly effective method for the detection of pathology. Saline infusion sonohysterography (SIS) has been shown to be as accurate as diagnostic hysteroscopy in the detection of intracavitary pathology in a large meta-analysis of 2278 procedures with a sensitivity and specificity of 95 percent and 88 percent, respectively (de Kroon & Jansen, 2006) (Figure 2.6). Endometrial polyps are the most commonly found focal pathology in infertile women and there is now increasing evidence that removal of these will improve outcome of IVF. Demirol and Gurgan (2004) have shown, in a randomized controlled study of 421 women, that removal of endometrial polyps will significantly improve the pregnancy rate compared with that found in the control group. Similarly, Perez-Medina (2005) showed that polypectomy prior to IUI significantly improved pregnancy rates.

Difficult embryo transfer

The apparent simplicity of embryo transfer has meant that this critical step in determining the success of IVF treatment is often overlooked. However, clinical experience and several studies have confirmed that a gentle and atraumatic transfer is associated with improved pregnancy rates, irrespective of embryo quality (Goudas *et al.*, 1998; Karande *et al.*, 1999). The use of ultrasound for correct placement, and maintaining a full bladder to correct any cervical angulation, have been shown to be of benefit. However, the difficulty of embryo transfer is primarily related to difficulty in negotiating the relatively stenosed or acutely angulated cervix (Egbase *et al.*, 2000). Most women undergoing embryo transfer are likely to be nulliparous and have an anteverted uterus, and therefore are likely to have a cervical canal that is less accommodating to instrumentation. However, not all nulliparous women and not all multiparous women will have difficult or easy transfers, respectively. In practice, the only way to predict the difficulty of a transfer is by performing a mock transfer.

Mock transfer may be done at any time in the cycle preceding the treatment cycle; however, the benefit of any screening test is the ability to take steps for correcting a problem when identified. Mansour *et al.* (1990) randomly selected 335 women to have a mock transfer at the start of IVF treatment to enable choice of “most-appropriate transfer catheter” or “no mock transfer.” They report that up to 30 percent of women in the “no mock transfer” cycle group had a difficult transfer, whereas none had a difficult transfer in the “most-appropriate transfer catheter” group. Additionally, the pregnancy rate was higher in the “most-appropriate transfer catheter” group (23 percent versus 13 percent). Although this approach seems to be promising in improving pregnancy rates, transfers deemed to be difficult are more likely to be done

Figure 2.7 From left: trial catheter, flexible embryo transfer catheter, Hard Wallace.

with a rigid catheter and, in contrast to the more flexible soft catheters, these have been shown to achieve a lower pregnancy rate (Mansour *et al.*, 1990) (Figure 2.7). Thus, several authors have reported the use of surgical dilatation prior to embryo transfer to facilitate easy transfer with a soft catheter. However, it is clear that if dilatation is done too close to the actual embryo transfer then this adversely affects the pregnancy rate and may indicate that the procedure of surgical dilatation does damage the endometrium for that cycle (Visser *et al.*, 1993; Groutz *et al.*, 1997). Abusheika *et al.* (1999) have shown that cervical dilatation done under general anesthetic prior to gonadotropin stimulation not only made transfer easier but also improved pregnancy rates: in their study they included 57 women who had previously failed IVF treatment and had a difficult embryo transfer procedure. Seventy percent of transfers after dilatation were classified as easy and the pregnancy rate was 36 percent. Additionally, in women in whom the transfer remained difficult after dilatation the pregnancy rate was four times lower than in those who subsequently had an easy transfer. A simplified approach was proposed by Serhal *et al.* (2003), who used hygroscopic dilators (Dilapan-S™) to overcome cervical stenosis in women with previous difficult embryo transfers and subsequent failed IVF treatment. The hygroscopic rod was inserted prior to gonadotropin stimulation and left *in situ* for four hours. Following cervical dilatation, 80 percent of women subsequently had an easy transfer and, overall, achieved a pregnancy rate of 55 percent. The two studies are comparable with respect to inclusion criteria and patient outcomes, and suggest that hygroscopic dilatation achieves a greater improvement in ease of transfer and better pregnancy rates.

Prevention of ovarian hyperstimulation syndrome

It is conventional practice to induce ovulation using urinary-derived hCG, as this has an LH-like effect. However, hCG has a longer half-life, a higher receptor affinity, and a longer duration of intracellular activity than LH, and, as a consequence, the effects of hCG last for up to six days (Casper, 1996). Additionally, hCG has been shown to have FSH-like effects and contributes to ovarian stimulation (Gerris *et al.*, 1995). As a result, hCG administration has been recognized as a triggering factor for ovarian hyperstimulation syndrome and thus a reduction in the dose or withholding of hCG has been suggested as a strategy to reduce the risk of this occurring (Whelan & Vlahos, 2000). It should be clear from the outset that the development of ovarian hyperstimulation syndrome requires a hCG trigger and without this the condition does not develop. Therefore, in women deemed to be at particular risk of the severest form, cancelation of the cycle and withholding of hCG could be considered. As nearly all cycles employ GnRH agonists, no endogenous LH surge would occur and this would result in a complete prevention of ovarian hyperstimulation syndrome. This is the only strategy that would completely prevent this; all other approaches only reduce the risk.

The most popular approach to reducing the risk of ovarian hyperstimulation syndrome is "coasting." This involves withholding both hCG and exogenous gonadotropins while maintaining GnRH agonists in women with high estradiol levels. Once the estradiol level has fallen to a more acceptable level, the hCG trigger is then given. The decline in estradiol levels indicates atresia of granulosa cells; however, this atretic phase may also have an impact on the oocyte itself, as coasting is associated with a lower yield of oocytes, poorer oocyte quality, and lower fertilization and pregnancy

rates (Aboulghar *et al.*, 1997; Whelan & Vlahos, 2000). The duration of coasting may also have an impact on endometrial function, as women who require coasting for more than three days seem to have reduced implantation and pregnancy rates even in the presence of good-quality oocytes (Ulug *et al.*, 2002). Additionally, a rapid fall in estradiol levels is also considered a poor prognostic indicator, and in this situation the oocyte yield is low and quality poor.

The IVF laboratory

There are several factors that need to be carefully controlled to ensure an efficient IVF laboratory. These are air pollution, temperature, sequential culture system, and quality control.

To control these issues, there is restricted entry to the IVF laboratory, as constant entry disrupts the airflow. Sterile conditions are maintained at all times, including masks, gloves, shoes, no nail varnish or perfume, and clean hands at all times. It is advisable to use a HEPA air filtration system where the air is filtered through 0.2 μm filters to remove volatile organic contaminants (VOC), inorganic molecules, smoke, bacteria, dust, alcohol fumes, odors, pesticides, pollen, and fibers (Cohen *et al.*, 1997).

The lights have to be dim as light causes vacuolization in oocytes and ultraviolet rays in fluorescent light is thought to be mutagenic to DNA and detrimental to embryo development. A study carried out in rabbit embryos has shown retarded growth and impaired cell proliferation (Schumacher & Fischer, 1988).

In another study by Takenaka *et al.* (2007), on mouse and hamster zygotes, it was concluded that cool white fluorescent light, which is richer in shorter-wavelength visible light, is more detrimental that warm white fluorescent light – with the highest-term fetuses derived from embryos that had total shielding from light. In this study the authors attribute the decrease in embryo viability to the increase in reactive oxygen species associated with short wavelength "cool" fluorescent white light (Takenaka *et al.*, 2007). In a study by Oh *et al.* (2007) it was reported that specific wavelengths of visible light increases blastomere apoptosis in hamster embryos.

The European Union (EU) Tissue Directive came into force in April 2007. This states that all IVF laboratories should have a background air quality that is grade D (i.e. 3 500 000 particles per m^3 air). All gamete and embryo manipulations must take place in air quality that is at least grade C (i.e. 350 000 particles per m^3 air). This is achieved by use of a vertical flow safety cabinet (Class 2) that protects both the operator and the sample. These are becoming mandatory in IVF laboratories for protection from infections (e.g. HIV, Hepatitis B and C).

The incubators used are very important. They should be set at 37°C + 6 percent CO_2 (in air), which mimics the physiological environment in terms of temperature and pH. The number of door openings should be restricted to maintain physiological conditions as the recovery time is up to 20 minutes. Overloading the incubator will result in suboptimal conditions owing to loss of temperature and change in pH.

Significantly better pregnancy rates and a higher percentage of eight cells is achieved with 5 percent oxygen and 6 percent CO_2 (Gardner *et al.*, 2002). This is more physiological and resembles the in vivo environment (Fischer & Bavister, 1993). In mouse and rabbit 5 percent O_2 and 6 percent CO_2 levels result in higher cell numbers and blastocyst rates (Giles & Foote, 1997; Orsi & Leese, 2001). No difference is seen for day two embryo morphology and pregnancy rates (Dumoulin *et al.*, 1995). Lower sex-chromosome mosaicism is seen in mouse embryos cultured in reduced oxygen (Bean *et al.*, 2002).

The IVF chamber

The active IVF chamber is an adapted neonatal unit (Figure 2.8). It is enclosed and maintains the temperature (37°C), pH (blow 6 percent CO_2) and humidity. It can be adapted to hypoxic conditions, and it is used for manipulation and handling of oocytes and embryos during retrieval, insemination, fertilization checks, and grading of embryos. This ensures maintenance of physiological conditions at all times.The newer versions of this chamber are compliant with the Class 2 air filtration required by the EU Tissue Directive.

Oocyte collection

Vaginal egg collection is performed in the operating theater as a day case procedure by transvaginal follicular puncture and needle aspiration.

Oocyte retrieval is undertaken 36–37 hours after hCG injection. Heparinized saline or flushing medium is used for flushing follicles (temperature 37–38°C). Follicular fluids containing oocytes are aspirated into 17 × 100-mm plastic tubes (Falcon™). Oocytes are identified under a dissecting microscope and placed in culture. Examination of follicular fluid is undertaken for the presence of granulosa cells and oocyte–cumulus complexes. Handling should be rapid, aseptic, and

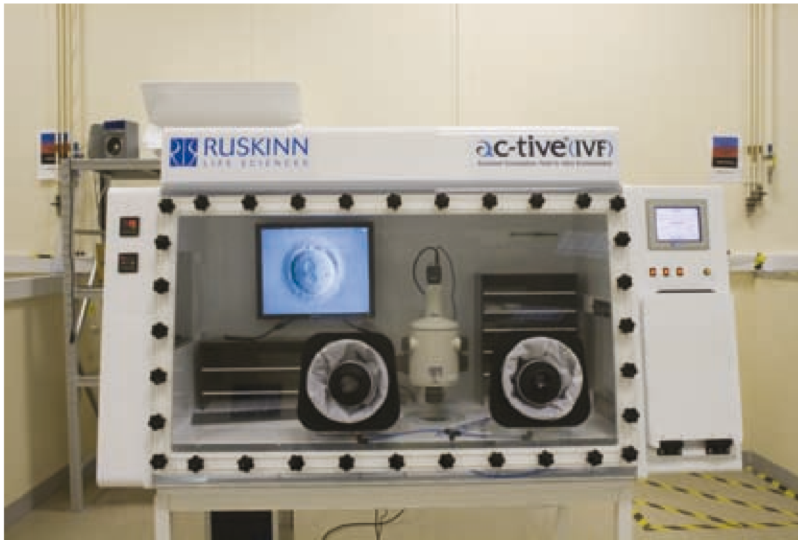

Figure 2.8 The "active" IVF chamber with controlled environment. Oocytes and embryos are in a temperature- and pH-balanced environment during manipulation and observation. Sealable arm ports permit access to dishes during observations.

take place in an IVF chamber (37°C, 6 percent CO_2). The oocytes are graded by size, cumulus expansion, and tightness of corona cells. They are cultured in four-well Nunc™ dishes and tubes (1 ml medium), which are prepared the day before for equilibration.

Oocyte culture dishes contain fertilization medium which is overlayed with sterile and washed paraffin oil, which is a physical barrier to microbes and airborne particles, prevents evaporation, delays gas diffusion, and helps minimize changes in pH, temperature, and osmolarity. These dishes are also equilibrated overnight before use. After the oocyte retrieval, the oocyte–cumulus complexes are incubated for two to four hours before insemination (IVF, ICSI).

Sperm preparation

On the morning of the oocyte retrieval (day 0), the patient's partner produces a sperm sample by masturbation. Semen prepartion is undertaken to remove seminal plasma, which consists of inhibitors that prevent capacitation of spermatozoa. Capacitation and the acrosome reaction are necessary for sperm to interact with the oocyte–cumulus complex to achieve fertilization. Prolonged exposure (>1 hour) to seminal plasma after ejaculation diminishes the fertilizing capacity of sperm in vitro. Therefore semen preparation should be done within an hour after production to isolate motile sperm from seminal fluid. There are four approaches to sperm preparation: direct swim-up from semen; dilution and washing; density gradient; and a combination of swim-up and density gradient.

The most common method of sperm preparation is density gradient. The gradient medium contains buffered saline containing silica particles that remove seminal plasma, debris, and blood cells, and selects against abnormal and immature sperm. It provides a high concentration of progressively motile sperm. It is undertaken by layering semen over gradients in a conical tube (45 percent + 90 percent) and centrifuging at 1000 rpm per 300 g for 20 minutes. The sperm are washed twice in fresh equilibrated culture medium to remove remnants of silica particles.

Culture medium

Most of the methods and culture media used in IVF were developed to culture animal embryos. In the early days of IVF, media such as Hams F10 and TC 199 were widely used for embryo culture. Subsequently, less complex media such as T6 and Earles balanced salt solution were found to be equally effective in supporting embryo development. More recently, commercial ready-to-use media have been produced which have been supplemented with serum and pyruvate, and are supplied in the correct osmolarity and pH. Most IVF laboratories now use sequential culture media which are all bicarbonate-buffered. The oocyte retrieval and sperm preparation are done in fertilization medium. This contains basic salts, glucose, ethylenediamine tetraacetic acid (EDTA), non-essential amino acids and human serum albumin (HSA). From days one to three embryos are cultured in cleavage medium, which contains basic salts, very low glucose or no glucose, EDTA, non-essential

amino acids, and HSA. From days three to six embryos are cultured in blastocyst medium, which contains basic salts, glucose, no EDTA, non- and essential amino acids, and HSA. The addition of hyaluronic acid to the culture media has been shown to increase pregnancy and implantation rates significantly (Gardner *et al.*, 1999; Schoolcraft, 2002). The addition of several growth factors to the culture media is being debated and trialed on animal models to validate its use in human IVF.

Insemination and ICSI

The number of sperm that are placed with each oocyte depends on the initial count and the motility of the sample. For samples with a good count and motility only 50 000 sperm per oocyte are used, and for poor samples with a reduced count or motility, 200 000 sperm are added to each oocyte.

In 1992 the first report of ICSI was published, where one sperm was injected directly into the cytoplasm (Palermo *et al.*, 1992). Improvements in the technique have been made whereby the sperm tail is broken and, on injection, puncture of the cytoplasm is ensured, in most cases by gentle aspiration of some of the cytoplasm. ICSI is an important procedure in PGD as for all molecular diagnosis ICSI must be used to achieve fertilization. Normal fertilization causes sperm to be embedded in the zona pellucida, which are released on zona drilling, and could lead to paternal contamination. Also, some men carrying chromosome abnormalities have poor sperm counts and will need ICSI to achieve fertilization. ICSI will be required for those male cystic fibrosis carriers who have congenital absence of the vas deferens, and sperm aspiration is required to obtain their sperm. Males with Y chromosome deletions will also require ICSI.

Microsurgical retrieval of spermatozoa

Microsurgical aspiration of spermatozoa involves either aspirating sperm from the epididymis (percutaneous epididymal sperm aspiration, PESA) or retrieval of testicular tissue (testicular sperm extraction, TESE). Testicular biopsy material may be taken using a biopsy gun (16 Gauge) or larger biopsy samples may be taken by making an open incision.

Processing of microsurgically derived spermatozoa

Sperm aspirated from the epididymis is collected in a buffered medium containing glucose. The aspirate is prepared by density gradient if the concentration of motile spermatozoa is good. This filters out red blood cells and achieves a cleaner sperm preparation for ICSI. However, if the numbers of motile sperm are very low then a single wash is performed in 3 ml of culture medium (fertilization medium) followed by resuspension in approximately 50 μL of medium. Testicular biopsies are processed by dissecting the tissue into smaller pieces of 1 × 1 mm in medium. Using microdissection the seminiferous tubules are teased out using hypodermic needles. Under a dissecting microscope the tubules are individually held using hypodermic needles and milked with a sterile blunted glass pipette. After milking of all the tubules the tissue debris is carefully separated out and the supernatant spun down in a centrifuge at 1200 rpm. The final resuspension of the pellet (if any visible) is carried out in 50 μL of fertilization medium. PESA- and TESE-processed sperm have to be allowed to equilibrate in the incubator for at least an hour prior to performing ICSI. Testicular sperm usually show very poor motility, which may lead to compromised sperm selection during ICSI. The use of 1 mg/ml pentoxifylline has been reported to enhance motility in sperm, leading to optimized sperm selection for ICSI (de Mendoza *et al.*, 2000; Kovacic *et al.*, 2006). The use of testicular sperm does result in a lower rate of fertilization and clinical pregnancy in comparison with ejaculated sperm (Göker *et al.*, 2002).

The meiotic spindle in the oocyte is temperature-sensitive, and fluctuations may induce depolymerization and hence nondisjunction of chromosomes, leading to aneuploidies (Wang *et al.*, 2001). Thus, during any manipulation of oocytes during ICSI it is imperative that the temperature and pH are maintained throughout the procedure.

Oocytes are denuded at 40 hours after hCG using equilibrated hyaluronidase (80 IU/L) under oil (equilibrated for four hours) for <1 minute. The oocytes are left for an hour after denuding before injection to overcome temperature and pH changes. The timing for ICSI is crucial. ICSI is performed 41 hours after hCG. Polar body fragmentation is observed later than 43 hours afterhCG. Hepes- or MOPS-buffered medium is used to prevent pH changes in ambient atmosphere. A maximum of three oocytes are placed per dish. The dishes are made three to four hours before ICSI and equilibrated at 37°C. The ICSI technique should be rapid (two to three minutes per oocyte) to avoid fluctuations in temperature (Figure 2.9).

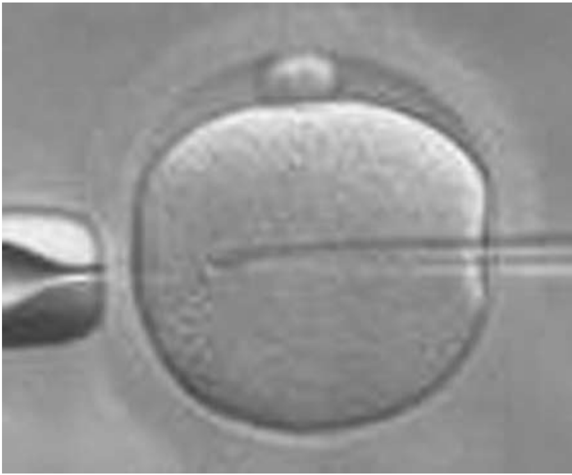

Figure 2.9 Intracytoplasmic sperm injection (ICSI). A metaphase II oocyte being injected at the 3 o'clock position with the polar body at the 12 or 6 o'clock positions.

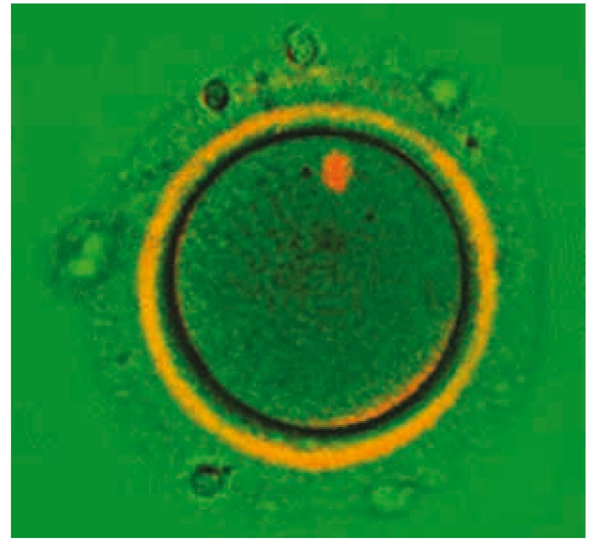

Figure 2.10 Spindle and zona imaging using ICSI Guard™ (Octax). Zona and spindle illumination under polarized light in a metaphase II oocyte.

Spindle imaging technology

Spindle imaging uses polarized light absorbed by birefringent structures within cells such as microtubules of the spindle and the zona pellucida (Figure 2.10). The polar body position is used as an indicator for the site of injection for ICSI to avoid spindle damage. However, spindle shifts of up to 180 degrees from the polar body have been reported (Moon *et al.*, 2003). Hence avoiding the spindle during microinjection would prevent any damage to the meiotic spindle and thus aneuploidy in embryos. Embryos derived from oocytes where the spindle may be seen have a much higher development potential (Wang *et al.*, 2001). The drawback of spindle imaging is the use of glass-bottomed dishes, which may be expensive and are potentially toxic owing to the adhesives used. In a study carried out by Rama Raju *et al.* (2007) spindle imaging and zona birefringence studies showed that the quantitative measurement of length and retardance of the meiotic spindle and zona pellucida had a positive predictive value in relation to embryonic development.

Safety of ICSI

There have been reports that ICSI is associated with an increase in birth defects. Kurinczuk and Bower (1997) reported a twofold increase (odds ratio 2.03; 95 percent confidence interval (95% CI) 1.4–2.93) in birth defects in 420 infants liveborn after ICSI and a nearly 50 percent higher incidence of a minor defect (odds ratio 1.49; 95% CI 0.48–4.66). Their results differed from the Belgian group (Bonduelle *et al.*, 2002), which used a narrow definition of what constituted a birth defect and concluded that there was no increase in the occurrence of major birth defects among infants conceived by ICSI. The European Society for Human Reproduction and Embryology (ESHRE) task force reported the outcome of 13 666 cycles of ICSI carried out in 1994 by 90 centers in 24 countries (ESHRE Task Force on Intracytoplasmic Sperm Injection, 1998). A total of 455 pre- and postnatal karyotypes revealed the presence of nine abnormal karyotypes. In four out of nine cases, the abnormal karyotype was related to paternal factors in severe male infertility. A widely accepted definition of major malformation was applied, that is, malformations that generally cause functional impairment or require surgical correction. Further research is needed to elucidate these results, but, meanwhile, they should be borne in mind when counseling couples about ICSI.

Fertilization check

On day one after oocyte collection, intact oocyte–cumulus complexes are dissected using either pipettes slighly larger than the oocyte or needles. For PGD it is essential to remove all the cumulus cells (both

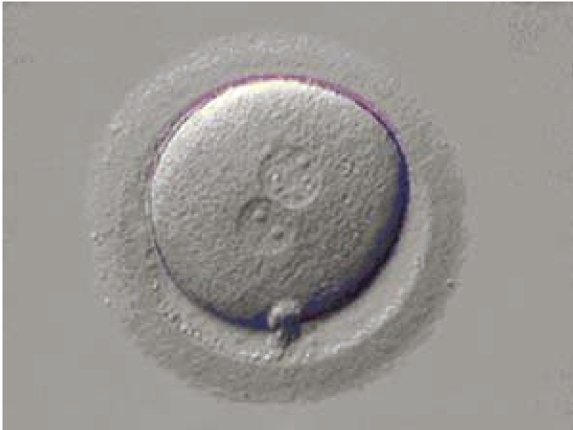

Figure 2.11 A zygote showing two pronuclei and two polar bodies. A fertilized zygote showing two equal-sized pronuclei with visible nucleoli and homogenous cytoplasm.

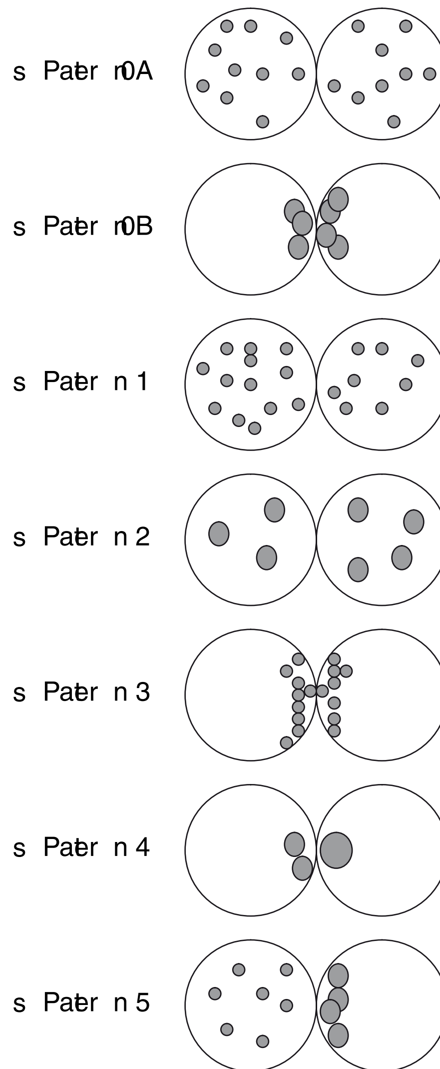

Figure 2.12 Nucleoli patterns and pronucleus scoring. Nucleoli positioning, size, number, and distribution have been shown to be important in relation to pregnancy rates. Pattern 0B gave the highest pregnancy rate. (From Montag and van der Ven (2001). With permission from Oxford University Press. Copyright © 2001 European Society of Human Reproduction and Embryology.)

for FISH and PCR diagnosis) as this could lead to maternal cell contamination. Normal fertilization is observed by the presence of two pronuclei at 18–20 hours postinsemination for IVF and 16–18 hours postinsemination for ICSI with the extrusion of two polar bodies (Figure 2.11). It is useful to look at the location of the pronuclei, sizes, and polarity (Garello *et al.*, 1999). Both pronuclei should appear within the same timeframe and they should be together. Zygotes with distant pronuclei at 16–18 hours after insemination could have damaged microtubules and may have a very poor rate of blastocyst development (Sathananthan *et al.*, 1991). The overall size of the pronuclei is also an important determinant of chromosomal normality. Pronuclei should be of approximately the same size. Grossly uneven pronuclei have an 87 percent incidence of chromosomal abnormality (Munné & Cohen, 1998).

The appearance of the cytoplasm of the oocyte is also evaluated for the presence of any vacuoles, refractile bodies, or excessive granularity.

Examining nucleoli

Nucleoli may be visualized within the pronuclei by use of an inverted microscope with Hoffman optics. The number and distribution of nucleoli have been assessed by several authors, who have reported an increased embryo implantation rate with certain patterns of nucleoli distribution (Scott & Smith, 1998; Tesarik & Greco, 1999; Montag & van de Ven, 2001). Montag and van der Ven (2001) reported a revised zygote scoring criterion. In this study the pregnancy rates were highest when transferred embryos showed Pattern 0B (Figure 2.12).

The accuracy of nucleoli scoring depends on the timing of observation after fertilization. ICSI cases are more reliant as it is easier to time the fertilization. It has been reported that pronuclear morphology is correlated with chromosomal complement and blastocyst rates (Kahraman *et al.*, 2002; Gianaroli *et al.*, 2003).

Figure 2.13 Morphological assessment of embryos based on fragmentation, cell numbers, and symmetry: *Level 1*– Even size blastomeres, little or no fragmentation. (<10 percent); *Level 2*+ Even size blastomeres, moderate degree of fragmentation (<20 percent); *Level 2* Uneven size blastomeres and/or moderate to heavy fragmentation (>50 percent); *Level 3* Totally fragmented (>80 percent).

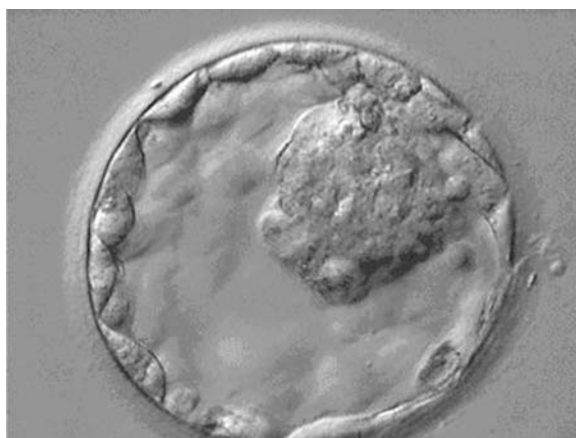

Figure 2.14 A Grade 3 AA blastocyst – a day five blastocyst showing a good-sized and compact inner cell mass. The trophectoderm is linear and cohesive with a good number of cells.

Cleavage- and blastocyst-stage examinations

On days two and three, embryos are examined and the number of cells determined. Each embryo is assigned a grade, based on the symmetry and extent of fragmentation of the blastomeres (Figure 2.13).

Embryos may be cultured up to the blastocyst stage (days five to six) but blastocyst culture requires the laboratory to have optimum culture conditions to enable blastocyst development. About 30–40 percent of all embryos should reach the blastocyst stage by day five or day six. Blastocysts are graded using the grading system developed by Gardner, which grades the degree of expansion and hatching status as "1" for an early blastocyst and "6" for a completely hatched blastocyst. For full blastocysts, graded "3" to "6," two other cell lines are assessed: the inner cell mass and trophectoderm. These are assessed between grades A and C according to cell number, compaction, and distribution. Therefore a Grade 3AA on day five is a high-scoring blastocyst that may elicit pregnancy rates of more than 60 percent (Figure 2.14).

Embryo transfer

In IVF and ICSI, patient transfer may be done on days two, three, five, or six. For PGD, biopsy is usually performed on day three (see Chapter 10) but may also be performed on polar bodies (see Chapter 9) and blastocysts (see Chapter 11). Embryo transfer may be undertaken as soon as the diagnosis is complete, especially if there are only one or two genetically suitable embryos. If there are more than two embryos diagnosed for transfer, it is advisable to wait until day five to see which embryo is developing better.

The embryo transfer procedure is performed under ultrasound control. The patient has a full bladder, the vagina is cleaned using saline, and the mucus is aspirated from the external os. The catheter is advanced through the cervix into the uterus so that the embryos will be placed approximately 1 cm from the uterine fundus. The length of the uterine cavity is known as it is measured during the dummy embryo transfer. In the laboratory, the inner catheter is loaded using sterile Biogel® (powder-free) gloves. The catheter is washed with 1 ml of pre-equilibrated medium. The embryos are picked up in 5–10 μl medium with 5 μl of air gaps on both ends. The total volume in the syringe must not exceed 20–30 μl. Only the inner catheter is loaded and fed through the outer sheath, which is *in situ*, and thus causes less temperature and pH stress as the embryos are not exposed while canulating the cervix. Embryos

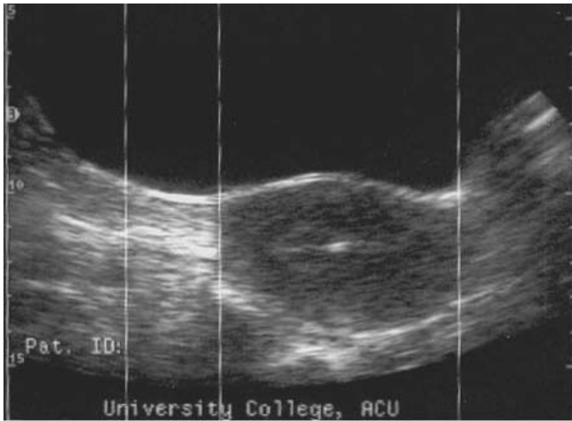

Figure 2.15 An abdominal ultrasound scan during an embryo transfer. The bright spot in the mid cavity of the uterus indicates the positioning of the fluid containing the embryos after an embryo transfer. The full bladder aids in straightening the uterus to facilitate easy canulation with the catheter.

can be damaged if an obstruction is encountered while canulating. The approach of the inner catheter is visualized on the scan (Figure 2.15). The embryos are released into the mid cavity and the patient remains lying down for 25 minutes.

In standard IVF or ICSI, embryos with the best morphology and the most advanced stage of development are chosen. A number of factors may be considered when deciding how many embryos to transfer, including the patient's age, cause, and history of infertility, and the number and grade of the available embryos.

Most IVF clinics in Europe have implemented single-embryo transfer (SET) for patients aged under 35. Two embryos are only transferred in older patients or for subsequent attempts. This significantly reduces the number of multiple pregnancies that result from IVF treatment.

Quality control in the IVF laboratory

Good quality control is crucial in an IVF laboratory. All batches of reagents, media, and/or plastic ware are tested in-house or by the manufacturer. The most common quality control test performed by most IVF consumable and/or culture media manufacturers uses a one-cell mouse embryo assay on a sensitive strain of mice (MEA). MEA testing certificates for batches of consumables and/or media that come into direct or indirect contact with gametes and/or embryos should be monitored and filed for reference. Products that do not have a MEA test performed should be tested in-house using a 76-hour sperm-survival test prior to clinical use. A control product should be used to compare test results. In Europe, commercially available products used for IVF should ideally be CE-marked (certified medical devices).

All consumables have to be disposable (no reuse). Borosilicate glass pipettes are double-washed and heat-sterilized (if not available already washed and sterilized). Incubators and IVF chambers need regular cleaning and swabbing to check for microbial contamination. Daily temperature and CO_2 checks are performed with independent and calibrated instruments to check the accuracy of displays. Flushing medium temperature should be checked at the needle tip. Heated stages, when any manipulation of oocytes and/or embryos is carried out, should be tested for temperature with a control dish of culture medium drops under oil and a K type thermocouple probe to get an accurate indication of temperature in a microdrop. All adjustments of temperature for heated stages, oven for warming flushing medium, and ICSI rigs, should be adjusted according to the above testing method.

The ultimate quality controls in any laboratory are the clinical end-points, such as fertilization, cleavage, blastocyst, and clinical pregnancy rates. These parameters have to be reviewed monthly to ensure the consistency of end-point parameters.

After embryo transfer and pregnancy

During the luteal phase, progesterone pessaries or injections, or further injections of hCG, are given to support the endometrium. If no period has occurred by 16 days after egg retrieval, a urine β-hCG test is performed to detect the pregnancy.

If a pregnancy test is positive an ultrasound scan is performed 10 days later to check that the pregnancy is intrauterine and the number of gestational sacs. Ectopic pregnancy occurs in 4–5 percent of IVF pregnancies (SART/ASRM, 1995). The miscarriage rate in a viable pregnancy (fetal heartbeat seen) approximates to that of spontaneous pregnancy, i.e. 8 percent. There is no increased risk of congenital abnormalities in babies born as a result of IVF or ICSI (Bonduelle *et al.*, 2002) nor reported in babies conceived with PGD (Harper *et al.*, 2008) and the risk is similar to that in natural conception. Ultrasound monitoring is continued through early pregnancy.

Treatment success

Even in fertile couples, IVF is more likely to fail than to succeed. In general, success rates for IVF depend on

the woman's age, duration of infertility, and whether she has had a previous pregnancy. Success rates of 25 percent mean that the pregnancy test will be negative in 75 percent of cases, and this may be devastating for the couple. It is at this time that support and counseling are most important. However, many clinics now report delivery rates of over 40 percent.

Cryopreservation

Cryopreservation of supernumerary good-quality embryos is vital to optimize pregnancy rates per cycle started without the need to superovulate the patient again. The success rate with frozen embryos is limited, and usually lower than with fresh embryos. There are many limiting factors that govern the success of frozen–thawed embryos. Some of these are the type of replacement cycle, i.e. natural or artificially prepared, slow freezing or vitrification, the stage at which embryos are cryopreserved, and the number of viable blastomeres post-thaw.

Slow freezing

It is important to keep the temperature controlled during dehydration and rehydration of embryos. Any temperature shocks are minimized with transient lowering or increasing of the temperature during the dehydration and rehydration processes. The embryos are thawed for at least four hours prior to transfer to assess compaction events (day three). Day two embryos and pronucleates are best thawed the day before. Blastocysts are thawed four to five hours beforehand to assess expansion of the blastocoel cavity. Freezing biopsied embryos gives very poor results and only a few pregnancies have been achieved (Wilton *et al.*, 2003).

Vitrification

"Vitrification" means solidification of a solution at low temperatures with no ice crystal formation. This is achieved by increasing the viscosity of the solution by using high cooling rates (Rall & Fahy, 1985). The rapid cooling rate would minimize any potential chilling injury and osmotic shock to the gametes and embryos. Vitrification has proven clinical value not only because of its simplified protocol but also because of better outcomes, such as survival and pregnancy rates (Kuwayama *et al.*, 2005; Zheng *et al.*, 2005). Vitrification appears to be quite a promising tool for cryopreserving biopsied embryos and pregnancies have been reported (Parriego *et al.*, 2007; Escribá *et al.*, 2008). The ideal stage of vitrification of biopsied embryos is the blastocyst stage (Escribá *et al.*, 2007) when, potentially, the embryonic junctions are well formed and may better sustain any cryoinjury.

Canceled cycles

As with all IVF, problems may occur during IVF cycles for PGD. Treatment cycles may be canceled before the egg collection if the follicular response is poor and, rarely, there are no oocytes at follicular puncture. It is possible that failed fertilization results in no embryos for diagnosis, or, after PGD, it may be discovered that all the embryos are affected with the disease, in which case there will be no embryos suitable for transfer. The ESHRE PGD Consortium initially tried to estimate the number of PGD cycles canceled prior to oocyte retrieval but since many centers only reported on cycles which reached oocyte retrieval, the data were very inaccurate (Harper *et al.*, 2008). Now, only cycles to oocyte retrieval are reported but all indications show cycles canceled after oocyte retrieval, post-ART, and cycles with no embryos to transfer.

For some diseases, such as cystic fibrosis, β-thalassemia, or sickle-cell, some of the embryos may be diagnosed as carriers of the disease and it may be advisable to replace them if they are of good quality.

Regulation, accreditation, and quality managemeent

Regulation

The Human Fertilisation and Embryology Authority (HFEA) is a government organization that regulates all licensed activities of licensed IVF centers in the UK. The HFEA carries out annual inspections of all IVF centers. The HFEA *Code of Practice* is a comprehensive manual that details what licensed centers are expected to do. PGD, preimplantation genetic selection (PGS), IVF, intrauterine insemination by donor (IUI (D)), and GIFT or surrogacy are all licensed activities. The performance of ICSI and biopsy practitioners is audited annually by the HFEA. The Authority requires that all necessary steps are taken to ensure that account has been taken of the welfare of any child who may be born as a result of treatment. The HFEA holds the central database of all reported live births from licensed treatments. In addition to clinical regulation, the HFEA governs embryo and stem cell research. For PGD every new diagnosis that a clinic wishes to carry out has to be approved by the HFEA. The HFEA is responsible for the implementation and compliance with EU

directives concerning tissues and cells of all licensed IVF centers in the UK.

Accreditation

The EU Directive sets standards of quality and safety for the donation, procurement, testing, processing, storage, and distribution of human tissues and cells. The main areas of compliance with the Directive include maintaining air quality in the laboratory (see above). Third-party agreements with all providers of IVF-related products or services have to be in place. Traceability includes batch-logging of all consumables in the laboratory which come into direct contact with gametes and embryos. Regular validation charts of culture conditions and temperature mapping are vital.

Quality management systems (ISO 9001, ISO 17025)

Quality management systems are important in an IVF unit to ensure reproducibility of all the protocols and methods performed by personnel in various departments. A quality policy forms the “mission statement” for the clinic’s activities personnel to. A quality manual outlines the structure of the documentation used in the quality system, and is a compilation of all policies, procedures, and documents that enable personnel to work toward the quality policy. Each document within the quality manual is checked, amended, version-controlled, and signed by the person in charge every year. Every quality system has to have a clear organization plan, which should clearly define the organization and management structure in the laboratory. The quality manager within the laboratory is responsible for ensuring that all the activities performed within the laboratory accord with the laboratory quality system.

Internal audit of performance is a key indicator for assessing the performance of a quality system. The key indicators to assess performance in any department should be reviewed regularly (preferably monthly). Key performance indicators for the laboratory could include fertilization rates, cleavage rates, blastocyst conversion rates, clinical pregnancy rates, and implantation rates. Yearly graphic plotting of monthly key performance indicators may be helpful in deriving an average for their future monthly comparison. Nonconformities of equipment or practice, and actions taken, are logged and monitored. An external quality auditor is hired annually to carry out audits for renewal of the International Organization for Stanardization (ISO) certificate.

Conclusion

Patients undergoing PGD differ from other couples referred for IVF in that they are usually fertile. Their inherited disorders may have resulted in an affected child or termination of pregnancy, or they may have suffered repeated miscarriages. PGD patients may be unaware of the procedures required for IVF, which is a prerequisite for PGD. Careful explanations should include discussion of potential risks and realistic assessment of success so that the couple can make an informed decision as to whether to proceed or to try a natural conception with prenatal diagnosis. As with all couples undergoing IVF, they should be offered counseling, with special consideration of the extra stresses they may be under. IVF for PGD differs from “ordinary IVF” in that ovarian stimulation is pushed in order to obtain the maximum number of oocytes and embryos for testing.

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Chapter 3

Genetic basis of inherited disease

Joep Geraedts and Joy Delhanty

Key points

- Genetic disease is divided into the following categories: monogenic disease, chromosomal abnormalities, mitochondrial disorders, and complex disease.
- In monogenic disease five simple modes of Mendelian inheritance may be distinguished: autosomal recessive, autosomal dominant, X-linked recessive, X-linked dominant, and Y-linked. The types of mutations giving rise to these disorders will be treated and a few examples will be given in detail as well as phenomena like anticipation, incomplete penetrance, variable expression, and pleiotropy.
- Two groups of chromosomal abnormalities may be distinguished: numerical and structural. Numerical chromosome abnormalities result from meiotic or mitotic division errors, which give rise to too many or too few chromosomes (aneuploidy). The majority of these are lethal before or during early pregnancy. Structural chromosome abnormalities follow from one or more chromosome breaks, which, after reunion of the wrong segments, result in translocations (reciprocal; Robertsonian), inversions, and deletions.
- The term "complex disorders" is preferred for all genetic conditions that are not strictly Mendelian or chromosomal in nature. Complex disorders might result from two or more genes (polygenic inheritance) as well as from more than one gene in combination with environmental factors. The latter situation was previously called "multifactorial inheritance."
- Mitochondrial inheritance shows completely maternal inheritance.

Introduction

Before considering the approaches to preimplantation genetic diagnosis (PGD) it is important to have a clear understanding of the genetic basis of inherited disease. Inherited disease may be caused at the level of the gene and the chromosome. In principle single-gene mutations can lead to genetic diseases which may be distinguished on the basis of five simple modes of inheritance: autosomal dominant, autosomal recessive, X-linked recessive, X-linked dominant, and Y-linked. In practice, only the first three are relevant. At the chromosome level a distinction may be made between numerical and structural chromosomal abnormalities. The first usually result from meiotic nondisjunction and are sporadic in the vast majority of cases. Chromosomal rearrangements are carried by a parent and may lead to the production of genetically unbalanced gametes. Besides these modes of nuclear inheritance there is also mitochondrial inheritance, which shows completely maternal inheritance.

In recent literature, the term "complex disorders" is preferred for all genetic conditions that are not strictly Mendelian or chromosomal in nature. Complex disorders might result from two or more genes (polygenic inheritance) as well as from more than one gene in combination with environmental factors. Disorders such as diabetes and schizophrenia are caused by the interaction of many different genes together with environmental influences. The latter situation is sometimes called "multifactorial inheritance." Technically it is not possible to offer PGD for this type of condition at present since the genetic basis is not fully understood and the exact environmental component is unknown.

Gene mutations and polymorphisms

Mutation simply means change. Change in gene function may be brought about by loss of the whole or part of the coding sequence of the gene (deletion) or by alteration in the bases that make up the DNA molecule (substitution). More rarely, gene duplication

can cause disease. Surrounding each gene are modifying sequences that affect gene expression; these too can mutate. Mutations also affect non-coding DNA sequences, usually regions of repetitive DNA that are associated with each gene. Mutations in these regions are unlikely to be disease-causing. Several forms of these variants may exist in the population and if they are common enough to be found in at least 1 percent of people they are known as "polymorphisms." Polymorphisms are very useful as genetic markers since a particular variant will tend to be inherited along with the gene mutation in each family (Wang *et al*., 2007).

Mendelian inheritance of monogenic disorders

The relevant genes may be carried either on the X chromosome or on the non-sex chromosomes (the autosomes). Autosomal conditions are either dominant or recessive. At the outset it is important to remember that chromosomes exist in pairs, one from each parent, which, in turn, means that a gene for a particular character is also present twice in each cell. The exception is the pair of sex chromosomes. In females one X chromosome is inherited from each parent. The X chromosome in males, which comes from the mother, has no counterpart. The Y chromosome, which is inherited from the father, is much smaller and carries few expressed genes, which are different from those on the X chromosome. It carries genes that are important for sex determination and fertility. The loss of part of this chromosome, or of the function of one or more of these genes, is therefore harmful. During the formation of the gamete recombination takes place between paired chromosomes after which both parental chromosomes segregate. This means that half of the genome becomes incorporated in the oocyte or sperm. At fertilization new combinations of maternal and paternal chromosomes will always be formed.

Examples of pedigrees showing these typical patterns of inheritance are shown in Figure 3.1. An autosomal-recessive mode of inheritance is suggested when there are affected children in a family with no known history of the disease. This happens when a gene carrier "marries" into the family. In dominant conditions affected children can be seen in every generation since the parent has a one in two chance of passing on the condition irrespective of the genetic status of his or her partner. In families with an X-linked recessive condition only males are affected, and they may appear in every generation because some of the mothers will be carriers of the gene but there is no male-to-male transmission.

Autosomal recessive inheritance

In recessive single-gene disorders a person who is a gene carrier has one abnormal copy of a particular gene in each of the cells along with one normal copy on the other chromosome. This carrier will not manifest the disease. This person is a heterozygote. If the partner is not a carrier the couple can safely produce children who will be unaffected. If by chance the partner is also a carrier for the same abnormal gene then the couple has a one in four chance of having a child with two copies of the abnormal gene. This child will then be affected by the disease in question. On average, half their children will be gene carriers like themselves and a quarter will have two normal genes (Figure 3.1 (a)).

The most common autosomal recessive disorder in Caucasian populations is cystic fibrosis (CF) (Sermon *et al*., 2007). Therefore in Europe this is by far the most frequent reason for referral to a PGD center. More than 1000 different mutations are known. In the majority of cases the mutation in this gene is a three base pair deletion known as p.F508del. In most cases both parents of an affected child are likely to be carrying this mutation (Figure 3.2 (a)). This situation makes single cell diagnosis relatively straightforward. However, there are a number of rare CF mutations that are not so easily detected. If the parents carry different mutations the child who inherits these two different abnormal genes will be affected. Such a child is called a "compound heterozygote" (Figure 3.2 (b)). Worldwide, the most common autosomal-recessive disease is β-thalassemia (Kornblit *et al*., 2007). In contrast to CF, there are numerous different common mutations in this gene. Certain types predominate in different populations, but it is still the case that most parents of affected children will be carriers of different mutations. This makes PGD more difficult because the affected embryos will be compound heterozygotes. Single-cell diagnosis is also technically more demanding than for CF since most changes are substitutions of one base for another rather than missing or additional bases.

Many inborn errors of metabolism are autosomal recessive (Scriver *et al*., 2001). Since one gene is responsible for the production of one enzyme, these diseases cause enzyme deficiency. If one gene coding for an enzyme is affected, 50 percent of the enzyme will still be produced from the wild-type gene. In general this level of enzyme activity is sufficient for normal life.

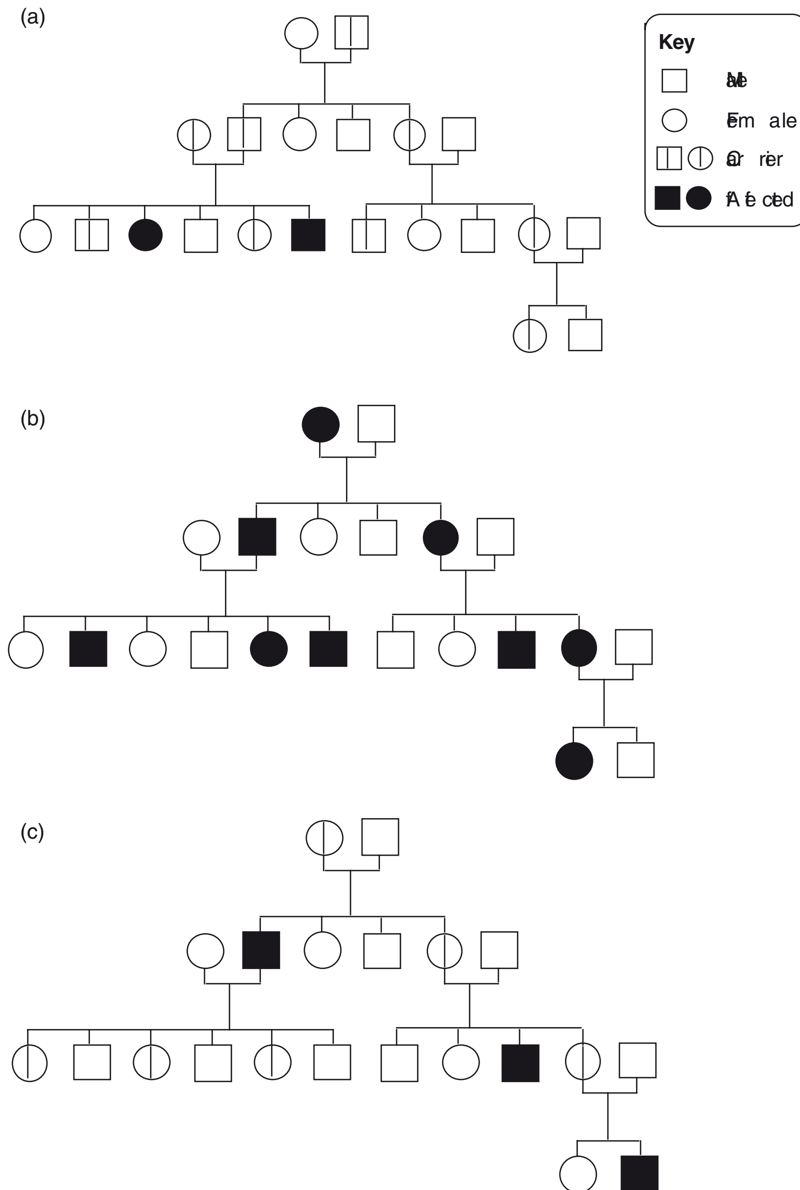

Figure 3.1 Modes of inheritance: (a) autosomal recessive, if both partners carry the mutation there is a 50% risk of offspring being carriers and a 25% risk that they will be affected; (b) autosomal dominant, affected individuals seen in every generation and there is a 50% risk that children will be affected; (c) X-linked recessive, only males are affected and females are carriers, 50% risk that the male will be affected and a 50% risk that the females are carriers.

Therefore both copies of the gene need to be affected in order to result in a severe clinical phenotype.

Autosomal dominant inheritance

Autosomal-dominant disorders require only one copy of the abnormal gene to be present in each cell for the disease to be expressed. The presence of a normal copy on the other chromosome is not sufficient to ensure normality. Generally, at birth dominant disorders are not so severe or life-threatening as recessives. In dominant disease, the gene carriers are usually able to have children despite being affected, as in the case of achondroplasia (dwarfism) or because the disease itself is of late onset (e.g. Huntington disease). On average, half the children of an affected mutant gene carrier will also be affected. From this it might be concluded that diseases which, when studying the pedigree, seem to follow a more complex pattern cannot result from simple point mutations in individual genes. However, complexity might result from incomplete penetrance and variable expression. Incomplete penetrance means that a number of genotypically affected carriers

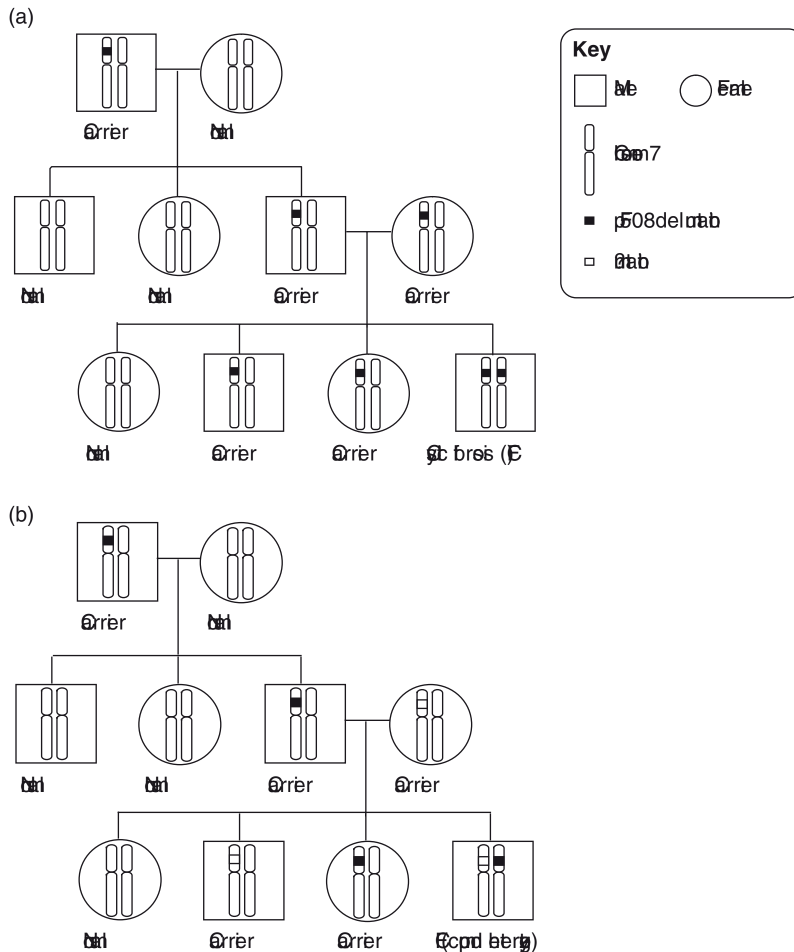

Figure 3.2 A cystic fibrosis (CF) family pedigree illustrating the typical inheritance of an autosomal recessive gene defect: (a) in this family the common CF mutation p.F508del is passed through healthy carriers in the grandparents' and parents' generations to the grandchildren, one of which is affected by the disease; (b) in this case the mother carries the p.F508del mutation whilst the father carries a different, much rarer mutation of the CF gene. The child affected by CF who has inherited both defective copies is essentially a carrier of each mutation and is termed a "compound heterozygote."

do not demonstrate any symptoms of the condition at all. Variable expression means that not all disease carriers show the same severity of the condition, and suggests that environmental factors or other genes are also involved. Furthermore, pleiotropy occurs, in which one mutation may cause more than one effect on development.

It should always be kept in mind that new mutations arising during gamete formation may be responsible for sporadic cases in a family. It is also possible that the legal father is not the biological father. Sometimes an individual is mosaic for the mutation. In that case it must have arisen as a somatic event after fertilization. Sometimes the mutation is confined to the germline.

Autosomal-dominant disorders that have led to requests for PGD include Marfan syndrome, which is a connective tissue disorder that predisposes to heart disease (Judge & Dietz, 2005). Other diseases include late-onset neurologic disorders and cancer syndromes. Among the latter, breast and colon cancer are the most frequent indications (Reed & Donnai, 2007). The breast cancer mutations BRCA 1 and 2 can either have no effect at all (non-penetrance) or result in breast cancer, on the one hand, or ovarian cancer, on the other, with variable risks (Sinilnikova *et al.*, 2006) as follows:

- risk of female carriers of a mutation in BRCA1/2 to develop breast cancer before the age of 70: 60–80 percent

- risk of female carriers of a mutation in BRCA 1 to develop ovarian cancer before the age of 70: 30–60 percent
- risk of female carriers of a mutation in BRCA 2 to develop ovarian cancer before the age of 70: 5–20 percent.

Germ line mutations in the tumor suppressor adenomatous polyposis coli (APC) gene predispose to the clinical familial adenomatous polyposis (FAP) syndrome, in which the development of hundreds of adenomas in the colon makes eventual progression to colorectal cancer inevitable. PGD is technically demanding because it is vital to be able to detect both the mutant and normal copies of the gene in a single cell with equal efficiency (Ao *et al.*, 1998).

One category of dominant disorders that presents particular problems for PGD is those that are caused by a variable increase in the number of copies of a particular trinucleotide repeat sequence (i.e. three base pairs, for example cytosine, guanine, guanine – CGG – and their paired bases on the opposite DNA strand) within the vicinity of a gene. The two most frequently requested disorders (myotonic dystrophy and Huntington disease) belong to this category.

Triplet repeat disorders

Myotonic dystrophy

Myotonic dystrophy, DM1, also known as Steinert disease, is a progressive muscular dystrophy caused by an expansion in the number of copies of a CTG repeat in the 3′-untranslated part of the DM1 kinase gene on chromosome 19. The repeat copy number varies from 5 to37 in normal individuals and from 50 to several thousands in people affected with the disease. Intermediate repeat numbers result in an unstable, premutation state. The expansion of the triplet repeat may lead to the phenomenon of anticipation, which means that the disorder starts at a younger age and becomes more severe with each generation. Mildly affected mothers can give birth to severely affected offspring with the congenital form of the disease (Harper, 2002). PGD identifies the normal allele, as the amplified allele is refractory to polymerase chain reaction (PCR) (Sermon *et al.*, 1997).

Huntington disease

Huntington disease is a progressive neuropsychiatric, late-onset disorder. It is characterized by involuntary movements and cognitive deterioration. The mean age of onset is 40 years, with death occurring, on average, 15 years later. The Huntington disease gene was isolated in 1993 and was the first to be shown to be attributed to a triplet repeat expansion. The increase in copy number of a CAG repeat in the vicinity of the gene on chromosome 4 disturbs its normal function. Expansion in the number of repeats beyond 36 results in disease manifestation (Walker, 2007).

PGD for Huntington disease is possible via three routes: direct testing, exclusion testing, and non-disclosure testing. Direct testing means testing of the expanded repeat or the study of the presence or absence of the mutated allele on the basis of the presence of parental alleles of the Huntington disease gene (Sermon *et al.*, 1998). Exclusion testing is based on examining linked polymorphic markers flanking the Huntington disease gene. This strategy allows identification of the maternally and paternally inherited alleles, and may subsequently be used to identify the low-risk (grandparental) allele in derived offspring. Apart from the small residual risk of recombination events, this method may be used to classify an individual as being at low or high risk for developing the disease (Jasper *et al.*, 2006). Non-disclosure PGD entails direct testing of the expanded repeat on the embryos without knowing the disease status. This approach is considered to be ethically problematic (Braude *et al.*, 1998).

Fragile X syndrome

Fragile X syndrome is a form of X-linked mental retardation caused by unstable expansion of a CGG repeat in the 5′ untranslated region of the FMR1 gene. In the normal population the number of repeats is polymorphic with different individuals possessing between 6 and 54 copies, and is usually inherited unchanged from parent to child. In families with Fragile X syndrome the number of copies of the repeat has a tendency to expand with each generation, which is an unstable, premutation, situation. Once the number of repeats exceeds 200 excessive methylation occurs, leading to transcriptional inactivation and absence of gene product. Males with repeat copy numbers in excess of 200 will show symptoms, and females, who also have a transcriptionally active copy of the gene, show variable disease manifestation.

Fragile X syndrome is a frequent cause of referral for PGD but is technically extremely demanding for a variety of reasons, including the 10-fold increase in premature ovarian failure (see Chapter 8) and the practical aspects of PCR (see Chapter 15).

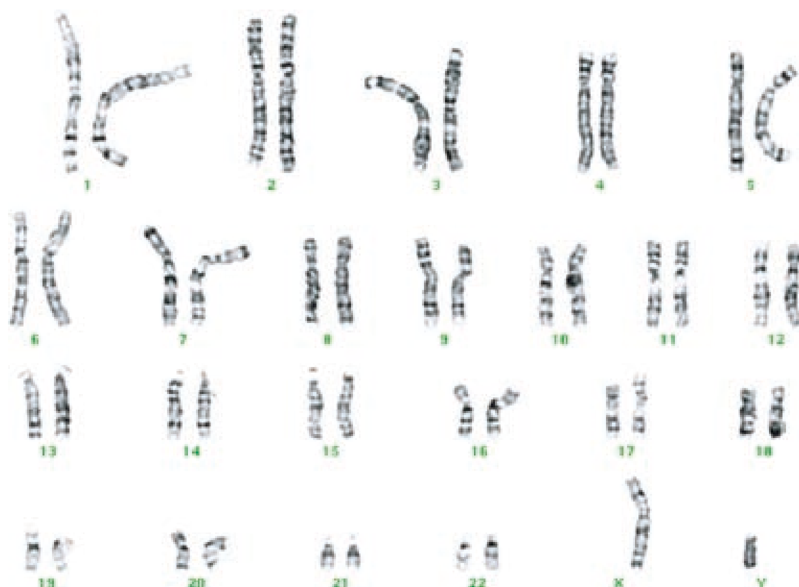

Figure 3.3 Human male karyotype.

X-linked inheritance

X-linked disorders (caused by mutation in genes that are carried on the X chromosome) may be either recessively or dominantly inherited. The vast majority are recessives and are carried by females who are themselves unaffected or only mildly so, because of the normal copy of the gene on their second X chromosome. Distorted X chromosome inactivation can result in symptoms in females, although much milder than in males. Half the sons (who receive their single X chromosome from their mother) will be normal; however, the others will be affected with the disease as their Y chromosome will not have the normal gene. Common X-linked recessive diseases include Duchenne muscular dystrophy and hemophilia. Since the molecular basis of these two diseases is understood it would be theoretically possible to carry out a specific PGD and diagnose affected males (see Chapter 14). However, the exact gene change is not always known in particular families so, in practice, it may be easier to offer sexing of the embryo with the transfer of females. Added to this is the fact that there are well over 1000 known X-linked disorders and for most of them the molecular basis is not known. For these families, all that can be offered in the way of prenatal diagnosis is sexing of the fetus and termination of all male pregnancies, half of which will be unaffected. PGD offers the chance to avoid this scenario (see Chapter 14).

Numerical chromosome abnormalities

Numerical chromosome abnormalities are abnormalities which deviate in number from the normal karyotype, which has 2n = 46 chromosomes; that is, two haploid sets (Figure 3.3). They may be divided into aneuploidy, an extra (trisomy) or missing (monosomy) chromosome of a pair, and ploidy abnormalities, in which the number of haploid sets of chromosomes is abnormal. An embryo may be haploid (1n = 23) triploid (3n = 69) or tetraploid (4n = 96). Aneuploidy is not normally inherited. Typically, the imbalance arises at meiosis I in the mother when two homologous chromosomes pass to the same pole of the spindle at anaphase. The mature oocyte formed after meiosis II will have either an extra or a missing copy of the chromosome, which will result in trisomy or monosomy in the embryo after fertilization. The most common numerical chromosome abnormalities are trisomies and monosomies, arising *de novo* as a result of meiotic non-disjunction during gametogenesis in parents with a normal karyotype. For all chromosomes, except the largest, the non-disjunction rate increases with maternal age. The cause of non-disjunction in the oocytes of older women is largely unknown. An increase in maternal age results in an increased embryonic aneuploidy rate as well as an increased frequency of spontaneous

abortion. This is reflected not only in a higher miscarriage rate but also in a lower success rate for IVF. The most frequent abnormality in spontaneous abortions is trisomy 16, representing about 30 percent of all such trisomies, but in cleavage-stage embryos trisomies for chromosomes 13, 21, and 22 are the most common. Trisomies for all chromosomes have been observed in spontaneous abortion, except trisomy 1. Autosomal monosomies are practically unknown in human miscarriages, since they stop development before or soon after implantation (Macklon *et al.*, 2002). On the other hand, monosomy X is a frequent finding in abortions. Non-disjunction may occur at either meiotic division. Trisomy 16, for example, always shows a maternal meiosis I error. Trisomy 18, on the other hand, results predominantly from errors during the second division. Premature centromere division at meiosis I, leading to separated chromatids, is an alternative mechanism for trisomy formation, occurring as frequently as whole-chromosome non-disjunction (Hassold & Hunt, 2001).

The abnormality most frequently resulting from abnormal fertilization is triploidy. There seems to be no effect of maternal age on triploidy and tetraploidy; the latter originates during preimplantation development. Only three autosomal trisomies are regularly observed at birth: trisomies 13, 18, and 21. They have an estimated prenatal survival of 3, 5, and 20 percent, respectively. The other autosomal trisomies have an estimated survival to term of less than 1 in 1000. Sex chromosome aneuploidies survive normally with the exception of 45,X, which is lethal in about 98 percent of conceptions (Macklon *et al.*, 2002).

Aneuploidy and gonadal mosaicism

Occasionally, couples present with a history of repeated conceptions involving trisomy for the same chromosome. The reason for this may be mosaicism in a parent with a second, trisomic, cell line present in addition to the normal population of cells. When the trisomic cells are present in the gonads this results in a high frequency of gametes which are unbalanced for the involved chromosome.

Aneuploidy for the sex chromosomes, leading to Klinefelter syndrome (47,XXY), triple X, or Turner syndrome (45,X), has a less severe effect on the phenotype of surviving individuals (in addition to the almost total lethality of 45,X conceptions, 50 percent of 47,XXY fetuses abort). Full Klinefelter syndrome leads to azoospermia, but epididymal sperm may sometimes be recovered and used for fertilization. Also, some infertile males are mosaic for a 47,XXY cell line; if this is discovered their embryos should undergo PGD as there is an increased risk of the production of XY sperm.

Structural chromosomal rearrangements

Structural chromosomal rearrangements are common in the human population. They are the result of simultaneous chromosome breakage, either within the same chromosome or in different chromosomes, and subsequent rejoining in an abnormal fashion. These events occur at the stage in which the chromosomes are decondensed. After condensation of the chromatin into microscopically visible chromosomes, these changes become visible. They usually occur before or during gamete formation so that every cell of the embryo that results from fertilization with that gamete carries the same rearrangement. Providing that the breakage in the chromosomes has not affected gene transcription the individual carrying the rearrangement will develop normally. The problems occur in oogenesis or spermatogenesis when the rearranged chromosomes have to pair with their normal homologs and abnormal products of segregation result.

Chromosomal translocations

The most common type of chromosome rearrangement is a translocation, which is the movement of a segment of chromosome from its normal position to a new site. Reciprocal translocations involve breaks along the arms of two chromosomes and exchange of material with reunion creating two abnormal derivatives (Figure 3.4 (a)). Robertsonian translocations involve breakage and reunion around the centromere of the "acrocentric" chromosomes; the minute short arms of these chromosomes are normally lost in the process so that the chromosome number per cell is reduced to 45, again with no phenotypic effect (Figure 3.5 (a)). Robertsonian translocations are unusual in that identical types occur repeatedly in humans, whereas reciprocal translocations are normally unique to the family. These factors make PGD much simpler for Robertsonian than for reciprocal translocations (see Chapter 12). In the case of reciprocal translocations a group of four chromosomes is formed at meiosis in the parental carrier, and this group may separate into two groups of two in three ways, only one of which will be balanced genetically

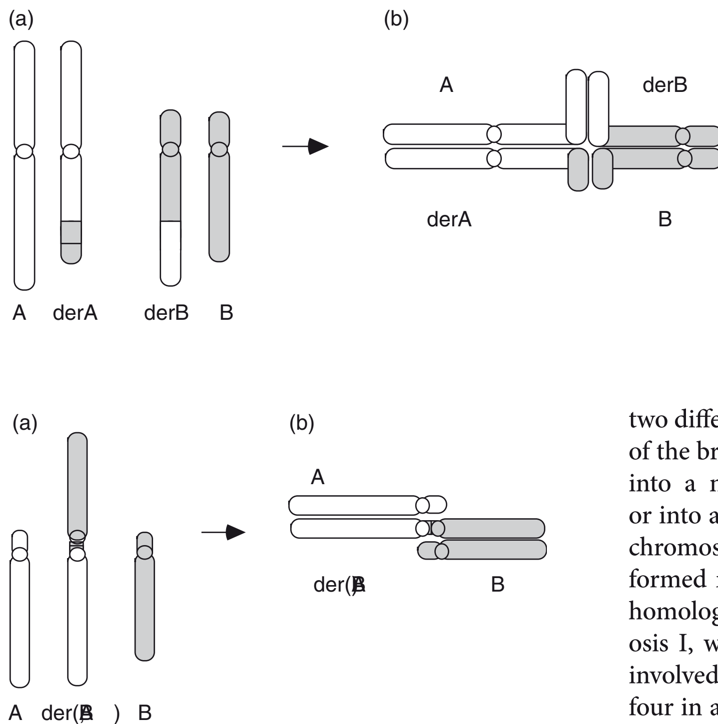

Figure 3.4 (a) Reciprocal translocation – reciprocal exchange of material between two non-homologous chromosomes; (b) cross-shaped arrangement (quadrivalent) adopted by reciprocal translocations during early meiosis allows pairing of homologous chromosomes. Chromosome rearrangements showing the normal homolog (A, B) with the rearranged or derivative chromosome (der A, der B).

Figure 3.5 (a) Robertsonian translocation – fusion of two acrocentric chromosomes with varying loss of centromeric and short-arm material; (b) pairing arrangement (trivalent) adopted by Robertsonian translocations during early meiosis allowing pairing of most homologous regions. Chromosome rearrangements showing the normal homolog (A, B) with the rearranged or derivative chromosome (der A, der B).

(Figure 3.4 (b)). It is also possible for three of the four chromosomes to go to one daughter cell and only one to the other (3:1 segregation). It is clear that there is a high risk of producing chromosomally unbalanced gametes but the exact risk depends on several factors, including the chromosomes involved, the position of the breakpoints, and the sex of the parent with the translocation (Kayser-Rogers & Rao, 2005).

In Robertsonian translocation carriers a group of three chromosomes is formed when pairing occurs in meiosis but, in the same way as for reciprocals, this group can segregate in three ways, only one of which is balanced (Figure 3.5 (b)). An example of a typical family with an inherited Robertsonian translocation is shown in Figure 3.6.

Insertions

Much rarer types of translocation occur, which are known as "insertions." These involve three simultaneous breaks, either within one chromosome or involving two different chromosomes. The segment freed by two of the breaks within one chromosome is then inserted into a new position within the same chromosome or into a second one (Figure 3.7). In the case of *intra*-chromosomal insertions, unbalanced gametes may be formed merely by recombination between the paired homologous chromosomes in the prophase of meiosis I, whereas for *inter*-chromosomal insertions the involved chromosomes come together as a group of four in a similar way to reciprocal translocations, and the type of gametes formed is again dependent on the segregation pattern.

Inversions

When two breaks occur within one chromosome the free segment may rotate through 180 degrees before rejoining (Figure 3.8). This produces an inversion of genetic material between the breakpoints, which usually has no phenotypic effect in heterozygous form when only one of a pair of chromosomes is involved. The problems for carrier parents occur in gamete formation (Kayser-Rogers & Rao, 2005). The genetic effects are different depending upon whether the centromere is included within the inverted segment. Classically, when the inverted chromosome pairs with its normal homolog there is loop formation. If crossing over takes place within this loop the chromatids involved will be genetically unbalanced owing to deletion of some genetic loci and duplication of others. Cross-over chromatids from inversions that include the centromere (pericentric) may lead to viable but congenitally abnormal embryos, whereas those from paracentric inversions (without the centromere) will either have two centromeres or none, a condition that is incompatible with further development of the gamete as the chromosomes will be lost or broken. For this reason paracentric inversions usually pass undetected from one generation to another as no affected offspring

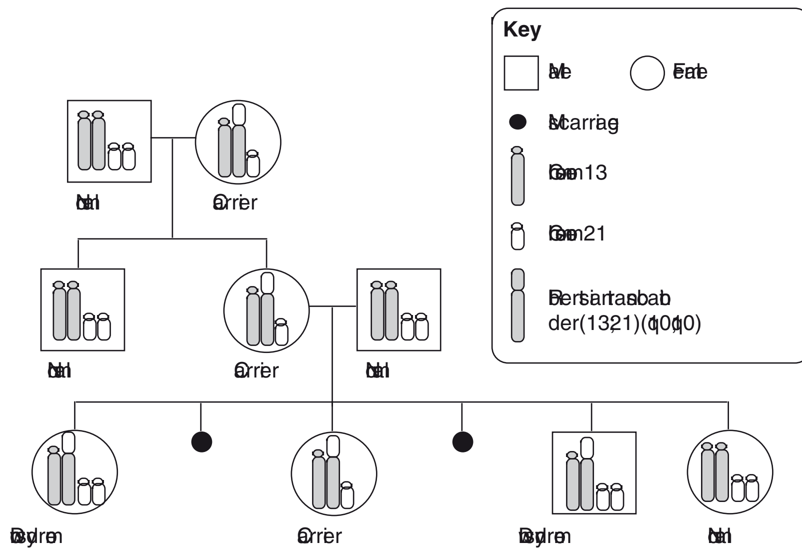

Figure 3.6 Pedigree of a family with a Robertsonian translocation between chromosomes 13 and 21. This balanced chromosome rearrangement was only detected in the healthy mother after the birth of two children with Down syndrome.

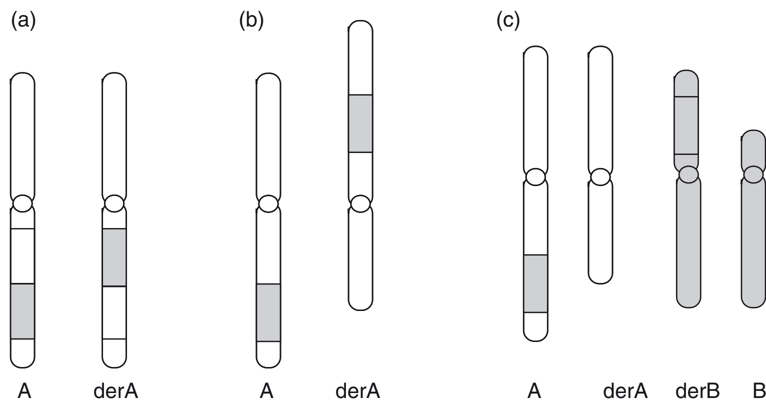

Figure 3.7 Insertions – translocation of a chromosome segment to another chromosome (*inter*-chromosomal) or to another position on the same chromosome (*intra*-chromosomal), either in the same orientation with respect to the centromere (direct) or reversed (inverted). Inserted segment is shown shaded.
(a) Within-arm intrachromosomal insertion; (b) between-arm intrachromosomal insertion; (c) interchromosomal insertion. Chromosome rearrangements showing the normal homolog (A, B) with the rearranged or derivative chromosome (der A, der B).

are born. However, couples with repeated births of chromosomally abnormal children as a result of a pericentric inversion in one parent have come forward to request PGD.

Ring chromosomes

Ring chromosomes are formed when genetic material is lost from both ends of a chromosome, creating an unstable situation which is resolved by the fusion of the broken ends (Figure 3.9). It is unusual to find ring chromosomes in a normal adult but they do occasionally occur as ring X-chromosomes or affecting an autosome if only the telomeres themselves are lost. Ring chromosomes are unstable during cell division as interlocking chromatids may be formed, leading to breakage and loss. Adult carriers are usually mosaics with a monosomic cell line in the case of the X-chromosome or a normal line if an autosome is involved. PGD has been requested in at least one case involving an autosome.

Mitochondrial disorders

Mitochondrial disorders are a group of diseases and syndromes commonly defined by lack of energy owing to

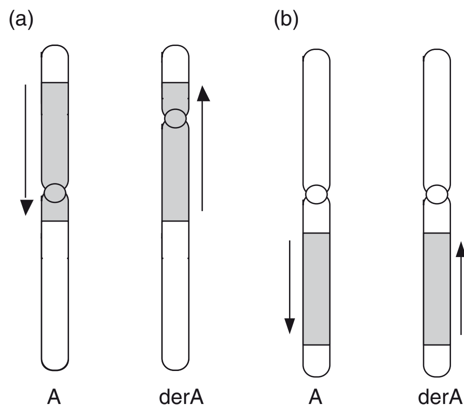

Figure 3.8 Inversions – inversion of a chromosome segment with (pericentric) or without (paracentric) involvement of the centromere. Inverted segment is shown shaded. (a) Pericentric inversion; (b) paracentric inversion. Chromosome rearrangements showing the normal homolog (A, B) with the rearranged or derivative chromosome (der A, der B).

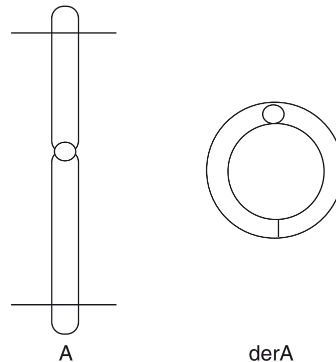

Figure 3.9 Ring chromosome – loss of telomeric material with fusion of the resulting free ends. Chromosome arrangement showing a normal homologue (A) with the rearranged derivative chromosome (derA).

defects in oxidative phosphorylation (OXPHOS). They affect at least 1 in 8000 of the general population, making them the most common inherited metabolic diseases. Clinical manifestations of OXPHOS diseases are extremely variable and range from a single affected tissue, like the loss of vision in Leber's hereditary optic neuropathy (LHON), to multisystemic syndromes like Leigh syndrome (subacute necrotizing encephalomyelopathy, LS), mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), neuropathy, ataxia and retinitis pigmentosa (NARP), and myoclonic epilepsy with ragged red fibers (MERRF) (Jacobs *et al.*, 2006).

The mitochondrial DNA is double-stranded, circular, and consists of 16 569 base pairs. The mtDNA encodes 37 genes, of which 13 genes encode proteins. Furthermore, there are 22 tRNA- and 2 rRNA-genes required for mitochondrial translation (Taanman, 1999).

Several unique characteristics discriminate mitochondrial from nuclear DNA:

- The mtDNA is a multicopy genome. A cell contains hundreds of mitochondria and each mitochondrion contains 5–10 copies of mtDNA. Dependent on the tissue and energy demand each cell contains between 500 and10 000 mtDNA molecules, except for mature oocytes, which contain beteen 100 000 and 600 000 mtDNA molecules.
- In a cell all mtDNA molecules may be identical (homoplasmy), or two types of mtDNA molecules, which differ in sequence, in the same cell, tissue, or even in the same organelle can coexist.
- The mtDNA is transmitted entirely through the maternal line.

Segregation of mtDNA diseases in families

The segregation of mtDNA disease in families is not straightforward, and is highly dependent on the nature and amount of the mtDNA mutation. A woman carrying an mtDNA mutation will transmit a variable amount of this mutation to her offspring. The percentage heteroplasmy of point mutations in the offspring is related to the mutation percentage in the mother, although extreme shifts in mutation percentages occur. The "mtDNA bottleneck" (Figure 3.10) is a restriction in the number of mtDNA molecules to be transmitted followed by an amplification of these founder molecules. The exclusive maternal transmission of mtDNA, the high mutation rate, and the lack of a good repair mechanism and recombination would lead to decay of the mtDNA. The stringent bottleneck has an evolutionary advantage as a sort of reset and acts to maintain a healthy mtDNA by filtering out mutations and minimizing heteroplasmy. Because this filtering happens very early during the development the chance to preserve age-related mutations in the early oocyte is small, although the low amount of mtDNA copies per mitochondria in the early developmental stages of the oocytes renders these oocytes vulnerable for mutational events.

Conclusions

Even for professional human geneticists it is impossible to keep up to date with the rapid progress that

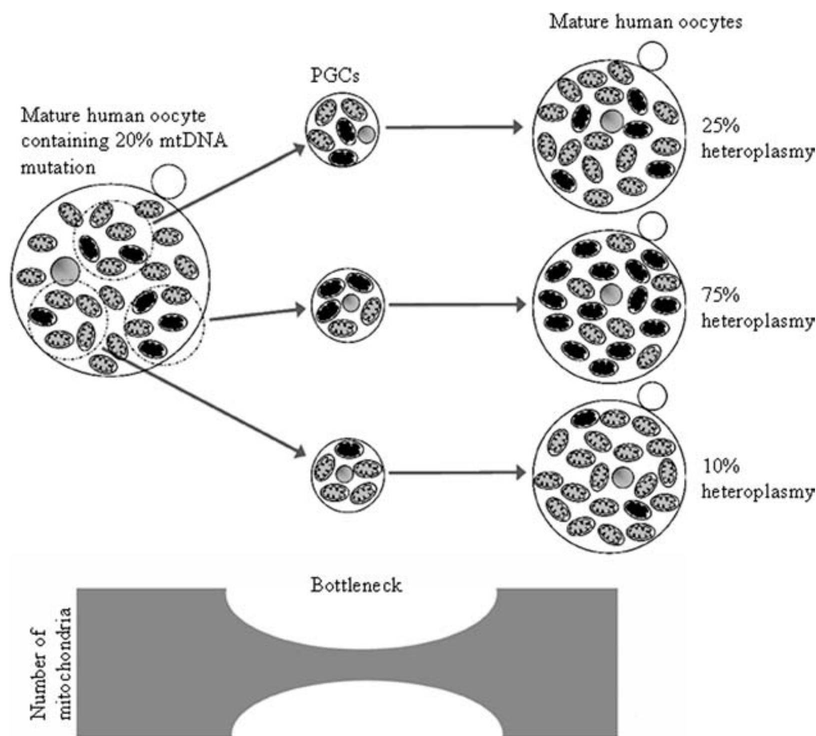

Figure 3.10 Drawing of the possible location and effect of the bottleneck on the transmission of an mtDNA mutation. The green-colored mitochondria represent the normal mtDNA and the red-colored mitochondria represent the mutated mtDNA. PGCs, primordial germ cells.

is being made in working out the molecular basis of inherited disease. Because of the widespread interest and importance of the subject, textbooks (such as the one suggested below) are regularly revised and they provide a good introduction to the field. The professionals rely on McKusick's *Mendelian Inheritance in Man*, now available online (OMIM) (www.ncbi.nlm.nih.gov/Omim), which is a comprehensive catalog of known single-gene disorders. Each described condition is given a number (the OMIM number) enabling geneticists to be sure which disease has been diagnosed, rather than relying on the name of the syndrome.

Further reading

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Chapter 4

Genetic counseling

Alison Lashwood

Key points

- Couples found to be at risk of transmitting a genetic or chromosomal disorder to their children need to have nondirective genetic counseling. This should include medical facts about the disorder and perception of their genetic risk, and the impact of the genetic disorder on the family. The couple can then choose the course of action suitable for them.
- Their reproductive options are: to remain childless, take a chance, or to consider prenatal diagnosis, preimplantation genetic diagnosis (PGD), gamete donation, or adoption.
- If the couple are considering PGD they will need a consultation with a PGD specialist who will outline the advantages and disadvantages of PGD. Issues requiring special attention may include discussion about the impact PGD may have on the couple and their family, for example when there is an affected child at home, their perception of PGD and its likely success, perception of their fertility, the welfare of the child in relation to genetic conditions that may cause ill health or early death in a parent, such as Huntington disease, and the impact of failure of treatment.
- The patients have to be fully informed about the misdiagnosis risks, the impact of multiple pregnancies, and the option of confirmatory prenatal testing.

Introduction

Preimplantation genetic diagnosis (PGD) is a complex process comprising many stages and involving a multidisciplinary team of health professionals. Centers offering such treatment will develop and deliver a service to patients and will be responsible for patient support before, during, and after a treatment cycle. PGD is highly invasive and demands a great deal of time and consideration by couples undertaking it. Practice will vary widely, although it has been acknowledged through the European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium that in order to offer a robust clinical service the centers providing PGD should involve the medical expertise of both assisted reproduction and clinical genetics departments (Bickerstaff *et al*., 2001). Recommendations have been made within the ESHRE PGD Consortium for all PGD services to adhere to guidelines that promote best practice (Thornhill *et al*., 2005). Within these guidelines specific acceptable practice requires that the counseling offered to couples requesting PGD is both nondirective and performed by an appropriately qualified professional.

Most couples who are referred for PGD have usually consulted a clinical geneticist in the first instance. This is an important area of practice as it affords couples the opportunity of specialist advice and support relating specifically to the genetic disorder within the family, and provides them with the knowledge to make a fully informed decision before they proceed to PGD. Every couple will present with their own individual agenda and it is the responsibility of the clinician to help assess the needs of the couple and discuss their future options with them (Harper, 2003). Genetic counseling has many facets, and there are many descriptions of what is meant by it, but for the purposes of this chapter the following is a useful definition from the American Society of Human Genetics (Ad Hoc Committee on Genetic Counseling, 1975). Genetic counseling may be described as in Table 4.1.

It is important not to confuse genetic counseling, which employs the use of "counseling skills," with therapeutic counseling that is long term and undertaken by recognized accredited counselors (Skirton & Patch, 2002). However, all health professionals should at least be aware of basic counseling skills as health and psychosocial issues are closely related. Clinicians who are

Table 4.1 Genetic counseling as defined by the American Society of Human Genetics

A communication process which deals with the human problems associated with the occurrence, or risk of occurrence, of a genetic disorder in a family. It involves an attempt to help the individual or family:
Comprehend the medical facts about a disorder
Appreciate the way in which heredity contributes to the disorder and to the risk of recurrence
Understand the options for dealing with the risk of recurrence
Choose the course of action which seems most appropriate to them
Make the best possible adjustment to the disorder in an affected family member.

Table 4.2 Skills needed for genetic counseling
Ask open questions
Notice nonverbal communication
Allow periods of silence
Clarify understanding as consultation proceeds
Summarize and paraphrase what the patient has said
Challenge if things are unclear

consulting with patients should be aware of the skills needed (Table 4.2).

A basic tenet of genetic counseling is that it is delivered in a nondirective manner. Kessler (1997), in his work on the impact of genetic counseling, explained the need to be nondirective. The aim of this is to present accurate information to patients in a way that they can decide the best course of action for themselves without conscious or subconscious pressure from the clinical professionals involved in their care. Families will still require the support of clinical genetics professionals, but their decisions are reaffirmed by the professionals rather than made for them. Many have argued that although this is a laudable aim, in reality true nondirective genetic counseling is not uniformly practiced (Michie *et al.*, 1997). Recommendations exist in the UK for the level of training and education required to ensure high standards of practice and an understanding of the need for a nondirective approach in genetic counseling (Skirton *et al.*, 1998).

Genetic counseling services will vary from country to country, but in many centers the professionals offering such a service will be either medically qualified clinical geneticists or genetic counselors. Genetic counselors will generally have education in either a nursing or a science discipline, usually having undertaken a Masters-level degree in a related field. In the UK, unlike North America, professional regulation is not yet in place, but a voluntary registration scheme based on the submission of a portfolio of evidence fulfilling required competencies has been in place since 2002 (Skirton *et al.*, 1998), and a scheme for national regulation is being developed.

The combined skills of genetic counselors and clinical geneticists from recognized accredited genetic centers and specialists in assisted reproduction should ensure that patients receive a high-quality service in PGD.

For the purposes of this chapter the reader should assume that genetic counseling will be provided by either genetic counselors or clinical geneticists.

Genetic counseling before PGD treatment

We now deal with the issues that are discussed at an initial PGD consultation, and describe the relevance and benefits of genetic counseling at this stage in the referral process. The issues generally addressed during such a PGD consultation are summarized in Table 4.3.

Couples should receive a written summary of the consultation, and accompanying patient information leaflets should be available to help them understand and retain the clinical details given (Thornhill *et al.*, 2005).

Family history and past experience

The first PGD consultation is important as it should help to establish how a couple are coping with their situation, whether PGD treatment is appropriate at the time, or whether they have psychological needs that may be negatively affected by treatment.

In 2002, Skirton and Patch said, “Psychological care of adult patients is as important as genetic information as guilt, blame, anxiety and hopelessness may accompany the feeling of risk in any of these situations.”

Those who seek genetic counseling initially usually do so because they fall into one of the following categories (Table 4.4).

For many, this means they have been bereaved by the loss of a child, pregnancy, or other family member. However, additionally it can signify the loss of reproductive freedom or the loss of health of that individual. Although these issues will have been addressed at the initial genetic consultation, revisiting the history is important to allow a couple to express their feelings and have the significance of their loss recognized. Telling one’s personal story was recognized by Brock

Table 4.3 Issues to be addressed during a preimplantation genetic diagnosis (PGD) consultation
The family history and reason for requesting PGD
Review of the diagnosis and understanding of risk
Review of other reproductive options
Explanation of the PGD treatment
Discussion of the advantages and disadvantages related to PGD

Table 4.4 Categories of patients requiring genetic counseling
Those who have had a child or pregnancy diagnosed with a genetic condition
Those who have a child or pregnancy with dysmorphic features or physical or developmental abnormalities
Those who have a genetic condition themselves
Another family member has a genetic condition
Those who have experienced recurrent miscarriages
Those who are carriers of an autosomal recessive condition based on their ethnicity

(1995) as a therapeutic process that helps adaptation to bereavement in the long term.

A consultation will present an opportunity for couples to tell their story, and may provide valuable information about their motivation for requesting PGD; it also allows assessment of how they are coping and helps the genetic counselor to establish if they have additional counseling needs. Flis-Treves *et al.* (2003) established that, aside from the clinical value, a PGD consultation had a major part to play in giving couples an opening to share their experience, thus helping them with their grieving process.

The impact of the history, and the affect this has had on the couple, will depend upon many issues so it is important for the genetic counselor to consider how long ago a couple lost their child, for example, or how many times they have terminated a pregnancy. Alternatively, the couple may recently have experienced the death of another relative, and together these factors may have implications for the timing of treatment or their coping mechanisms. Anniversaries of loss are important and can be incredibly difficult periods for those coping with grief. It may be in the interests of a couple not to undertake PGD treatment that may coincide with a time when they may expect to have to face specific memories or acute grief such as the date of their child's death. Lavery *et al.* (2002) reviewed the experiences of 67 couples who had undergone a PGD cycle and found that 41 percent reported the treatment as being extremely stressful. Of those who had experienced both prenatal diagnosis (PND) and PGD, 35 percent indicated that they found PGD the more stressful of the two processes. It is therefore imperative that any potential impact of treatment must be considered as a couple's coping mechanisms may be less robust at such a time and this could affect how they manage a PGD cycle psychologically.

Some couples or individuals will experience feelings of guilt associated with their reproductive history, apportioning blame to the carrier of the single gene disorder or chromosome rearrangement. The feelings of guilt and blame may differ with the disorder and mode of inheritance. James *et al.* (2006), in their study of X-linked carrier women, found that being such a carrier was perceived as more stigmatizing – with associated increased guilt – compared with those who were carriers of recessive conditions, probably as in the latter scenario the burden of guilt is shared. While investigating attitudes in women with dominant conditions Faulkener and Kingston (1998) found that those with myotonic dystrophy reported high levels of guilt relating to the risk to their offspring, which, in turn, affected their reproductive decisions. Although such thoughts are frequent and recognized, if a couple or individual do not adapt to the situation this may also indicate a need for referral for therapeutic counseling.

Outcome of genetic counseling

Many years of research into the value of genetic counseling and the impact on outcome has been hampered by a poor understanding of what outcome measures should be used. Measuring patient satisfaction or a simple cost–benefit analysis without taking account of the greater social impact of genetic counseling is of limited use. Clarke (1993), Chadwick (1994), and McAllister *et al.* (2007) have suggested that genetic services should be evaluated by the impact of genetic counseling on the patient's perspective of the disorder, and this research group is using the results of their initial qualitative study to develop useful outcome measures for clinical genetics. However, it is clear from many reports that one of the key elements for patients' understanding and satisfaction following genetic counseling is whether or not their perceived needs are met at the consultation (Sorenson *et al.*, 1981; Hopwood, 2005). Therefore, in practice it is beneficial both to offer a couple, at the start of an appointment, the chance to "tell their story" and also to ask them to explain what information they want from the consultation. This will enable the genetic counselor or geneticist to address the issues that may

be of greatest importance to them and prevent any misunderstanding of their needs.

Accurate information and confirmation of risk

It is essential that couples are aware of current information in relation to the genetic disorder itself. Some individuals may have consulted with clinical genetics professionals several years previously, when they were tested for their carrier status, perhaps as part of active cascade testing (Super *et al*., 1995) or family studies. Clinical genetics is a very dynamic field and with advances in the molecular basis of genetic conditions more accurate defining of carrier status is possible. For example, let us look at the case of a woman who had a brother and a maternal uncle with Duchenne muscular dystrophy, an X-linked condition caused by a mutation in the dystrophin gene (OMIM #310200). Initially, when tested, no mutation was detected in the affected brother, so polymorphic markers in the region of the dystrophin gene on Xp21 were used to establish whether the woman had inherited the same maternal X chromosome as her brother or the opposite maternal X chromosome. As she carried the same X chromosome the woman was given a high risk of being a carrier (95 percent), with a small risk of error due to recombination (Lindlöf *et al*., 1986). With further development of mutation analysis in Duchenne muscular dystrophy, and the detection of the causative mutation in her brother, her carrier status could be more accurately defined. Molecular testing revealed that the woman did not carry the mutation in the dystrophin gene, and therefore she was not a carrier and would not have affected children.

In addition, some chromosomal rearrangements initially believed to carry reproductive risk in the form of miscarriage or physical or mental disability, where family members may have considered prenatal testing, may be reevaluated and thus now represent normal variants without reproductive risk (Mackie Ogilvie & Scriven, 2004).

In both the above examples it is possible that without access to contemporary genetic counseling, such individuals may have opted for PGD on the basis of out-of-date information which led them to believe they were at high risk of abnormality, whereas, in fact, the converse applied. The author reports direct and recent experience of a similar case history where the redefining of risk has prevented a second cycle of PGD as the carrier status of the woman has been confirmed, indicating that she is not a carrier.

Review of risks

Patients requesting PGD will be given many risk or chance figures. The risk of inheritance, side effects of treatment, misdiagnosis, the chance of success, or of multiple pregnancies, will all be discussed during a consultation.

Many of those who have consulted with clinical geneticists will come away with a clear understanding of the genetic basis of the disorder affecting their family and the risks of occurrence and recurrence. Explaining risk in the context of genetic counseling is an essential and basic component of the aims of a consultation. It is therefore essential that the risk information is presented in a way that is understood by the patients and that factors which may influence understanding are taken into account.

There may be marked differences in perceived risk and actual risk, as described by Marteau *et al*. (1991) looking at women's uptake of amniocentesis for raised maternal age. McAllister (2003) found that some individuals may even construct a theory as to the background of their inheritance, which may help them to cope with their risk but may be a barrier to their understanding of risk. An awareness and exploration of such beliefs by the genetic counselor would be beneficial to help ensure accurate risks are understood.

The presentation of risk in either numerical or descriptive terms has been reviewed in several studies (Marteau *et al*., 2000; Hopwood *et al*., 2003), and the overall conclusion is that recall and understanding are better if a numerical odds risk is given. It is therefore better to give the risk as 1 in 400, for example, rather than a descriptive "low chance." However, it is clear during discussion with couples requesting PGD that a significant number have not completely understood the inheritance mechanism and the risks attributed to this (Rona *et al*., 1994). There may be many reasons behind this, but it is vitally important that this understanding is addressed and clarified with couples where necessary. Michie *et al*. (2005), in an observational study looking at the communication of risk in genetic counseling, noted that 43 percent of patients did not respond to indicate their understanding or nonunderstanding of the risk given. Their understanding of the risk was only checked in 9 percent of cases by the genetic counselor, raising concern that some patient decisions are taken without full understanding of the associated risks.

A genetic counseling consultation at the start of the PGD process can help to establish understanding

Table 4.5 Options for couples at risk of transmitting a genetic disease to their child

Having none or no further children
Taking a chance, becoming pregnant spontaneously with no confirmatory testing in pregnancy
Prenatal diagnosis (chorionic villus or amniocentesis) in a pregnancy
Preimplantation genetic diagnosis
Gamete donation (egg or sperm)
Adoption

of risk and ensure couples are making their decisions based on accurate information.

Reasons for requesting PGD

The reproductive options for couples at risk of genetic disease will usually have been addressed by the genetic clinician prior to referral for PGD. Couples will have reviewed their potential options (Table 4.5).

Couples requesting PGD do so for a variety of reasons. Asking couples the reason behind their request is important when establishing whether their expectations may be met.

Prenatal diagnosis

Many couples will have experienced the loss of a child or a pregnancy and possibly had PND in subsequent pregnancies (see Chapter 5). This may have ended in the termination of an affected pregnancy. Past studies have shown that termination of pregnancy for fetal abnormality or "genetic termination" carries with it potentially serious psychological consequences in both the short and long terms (Donnai *et al.*, 1981; Seller *et al.*, 1993). Termination of pregnancy for genetic reasons is different from social termination as it means the loss of a generally wanted and planned pregnancy. Many women requesting termination for abnormality will have reached the second trimester. In one study of 84 women having undergone termination in the second trimester, 20 percent reported psychological difficulties affecting their general well-being two years after the termination (White-van Mourik, 1992). Korenromp *et al.* (2005) found more adverse outcomes where there was less support from partners, if the abnormality was not necessarily lethal, and when the gestation of pregnancy was advanced.

For many patients the time between taking a positive pregnancy test and undertaking PND is a huge emotional burden that they know may end unsuccessfully. Such couples see PGD as relieving them to an extent of the burden of having PND and termination of pregnancy, and also as giving them the chance of knowing from the earliest possible time that a pregnancy is unaffected. A study by Snowden and Green (1997) reported on attitudes of male and female carriers of recessive disorders to the availability of PGD and other reproductive options. The results indicated that the early knowledge of an unaffected pregnancy, the biological link with both parents, and the avoidance of termination of pregnancy were all deemed important advantages of PGD.

Social, moral, or religious beliefs all play a major part in many couples' reproductive decision-making, and termination of pregnancy may not be an acceptable option. Eighty-six percent of women in one study stated that avoidance of termination of pregnancy was the main advantage of PGD (Pergament, 1991). Palomba *et al.* (1994) suggested that "preimplantation diagnosis is the chosen option when there is a strong desire for pregnancy together with an equally strong desire to avoid abortion."

Infertility related to genetic disorder

Some of the couples seen in clinical genetics clinics will have fertility problems related to the monogenic or chromosomal abnormality that they carry. For example, men affected by classical cystic fibrosis (CF) (OMIM #277180) will have congenital bilateral absence of the vas deferens (CBAVD). In addition, some men who have congenital bilateral absence of the vas deferens will be carriers of non-classical CF and be heterozygous or homozygous for causative mutations (Daudin *et al.*, 2000; Ratbi *et al.*, 2007). Alternatively, a man with oligozoospermia may carry a chromosomal translocation (Gardner & Sutherland, 2004). These couples may require assisted reproduction to address their primary infertility problems, and therefore the additional step of embryo biopsy would seem reasonable to give the couple the best chance of an ongoing unaffected pregnancy. However, the significance of a genetic disorder and their view of the risk and alternative options, such as fertility treatment followed by prenatal diagnosis, should be discussed in full with the couple.

Special genetic counseling issues

Late-onset disorders

Many centers offering PGD are now able to do so for genetic disorders that may not affect an individual until adulthood, for example Huntington disease. Huntington disease is an autosomal dominant

inherited disorder. It is a late-onset, degenerative, neuropsychiatric disorder with symptoms including choreiform movements, cognitive impairment, and associated psychiatric illness. The average age of onset is between 35 and 50 years and the course of the disease is about 20 years' duration. In the early stages of the disease it is possible for an affected individual to suppress the movements, but as the disease progresses, control is limited (OMIM 143100). The gene IT 15 was isolated and found to comprise an unstable expanded trinucleotide repeat sequence in affected individuals (Huntington's Disease Collaborative Research Group, 1993). The mutation is common in all those affected and any individual at risk may now be tested for the gene from the age of 18 years (Craufurd & Tyler, 1992). Mutation testing allowed at-risk individuals who wished to be tested to do so independently of DNA availability from affected family members.

Those who request PGD for Huntington disease usually comprise two categories, as follows.

- The prospective parent has undergone presymptomatic testing and is a Huntington disease gene carrier (Tyler *et al*., 1992).
- The prospective parent is at 50 percent risk of Huntington disease and has decided against presymptomatic testing. Those who opt not to undergo testing often state the overriding reason behind nonparticipation is concern about their ability to cope with a bad news result (Bloch *et al*., 1989; Tibben *et al*., 1993).

Offering PGD for late-onset disorders presents the clinician and the couple with some additional specific considerations.

Welfare of the child

The potential for future ill health in a prospective parent raises an additional specific issue that needs to be addressed during genetic counseling. In the UK, practicing clinicians are bound to practice in accordance with the 1990 Human Fertilisation and Embryology Act (HFEA 1990). As part of this Act the welfare of any child born following any form of assisted reproductive technologies (ART), including PGD, must be taken into consideration. When the prospective parent either has a genetic disorder or may become symptomatic at a future stage, an open discussion with the couple is required. Addressing issues about the future care of the affected partner in addition to the care of any children born as a result of PGD would be beneficial. Asking the couple to consider what additional support they may have now or in the future may enable them to think about planning for the future. If at any stage it was felt that the impact of a parent's illness may be a risk for a future child, it should be the responsibility of the clinician to address this and if necessary may lead to refusal of treatment. This may create difficulties for staff working within PGD centers while they balance social aspects with medical aspects relating to the welfare of the child. This professional tension has been studied by Ehrich *et al*. (2006), who, through clinical observation and interviews in two PGD units, concluded that staff should deal with such issues along a continuum that considers both social and medical issues related to the patient, the "state," and the boundary of professional accountability.

In many countries the same legal framework will not exist, but it is important to consider these issues for the sake of the health and welfare of the couple and their children.

Confirmation of diagnosis

In accordance with best practice guidelines all couples who become pregnant following PGD are offered confirmatory prenatal testing. Uptake of this is variable. One of the problems associated with an absence of prenatal confirmation for Huntington disease PGD was that confirmatory testing at birth could result in mutation detection in a child if a misdiagnosis has occurred. It is widely accepted that testing children for late-onset disorders, in which no intervention prevents or ameliorates the symptoms, is not advocated (Bloch *et al.,* 1990; Clinical Genetics Society, 1994; British Medical Association, 1998). In PGD practice this means that if a couple decline confirmatory PND then testing at birth could represent a disclosure of the child's Huntington disease status to which that child has not consented. Many centers feel that if a couple will not consider termination of such a pregnancy then it is not appropriate to offer a PND test (Tyler *et al*., 1990; Thies *et al*., 1992). Although the risk of misdiagnosis is low this issue requires discussion before the start of treatment.

Impact of PGD on affected children

This is an issue that rarely occurs in the realms of ART, but some couples requesting PGD will be caring for children affected with specific genetic disorders. As a result genetic counseling related to PGD must address this. The impact of the treatment schedule, risk of

ovarian hyperstimulation syndrome, and multiple pregnancies may be further complicated when couples are also responsible for potentially the full nursing care of another child. Establishing support networks is important to ensure back-up if there are complications associated with treatment.

Multiple pregnancies

The frequency of multiple pregnancies in PGD couples has reduced from 25 percent to 20 percent over two time periods: 1999–2003 and 2003–2004 (Sermon *et al*., 2007). It is, however, still a significant factor to be considered in treatment. Decision-making about the number of embryos for transfer in PGD is important and requires careful discussion between patient and clinician. Studies are now demonstrating improvements in live-birth outcome using single blastocyst transfer (Papanikolaou *et al*., 2005), and that the use of single embryo transfer, in particular in women under the age of 36 years (Donoso *et al*., 2007), is resulting in fewer multiple pregnancies without compromising delivery rates.

In a study by Bryan (2005), and from the author's own experience, it appears that couples may not have fully considered the impact of multiple pregnancies. Their overriding desire for a child may have affected their decision. Newton *et al*. (2007) looked at factors affecting the number of embryos transferred in a population of infertile women and saw a marked difference between cautious and less-cautious patients. The former balanced the risk of success with the implications of multiple pregnancies, whereas the less-cautious may be more influenced by age or their desire for twins. In conclusion, they felt that counseling in relation to embryo transfer may need to be adapted depending on the level of caution displayed by the patient.

One of the aims of genetic counseling should be to ensure that couples are completely aware of the facts and understand the implications for their family and the health of a potential PGD baby. The issues that may have an impact will be whether or not they have children already, with or without disability, the financial impact of a multiple pregnancy, and how a multiple pregnancy would affect the availability of confirmatory prenatal diagnosis.

Success rates

According to European and local data (Harper *et al*., 2003), the overall success rate of PGD per oocyte retrieval is approximately 18–25 percent. Given that the majority of patients are fertile, the comparative odds of success are low. For those who have requested PGD because the alternatives, including prenatal diagnosis, are unacceptable to them, the low success rate is tolerated. However, this situation must be addressed in relation to the impact a failed cycle can have on a couple and how they cope with this.

Perception of fertility

One area that has not yet been explored in the literature is that of couples requesting PGD and the perception of their fertility. In most cases couples have proven fertility and their request for PGD is generated from a concern over the acceptability of alternative reproductive options. However, there are also a number of couples who have initially given their reluctance to consider termination of pregnancy as the reason behind their request for PGD, who, on closer questioning, raise concern over their ability to conceive. It is important to establish this as, for some couples, the perception of infertility may be misplaced and thus PGD may not be the most acceptable way forward. One PGD center has reported data looking at 123 couples referred for PGD who became pregnant while waiting for treatment. Among several reasons given, some couples indicated that it was the need to confirm their fertility that led to the pregnancy (Flis-Treves *et al*., 2005). This may be an area that warrants further research.

Role of genetic counseling in later stages of PGD

The role of genetic counseling will extend past the initial PGD consultation and may involve providing a supportive role during treatment. Other genetic counseling input may be required. Once molecular or chromosomal analysis is complete, further explanations of the outcome of the results may be needed.

If PGD is successful a couple may want to consider confirmatory prenatal testing. The genetic counselor will be in a position to discuss the options, arrange the test, and discuss the results.

After delivery obtaining pediatric follow-up data is important, or organization of confirmatory postnatal testing is required if prenatal testing was declined.

Conclusion

PGD is a complex and extensive process, but is managed well using a multidisciplinary approach. The wide-ranging psychosocial impact of PGD is just as important as the complexity of the procedure and

analysis itself. Ensuring that couples have access to professionals involved in both genetic counseling and assisted reproductive medicine provides them with accurate contemporary information about genetic risk and reproductive options. This approach helps us to assess their coping mechanisms and affords the couple an opportunity to make an informed decision about proceeding with PGD. In addition, it will provide a high-quality care package throughout treatment.

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Chapter

5 Prenatal screening and diagnosis

Anna L. David and Charles H. Rodeck

Key points

- Prenatal diagnosis of congenital birth defects or genetic disorders is based on screening a low-risk population using noninvasive tests. The various strategies available include screening by maternal age, genetic and obstetric history, maternal serum, and ultrasound. Magnetic resonance imaging (MRI) may be used to better visualize certain fetal organs, such as the brain.
- Ultrasound may be used in combination with soft markers of aneuploidy, such as increased nuchal translucency, to provide a risk assessment for an individual woman. It may also be used to identify structural abnormalities known to be associated with aneuploidy, such as exomphalos, for example, a hernia of the anterior abdominal wall.
- Measurement of specific biochemical markers in the maternal serum may be combined with the maternal age-related risk of Down syndrome to provide a risk assessment in an individual woman. Screening tests that combine ultrasound and serum markers have the highest detection rate for Down syndrome.
- Invasive tests are used to obtain fetal cells, fluids, or tissues for prenatal diagnosis and include amniocentesis, chorionic villus and fetal blood sampling, and sampling of fetal tissues such as the skin for prenatal diagnosis of congenital epithelial disorders, for example. The technique, miscarriage risk, and optimum gestational age will be discussed for each method.
- For chromosome analysis, a karyotype, or rapid methods that use fluorescent *in situ* hybridization (FISH) or quantitative fluorescence polymerase chain reaction (QF-PCR) may be performed.
- A more recent development is the use of free fetal DNA in the maternal circulation to test for Rh-positive fetuses in Rh-negative women who are at risk of developing Rh alloimmunization. Free fetal DNA has also been used in the prenatal diagnosis of certain paternally inherited genetic disorders and to sex fetuses where the mother is a carrier of an X-linked condition.
- Potential future developments in prenatal diagnosis, such as noninvasive diagnosis of aneuploidy using maternal circulating nucleic acids, will be discussed.

Introduction

Preimplantation genetic diagnosis (PGD) is considered as an alternative to prenatal diagnosis and the same types of patients are treated. Patients referred for PGD may have previously undergone an invasive prenatal diagnosis procedure and terminated an affected pregnancy. After PGD it is common to advise patients to undergo prenatal diagnosis to confirm that the pregnancy is normal. However, in practice, most patients decide not to embark on an invasive prenatal diagnosis procedure after PGD. Noninvasive tests can now be used in certain cases, such as free fetal DNA to diagnose certain paternally inherited genetic disorders and to sex fetuses where the mother is a carrier of an X-linked condition, and screening for those at risk of age-related aneuploidy and certain chromosome abnormalities.

Prenatal diagnosis of certain congenital birth defects is now part of routine antenatal care. It first became feasible in the 1960s for families at risk of X-linked inheritance to confirm a female fetus by identifying the Barr body in amniocytes taken by amniocentesis. The prenatal diagnosis of Down syndrome by amniocentesis was reported in 1968 (Valenti *et al.*, 1968) and elevated levels of α-fetoprotein (AFP) in amniotic fluid were associated with anencephaly in 1972 (Brock & Sutcliffe, 1972). Fetal hemoglobinopathies were subsequently diagnosed

Table 5.1 Screening tests used in prenatal diagnosis

Screening tests
Maternal age
Genetic history
Previous obstetric history
Abnormal maternal serum screening
Measurement of nuchal translucency (NT) in first trimester using ultrasound
Absent nasal bone in first trimester using ultrasound
Fetal Doppler blood flow measurement
Anomaly ultrasound scan in second trimester

by chorionic villus sampling (CVS) in 1982 (Old *et al.*, 1982). Since then, ultrasound technology has developed as both a screening and diagnostic tool. The development of cytogenetics, biochemistry, and molecular biology has now facilitated prenatal diagnosis of an ever increasing number of inherited genetic disorders. A more recent development is the use of free fetal DNA in the maternal circulation to test for Rh-positive fetuses in Rh-negative women who are at risk of developing Rh alloimmunization. This has been extended to certain genetic conditions, and may in the future be used to screen for or even to diagnose fetal aneuploidies.

Prenatal diagnosis has been rapidly accepted within antenatal care in accordance with modern society's changing attitudes to childbearing, congenital defects, and religious beliefs. In this changing climate, with the reluctance to accept physical handicap, the ethical issues implicit in prenatal diagnosis should not be ignored. Maternal autonomy allows a potential mother to limit perceived suffering for herself, her unborn child, and her family, and this must be balanced against the ethical standpoint of the unborn fetus. Furthermore, economic constraints will be increasingly important as medical resources are challenged by the rapid growth of these technologies and choices.

Prenatal diagnosis has an important role in confirming normality and in offering women the widest options after diagnosis of abnormality. This may be to terminate the pregnancy and prevent handicap (secondary prevention) or to prepare the mother and family for care of the affected baby, prior to delivery. Prenatal diagnosis of certain abnormalities will optimize antenatal management and may provide an opportunity to offer intrauterine treatment and limit progression of disease before birth.

This chapter will outline the principles and techniques of prenatal diagnosis as currently practiced and discuss some potential future developments in prenatal diagnosis.

Screening versus diagnostic tests

The prevalence of congenital abnormalities, from major to minor, is in the region of 2 percent (Baird *et al.*, 1988). Prenatal diagnosis of congenital birth defects or genetic disorders is based on screening a low-risk population. In the past standard screening policies to detect Down syndrome (trisomy 21), for example, relied on offering fetal karyotyping to pregnant women over the age of 35 years, who have a higher maternal age-related risk (Hook, 1981). However, this failed to significantly reduce the incidence of Down syndrome babies that were born, as the majority of affected babies are born to women under the age of 35 (Walker & Howard, 1986). This stimulated the development of screening tests to identify high-risk pregnancies with increasing sensitivity.

Screening tools that are currently used in antenatal care are shown in Table 5.1. In prenatal screening for Down syndrome, the maternal age-related risk may be altered by results of maternal serum screening, nuchal translucency measurement, the absence of the nasal bone, and first-trimester fetal Doppler (see below), and the best screening strategies use a combination of the above. Ultrasound examination of the fetus in the second trimester can identify structural defects that are known to be associated with aneuploidy, such as a cardiac abnormality such as Tetralogy of Fallot. For these pregnancies and those screened to be at high risk of aneuploidy, an invasive diagnostic test for fetal karyotyping must then be considered, with the knowledge that invasive testing carries a risk of losing the pregnancy (Table 5.2). Using screening before applying an invasive diagnostic test therefore limits the number of invasive prenatal diagnostic tests that are offered. Screening tests should have a high sensitivity (detection rate) and high specificity (low false-positive rate). Diagnostic tests must be as safe and accurate as possible.

Table 5.2 Prenatal diagnostic tests

Prenatal diagnostic tests	
Noninvasive tests	Free fetal DNA
	Ultrasound – two-, three-, and four-dimensional
	Magnetic resonance imaging (MRI)
Invasive tests	Amniocentesis
	Chorionic villus sampling (CVS)
	Fetal blood sampling
	Fetal tissue sampling

Ultrasound in prenatal diagnosis

Ultrasound technology has developed rapidly. Routine ultrasound screening in two stages is recommended in the UK (Royal College of Obstetricians and Gynaecologists, 2000). With improved technology, in particular with the use of transvaginal probes (Achiron & Tadmor, 1991), it has become possible to assess fetal anatomy in the late first and early second trimesters (Timor-Tritsch *et al.*, 1988; Cullen *et al.*, 1989). The introduction of maternal serum biochemistry and nuchal translucency screening for Down syndrome drove the need for routine 12 weeks' scanning to confirm gestational age for correct interpretation of the test. Early diagnosis of pregnancy failure, ectopic, and multiple pregnancies have also stimulated this development. The first scan in early pregnancy, referred to as a booking scan, is undertaken ideally before 15 weeks and its purpose is to establish the correct gestational age, viability, fetal number, and, in multiple pregnancies, the chorionicity/amnionicity and detection of gross fetal abnormalities (Royal College of Obstetricians and Gynaecologists, 2000). The routine second-trimester "20-week" anomaly scan is usually performed between 18 and 22 weeks of gestation when the fetal heart, face, and kidney structures become more discernible. Quality standards for the diagnosis of abnormalities, description of markers of aneuploidy, record-keeping, information-giving, and care of the pregnant woman are established and should be audited regularly in units that provide this service (RCOG, 2000).

There is continuing debate as to whether prenatal diagnosis early in pregnancy may be advantageous by allowing the patient to undergo surgical rather than a medical termination of pregnancy (Statham *et al.*, 2007). Healthcare professionals place a higher value on earlier tests than pregnant women (Bishop *et al.*, 2004) and it is clear that a false-positive diagnosis results in high maternal anxiety (Susanne *et al.*, 2006). Therefore clinicians should aim to provide the best screening test possible for the individual woman.

First-trimester ultrasonography

Diagnosis of structural anomalies in the first trimester

First-trimester sonographic assessment of fetal anatomy has become an important component of prenatal diagnosis. It requires a good understanding of embryological development, to ensure that what the sonographer regards to be abnormal is not a developmental stage, for example to differentiate between a physiological abdominal hernia and exomphalos. The optimal gestational age for early fetal anomaly scanning will depend on the time that the majority of fetal structures may be visualized, at or after 12 weeks' gestation, although over 80 percent of the most common structural defects are present by this gestation. The completeness of the examination of the fetal organs in the first trimester is limited by fetal size and maternal habitus (Souka *et al.*, 2006). Either the transvaginal (TV) or the transabdominal (TA) route may be used, although most authors report better overall visualization using TV sonography (Cullen *et al.*, 1989; Achiron & Tadmor, 1991). In one study, use of the TV approach increased successful examination of the fetal anatomy from 72 percent to 86 percent of the fetuses, and was particularly helpful in examining the face, kidneys, and bladder (Souka *et al.*, 2006). Prospective studies show that up to half the major structural defects may be detected by the 11 to 14 weeks' ultrasound scan in low-risk (Hernadi & Torocsik, 1997; Economides & Braithwaite, 1998; Carvalho *et al.*, 2002; Taipale *et al.*, 2004; Souka *et al.*, 2006) and high-risk populations (Chen *et al.*, 2004). However, diagnostic criteria and limitations for specific anomalies must be clearly established. Additional training of sonographers and sufficient time for the scan will also be required before first-trimester ultrasound screening can be introduced universally. A normal sonographic assessment at 12 weeks' gestation may be reassuring, yet such reassurances must be guarded

in view of the failure to detect some anomalies that are sonographically gestation-dependent, for example duodenal atresia, hydrocephalus. The natural history of some conditions detected in the first trimester remains unclear, for example bright echogenic kidneys, choroid plexus cysts. Furthermore, some findings resolve spontaneously with no long-term consequences, for example cystic hygroma. Sonographic findings in the first trimester may not have the same clinical significance as those detected in the second and may lead to considerable parental anxiety. Therefore the place of late first-trimester sonography as the sole anomaly assessment has yet to be proven and standard TA sonography in the second trimester (at 18–22 weeks' gestation) is still recommended.

The use of nuchal translucency in the first trimester

An increased nuchal fold (NF) in the second trimester was reported to have an association with aneuploidy by Bronshtein *et al.* (1992) and an increased nuchal translucency (NT) in the first trimester, with Down syndrome by Szábo and Gellén (1990). The NT is the maximum thickness of the subcutaneous translucency between the skin and the soft tissue overlying the cervical spine of the fetus (normal NT measurement <3mm) (Figure 5.1). NT may conveniently be assessed at the dating scan between the eleventh and thirteenth weeks of gestation, and has proved to be a useful screening test for aneuploidy: the thicker the NT the higher the risk of trisomy 21 (Pandya *et al.*, 1995). An accurate NT measurement is dependent on the distinction of both fetal skin and amnion as, at this gestation, both structures appear as thin membranes. Increased NT is also recognized to be associated with structural defects of the cardiovascular or skeletal systems that are not necessarily amenable to prenatal diagnosis in the first trimester. An increased NT has been used to screen for fetal cardiac defects in a low-risk population (Hyett *et al.*, 1999) and following the identification of increased NT in a karyotypically normal fetus a detailed second-trimester cardiac scan should be performed.

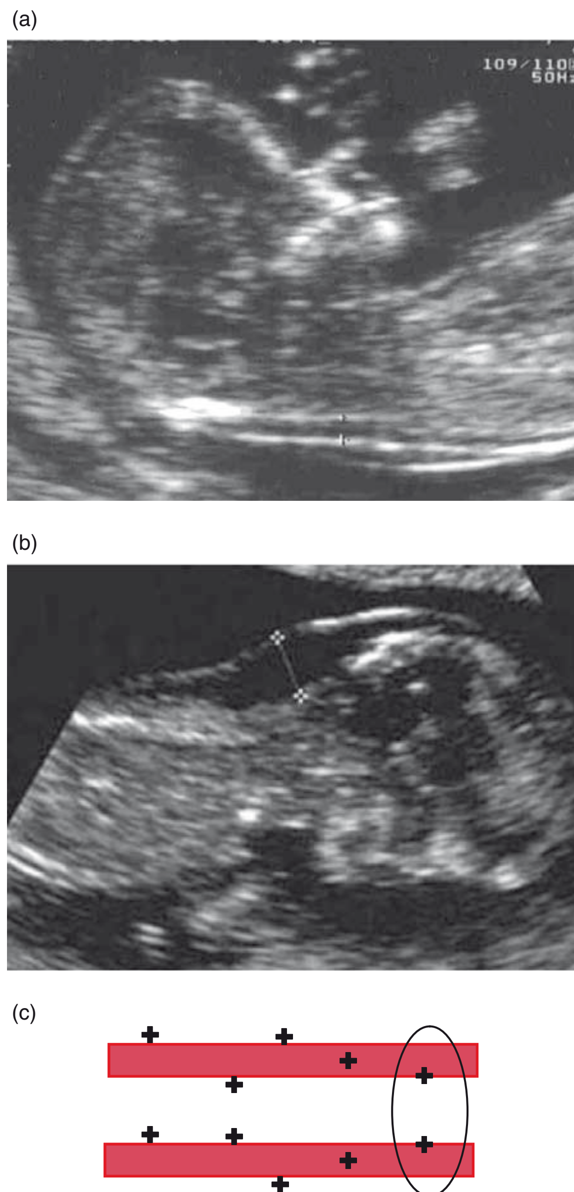

Figure 5.1 Ultrasound images and diagram of nuchal translucency (NT) measurement in the first trimester: (a) a fetus with an NT within the normal range for gestation (2.1 mm); (b) a fetus with an enlarged NT; a chorionic villus sample was taken and the karyotype was trisomy 21; (c) a diagram to illustrate that the callipers should be crosses and should be placed "on-to-on" when measuring the NT correctly (circled).

Other markers of aneuploidy in the first trimester

Absence or hypoplasia of the nasal bone has been known to be associated with Down syndrome since John Langdon Down first described his syndrome in 1866. The fetal nasal bone may be visualized by sonography at 11 to 13 + 6 weeks of gestation, as the bottom of three lines when the fetal profile is viewed in a mid-sagittal section and the ultrasound transducer is held in parallel to the longitudinal axis of the nasal bone (Figure 5.2) (Cicero *et al.*, 2001). Absent nasal bone is not an absolute marker of aneuploidy since it depends on ethnicity,

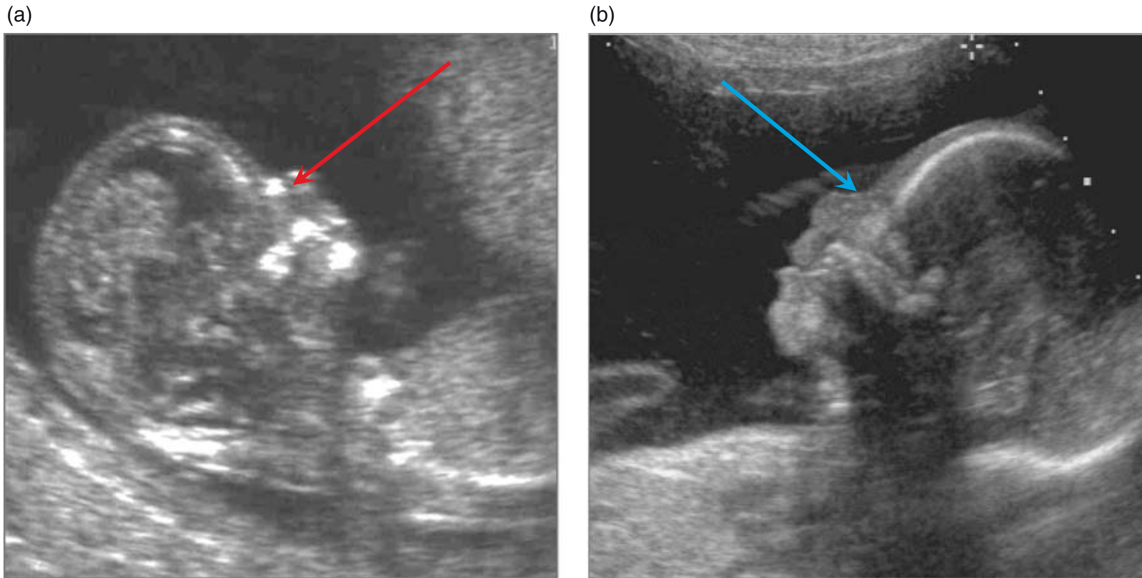

Figure 5.2 Ultrasound images illustrating (a) the presence of a nasal bone (red arrow) and (b) an absent nasal bone in the first trimester (blue arrow). A chorionic villus sample was performed in the fetus where the nasal bone was absent and the fetal karyotype was trisomy 21.

being absent in 2.2 percent of Caucasian, 9.0 percent of Afro-Caribbean, and 5.0 percent of Asian chromosomally normal fetuses. Data from studies investigating it as a screening tool for Down syndrome show that the detection rate is 69 percent with a false-positive rate of 1.4 percent (Nicolaides, 2004). There can be difficulties with the fetal position preventing adequate examination of the nasal bone, and in one study successful examination was reported as being possible in only 76 percent of fetuses (Malone *et al.*, 2004), although other studies report over a 97 percent detection rate (Nicolaides, 2004). Again, adequate training and sufficient time for sonographic examination are important. Fetal Doppler in the first trimester may also prove useful in screening for aneuploidy. Tricuspid regurgitation (Faiola *et al.*, 2005) and raised pulsatility index in the ductus venosus (Borrell *et al.*, 2003) are seen more frequently in Down syndrome fetuses and may be used to improve sonographic screening.

Second-trimester ultrasonography

Diagnosis of structural anomalies in the second trimester

The routine second-trimester "20-week" anomaly ultrasound scan is usually performed between 18 and 22 weeks of gestation, and includes a series of predefined images to obtain standardized measurements (Royal College of Obstetricians and Gynaecologists, 2000). Within this, an evaluation is made of the key structures, including the head shape and internal structures, the spine, abdominal shape and contents, the heart in the four-chamber view, and the extremities (Figure 5.3). Studies show that this detects 60–80 percent of major and 35 percent of minor congenital malformations (Chitty *et al.*, 1991; Luck, 1992) and in clinical practice up to 50 percent of significant abnormalities may be detected (Boyd *et al.*, 1998). An optimal scan includes an assessment of the cardiac outflow tracts, face, and lips in addition to the above examination. However, ensuring the outflow tracts are intact takes additional time and, in up to 26 percent of cases, the woman may be required to return for a further check or review (Rustico *et al.*, 1995). An increased sensitivity for ultrasound diagnosis is achieved in high-risk pregnancies where a specific fetal part is surveyed, for example following a high maternal serum AFP (MS-AFP) suggestive of a neural tube defect (NTD) or a family history of a cardiac abnormality. Prenatal diagnosis of NTD and cardiac defects will be discussed here as examples of fetal structural malformations detected by ultrasound.

The diagnosis of NTD has now been reported to be almost 100 percent with the combined use of MS-AFP and high-resolution ultrasound (Chan *et al.*, 1993). The cranial signs of NTD have been well reported and

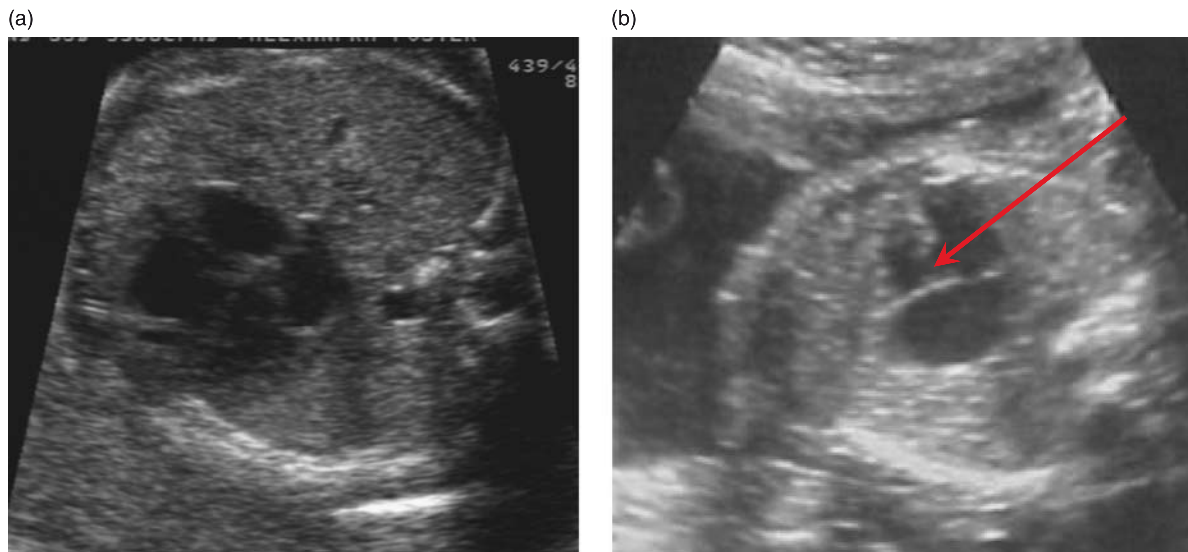

Figure 5.3 Ultrasound images illustrating (a) a normal four-chamber view of the heart and (b) an abnormal four-chamber view of the heart showing a large ventricular septal defect (arrowed).

include scalloping of the frontal bones to give the cranium a characteristic "lemon" shape and a cerebellum that appears "banana"-shaped or it cannot be seen at all (Nicolaides *et al.*, 1986). Using these signs alone without MS-AFP, the detection rate achieved for open spina bifida was up to 80 percent in a prospective regional study (Roberts *et al.*, 1983). The widespread use of the triple and quadruple test to screen for Down syndrome also ensures the MS-AFP screen for NTDs is done, and can lead to their diagnosis several weeks before the 20-week anomaly scan.

Congenital cardiac defects are common and may affect up to 1 percent of neonates; some are amenable to prenatal diagnosis. Improved gray-scale imaging has allowed more detail of intracardiac structures and color-flow Doppler imaging, in combination with three-dimensional (3D) ultrasound technology, has advanced the diagnosis of valvular abnormalities and anomalous cardiac connections. The routine second-trimester ultrasound assessment includes a standard four-chamber view of the heart, which demonstrates the four cardiac chambers, the ventricular and atrial septa, and valves of the chambers (De Vore, 1985). An inability to obtain this image will detect 25 percent of fetal cardiac abnormalities, which includes the majority of the most serious cardiac anomalies. Specific training for ultrasonographers in the detection of cardiac abnormalities may result in above-average detection rates in a low-risk population (Sharland & Allan, 1992). Specialist fetal echocardiographers increase the sensitivity of the fetal cardiac scan. Women at high risk for fetal cardiac anomalies are referred prenatally for assessment, for example diabetics or those with a family history of cardiac malformation. It is important to remember that cardiac abnormalities have also been strongly associated with chromosomal anomalies (Allan *et al.*, 1991) and therefore fetal karyotyping may be discussed in these cases.

There are other major structural defects that may be associated with the common trisomies 13, 18, 21, and 45 XO (Turner syndrome). Some of these are strong associations, for example duodenal atresia with trisomy 21, cystic hygroma with Turner syndrome (45 XO).

Second-trimester identification of "markers"

There are also more subtle features of aneuploidy that may be diagnosed by ultrasound, which otherwise have no consequence to the pregnancy outcome in the longer term and are termed "markers". These may be readily identified within the routine second-trimester anomaly scan. Detection of these "markers" may modify risk estimates of aneuploidy initially determined by maternal age and serum screening. The majority of studies reporting the associations of markers with aneuploidy have been based on selected populations,

Table 5.3 Association of second-trimester ultrasound-diagnosed major congenital abnormalities and aneuploidy

Ultrasound-diagnosed major congenital abnormalities	Trisomy 21	Trisomy 18	Trisomy 13	45 XO
Cardiac defects	++	++	++	++
Duodenal atresia	++	–	–	–
Radial aplasia	++	–	–	–
Exomphalos	–	++	+	–
Congenital diaphragmatic hernia	–	++	+	–
Renal anomalies	–	++	++	++
Holoprosencephaly	–	–	++	–
Growth restriction	–	++	++	–

Table 5.4 Association of minor second-trimester ultrasound markers and aneuploidy

Ultrasound-diagnosed markers	Trisomy 21	Trisomy 18	Trisomy 13	45 XO
Mild pyelectasis	++	+	+	–
Hyperechogenic bowel	++	++	–	–
Short femur	++	++	+	–
Mild ventriculomegaly	++	++	+	–
Choroid plexus cysts	+	++	–	–
Nuchal edema >6 mm	++	+	+	+
Sandal gap	++	–	–	–
Clinodactyly	++	–	–	–
Absent or hypoplastic nasal bone	++	–	–	–

and extrapolation of the associated risks to a low-risk population may be inappropriate.

As an example, mild pyelectasis (renal pelvis anterior–posterior diameter 5–10 mm) has been associated with fetal aneuploidy, in particular Down syndrome, but in the presence of a normal karyotype is of minor significance (Figure 5.4). This was first reported in 1990 (Benacerraf *et al.*, 1990) and others have confirmed this association (Wickstrom *et al.*, 1996). In a low-risk population the overall incidence of aneuploidy with isolated mild pyelectasis was reported to be one in 238 (Scott & Renwick, 1993). However, pyelectasis in the presence of other markers or structural malformations may increase the risk of chromosomal abnormalities by as much as 30 times the age-related risk (Thompson & Thilaganathan, 1998). Scoring systems have been devised to take into consideration the finding of sonographic markers. The Benacerraf index scoring system assigns a score of 1 or 2 for the presence of a soft marker according to the strength of its association with aneuploidy, and the total score is used to assess the risk of aneuploidy (Benacerraf *et al.*, 1994). Using this method the authors reported a detection rate of 73 percent for a false-positive rate of only 4 percent. Incorporating maternal age into the score increased the sensitivity but reduced the false-positive rate (Bromley *et al.*, 1997). An alternative age-adjusted ultrasound risk assessment (AAURA) multiplies the a priori risk based on maternal age, with likelihood ratios resulting from the presence or absence of sonographic markers, and reported a 74 percent detection rate with a false-positive rate of 14.7 percent (Nyberg *et al.*, 1998).

Because so many individual risk factors need to be considered, precise numerical adjustments can be difficult to achieve for sonographic markers. In practice, for the high-risk pregnant woman, the failure to detect these markers can reassure her that her pre-ultrasound risk is not increased, but it should not be used to reduce a high risk when it has been determined by a high-yield screening method such as the combined or integrated test (see later) (Weisz *et al.*, 2007a). The low-risk patient with a single marker may be reassured that, although one marker was found, her pre-existing risk is low and that other markers were not detected.

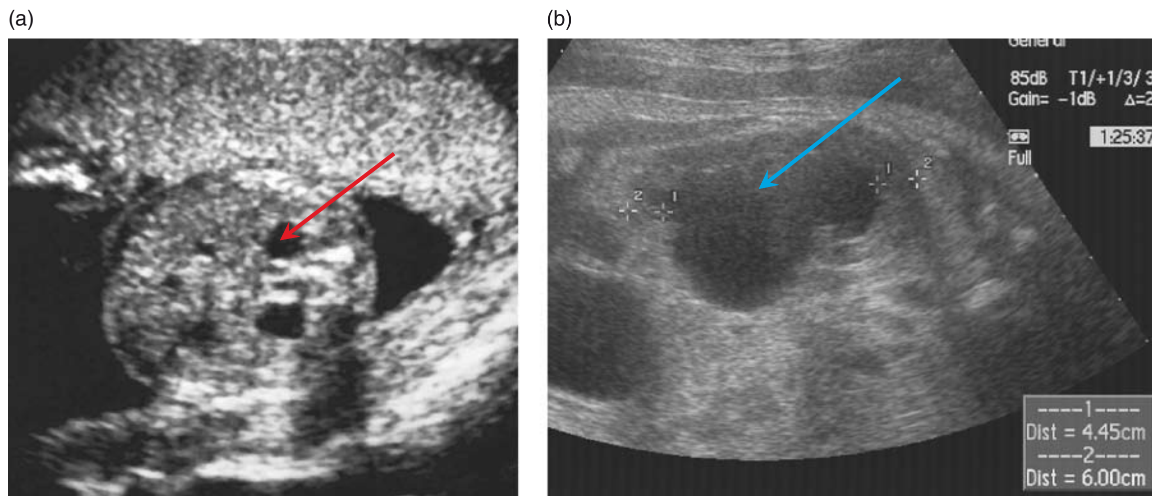

Figure 5.4 (a) Ultrasound image of fetal kidneys showing mild bilateral pyelectasis (red arrow) at 20 weeks of gestation, which is a marker of trisomy 21. (b) Ultrasound image showing severe unilateral hydronephrosis (blue arrow) with echogenic renal cortex but no dilated ureter at 35 weeks of gestation. This is not generally a marker of trisomy 21 and probably represents pelvi-ureteric junction obstruction.

Three- and four-dimensional ultrasound

Fast computers have enabled three- and four-dimensional (3D and 4D) ultrasound to become a reality in clinical practice. A number of different views of the fetus are available with 3D ultrasound, such as surface rendering to display the skin surface, transparency mode (which examines the sub-surface), and maximal intensity mode (which delineates the bony structure). Four-dimensional ultrasound continually updates the volume under examination in real time, creating the impression of a moving structure.

There is continuing debate as to how 3D and 4D ultrasound examination may enhance two-dimensional ultrasound (2D) in prenatal diagnosis; 3D may provide better assessment of congenital heart abnormalities, especially when combined with Doppler blood flow examination (Timor-Tritsch & Platt, 2002). Abnormalities of the central nervous system, such as absence of the corpus callosum, and assessment of residual lung volume in congenital diaphragmatic hernia may also be better achieved using 3D ultrasound. The surface rendering display may be used to show parents what specific fetal abnormalities look like, such as for example, cleft lip and palate, spina bifida, and syndactyly, as part of the counseling process. Figure 5.5 contains 3D sonograms illustrating syndactyly or “mitten hands” in a fetus affected by Apert syndrome (David *et al.*, 2007). When 3D volumes were compared with a standard 2D examination in one institution, the acquisition and interpretation time of the examination was significantly shorter in the group examined using 3D alone (Benacerraf *et al.*, 2005). It is likely that 3D and 4D examinations will become a standard adjunct to the current 2D ultrasound scan.

Magnetic resonance imaging

The introduction of ultrafast imaging methods such as echo planar imaging and single shot fast spin echo techniques allow the acquisition of single, high-resolution images in less than one second and have made magnetic resonance imaging (MRI) examination of the fetus a real possibility (Griffiths *et al.*, 2005). The long-term effects of exposure to MRI imaging on the fetus are currently unknown, although there do not appear to be any adverse effects in the short term (De Wilde *et al.*, 2005). MRI appears to be particularly useful for examination of the fetal brain, in one study increasing the diagnostic accuracy in 48 percent of fetuses with brain abnormalities and changing the counseling in 36 percent of cases (Whitby *et al.*, 2004). See Figure 5.6.

Maternal serum screening in prenatal diagnosis

The analysis of specific biochemical markers in the maternal serum, derived from the feto–placental unit, is a noninvasive technique to identify pregnancies at high risk of certain birth defects. An accurate assessment of

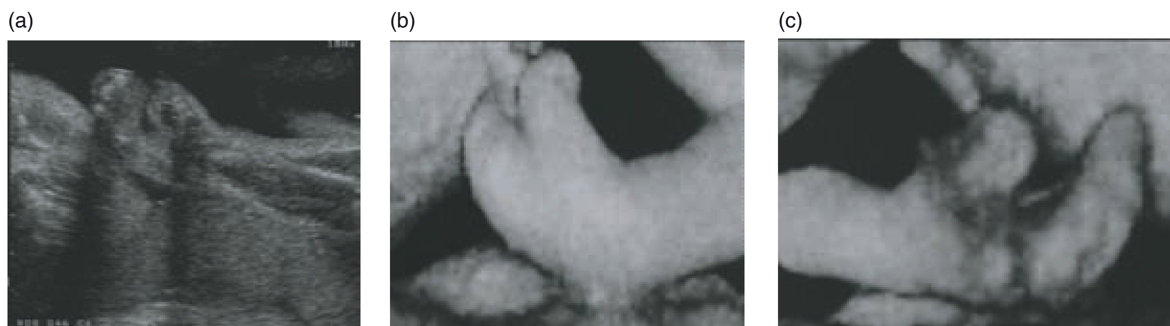

Figure 5.5 Two-dimensional (2D) (a) and three-dimensional (3D) (b and c) sonograms illustrating syndactyly or "mitten hands" in a fetus affected by Apert syndrome, a rare autosomal dominant genetic syndrome caused by a mutation in the *FGFR2* gene (David *et al*. (2007), with permission from Wiley. Copyright © 2007 John Wiley & Sons, Ltd.).

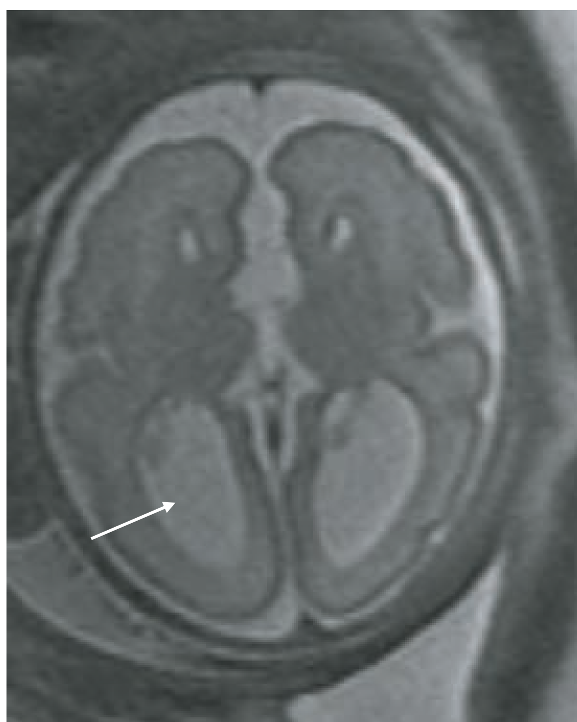

Figure 5.6 At 30 weeks of gestation severe ventriculomegaly (18 mm) was diagnosed on ultrasound. Fetal magnetic resonance imaging (MRI) revealed agenesis of the corpus callosum. This axial T2 weighted magnetic resonance image of a 30-week fetus shows the typical imaging features of this condition. The posterior horns appear ballooned due to a persisting fetal configuration of the ventricles termed colpocephaly (arrow). Picture courtesy of Dr. C. Hagmann, University College London, UK.

fetal age, by first-trimester fetal crown–rump measurement, is paramount to interpret these values (Wald *et al*., 1992). Maternal serum biochemistry results are usually interpreted in multiples of the median (MoM) of the normal value at the specified gestational age. This statistical tool is less susceptible to the distorting effects of extreme values or to the variation between laboratories (Wald & Cuckle, 1980). Currently, there are a number of strategies in clinical practice in the UK that use maternal serum biochemistry markers alone or in combination with ultrasound findings.

Detection of neural tube defects

AFP is produced primarily from the fetal liver, and levels in the fetal blood are very high. Any interruption in the fetal skin integrity or change in the functional performance of the feto–placental unit will alter amniotic and therefore maternal serum levels of AFP. Fetal structural abnormalities such as abdominal wall defects (gastroschisis, exomphalos) and open NTDs elevate MS-AFP, and falsely elevated MS-AFP may be attributed to an underestimation of fetal age, multiple pregnancy, or fetal demise. A maternal serum threshold of 2.5 MoMs correctly identified 79 percent of the fetuses with open spina bifida (UK Collaborative Study, 1982).

Detection of Down syndrome

The first maternal serum biochemical marker to be associated with Down syndrome was AFP, which was found to be reduced in the early second trimester in affected pregnancies (Merkatz *et al*., 1984). The measurements were converted to MoMs and used in combination with maternal age (which is independently associated with Down syndrome) to establish risk. High levels of human chorionic gonadotropin (hCG, particularly the free β-subunit), low levels of unconjugated estriol, and high levels of inhibin have been used to increase the sensitivity of maternal serum screening to detect Down syndrome. The different combinations of markers within current screening tests are shown in Table 5.5.

Table 5.5 Screening tests for Down syndrome currently in use and their detection rates at a specific false-positive rate (FPR) (Weisz & Rodeck, 2006; Malone, 2005; Wald *et al.*, 2003)

Screening test	Age	NT	PAPP-A	Free β-hCG	AFP	E3	Inhibin A	DR (%)	FPR (%)	Cut-off
Double	✓			✓[2]	✓[2]			71	5	1:250
Triple	✓			✓[2]	✓[2]	✓[2]		69–77	5	1:250
Quadruple	✓			✓[2]	✓[2]	✓[2]	✓[2]	81–83	5	1:250
NT	✓	✓						69–82	5	1:250
Combined	✓	✓	✓[1]	✓[1]				90	5	1:250
Serum integrated	✓		✓[1]	✓[2]	✓[2]	✓[2]	✓[2]	88	5	1:150
Integrated	✓	✓	✓[1]	✓[2]	✓[2]	✓[2]	✓[2]	93–5	5	1:150

NT, nuchal translucency; PAPP-A, pregnancy-associated plasma protein A; free β-hCG, free beta-human chorionic gonadotropin; AFP, alpha fetoprotein; DR, detection rate; E3, estriol; FPR, false positive rate.

Note: ✓[1] indicates the maternal serum is tested in the first trimester (11–14 weeks of gestation); ✓[2] indicates the maternal serum is tested in the second trimester (15–22 weeks of gestation). Cut-off levels indicate the risk above which a woman is offered an invasive test for prenatal diagnosis.

First-trimester screening for Down syndrome is more recent. Studies show detection rates ranging between 82 percent and 77 percent for a false-positive rate of 8.3 percent, 5 percent, and 4 percent (Snijders *et al.*, 1998; Nicolaides, 2004; Avgidou *et al.*, 2005). NT alone is used to screen for Down syndrome in multiple pregnancies. The pregnancy-specific risk is calculated for dichorionic pregnancies by summing the individual risk estimates, and for monochorionic pregnancies by averaging the likelihood ratios derived from the NT measurements in each fetus (Weisz & Rodeck, 2006).

Tests that combine serum and ultrasound markers have the highest detection rate. In the combined test (Wald & Hackshaw, 1997) NT measurement is combined with serum levels of pregnancy-associated plasma protein A (PAPP-A) and hCG in the first trimester (11 to 13 + 6 weeks of gestation). This results in a significantly lower false-positive rate than with NT measurement alone and has led to the conclusion that there is no justification for retaining the NT alone in antenatal screening in singleton pregnancies (Wald *et al.*, 2004).

The integrated test was found to have detection rates of 93 percent and 95 percent at a 5 percent false-positive rate (FPR) or 85 percent for a 1 percent FPR in studies in Europe (Serum, Urine and Ultrasound Screening Study, SURUSS) (Wald *et al.*, 2003) and in the USA (First and Second Trimester Evaluation of Risk trial, FASTER) (Malone, 2005). The advantage of this test is the low odds of an affected pregnancy rate (OAPR) of 1:6 in the SURUSS (Wald *et al.*, 2003). This means that for women who have the integrated test, fewer will need to undergo invasive testing, and thus avoid the inherent miscarriage risk, and fewer women will be made anxious by a false-positive result. Some have made an issue of "withholding" first-trimester results while waiting for the second-trimester markers to be integrated into the overall result. This misses the point that the integrated test is a single test in two stages. Its power is that it gives single risk figures based on seven markers producing a very low FPR. In the FASTER study (Malone, 2005) stepwise sequential screening was suggested as an alternative strategy whereby women with a high-risk combined test are offered CVS, while the remaining low-risk women continue to have the quadruple test at 15 weeks of gestation and a new risk estimate is calculated at that time. Another strategy is contingency screening whereby a first-trimester test is used to triage screened women into three groups: high-risk screen-positive women, who are immediately offered a diagnostic test; a screen-negative group, who receive no further screening; and a lower-risk screen-positive group, who continue with screening tests into the second trimester. These and other strategies are more complicated, and need to be fully evaluated before they are introduced into screening practice (Wald *et al.*, 2006).

The current Down syndrome screening program in the UK is designed to provide equal provision and quality audited standards. In October 2003 the National Institute for Clinical Excellence (NICE) recommended that screening programs should have a detection rate of greater than 75 percent with a

false-positive rate of less than 3 percent by April 2007. At present, the tests that meet these criteria are the combined test, the integrated or the serum integrated test (if NT measurement is unavailable), and the quadruple test for women who attend for screening after the first trimester.

Invasive tests for prenatal diagnosis

Invasive tests in pregnancy are used to obtain fetal cells, fluids, or tissues that are used for prenatal diagnosis of aneuploidy, fetal congenital disease, and fetal infection (Table 5.2). The choice of invasive test is determined by the indication and fetal gestation balanced against the safety of the procedure. Patients who have diagnostic invasive procedures are screen positive or at increased risk after a screening test.

Amniocentesis

Amniocentesis is performed from 15 weeks' gestation but not before. It is used to determine the fetal karyotype, fetal DNA, for investigation of fetal infection or to detect inborn errors of metabolism by biochemical assay. It is now rarely used in the evaluation of NTD (measuring amniotic fluid AFP) with the advent of high-resolution ultrasound (see above).

Following confirmation of gestation and viability by ultrasound, fetal position and placental site are determined. A pool of liquor, free of fetal parts and ideally avoiding the placenta, is visualized. Using an aseptic technique and under constant ultrasound visualizaton, a fine 22 gauge spinal needle with a stylet (to avoid maternal contamination) is passed directly into the amniotic sac (Figure 5.7). Most operators use a freehand technique, although a few use a needle guide. Local anesthetic is not usually required for this procedure because the needle used is so fine. Amniotic fluid is aspirated (18–20 ml) and the needle is withdrawn. All women with an Rh-negative blood group should be given Anti-D immunoglobulin (1250 IU) to prevent isoimmunization.The best estimate of the true risk of miscarriage after amniocentesis is based on the only randomized controlled trial, which showed that the pregnancy loss rate up to 28 weeks was 1.7 percent in those randomized to aminocentesis, compared with 0.7 percent in the control group (Tabor *et al.*, 1986). A recent analysis of 3096 patients undergoing amniocentesis as part of the FASTER study showed no significant effect of mid-trimester amniocentesis on fetal loss before 24 weeks of gestation (Eddleman *et al.*, 2006). However, a retrospective analysis of 29 papers

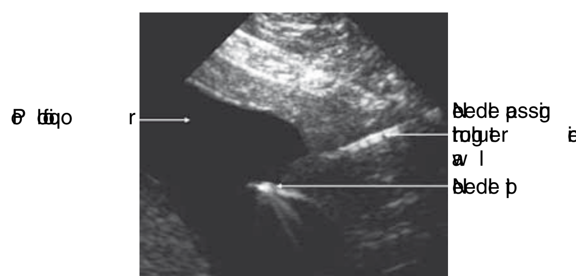

Figure 5.7 Ultrasound image of an amniocentesis. The tip of the 22 Gauge needle (arrowed) may be seen in a pool of liquor.

containing 68 119 mid-trimester amniocentesis procedures found a fetal loss rate of 1.7 percent by 28 weeks of gestation, supporting the data from Tabor *et al.* (1986) (Seeds, 2004). Most units continue to counsel women undergoing amniocentesis of an approximate one in100 risk of fetal loss.

Short-term complications include a failure of the amniotic membrane to seal, leading to persistent amniotic fluid leakage or chorioamnionitis. There is a remotely small risk of fetal injury, which should be avoidable by continuous ultrasound guidance during the procedure, and a significant risk of feto–maternal hemorrhage. Longer-term complications may include respiratory problems in the neonate (Tabor *et al.*, 1986). If amniocentesis is performed as early as 11–12 weeks of gestation there is an increased fetal loss rate, complication rate, and a higher risk of talipes equinovarus when compared with amniocentesis over 15 weeks of gestation (CEMAT Group, 1998). Early amniocentesis also has a higher risk of miscarriage when compared with CVS at the same gestational age (Philip *et al.*, 2004).

Chorionic villus sampling

The indications for CVS are similar to those for amniocentesis. It is particularly useful in the prenatal diagnosis of genetic diseases because it provides a larger amount of fetal DNA when compared with amniocentesis. It may be performed via the abdomen (TA) or through the cervix (transcervical, TC) and the preferred route and technique is determined by the placental localization, the gestation, safety of the procedure, and operator experience. The TC approach to the placenta is limited to 10–13 weeks of gestation, and by the distance to, and localization of, the placenta. The TA approach may be performed at

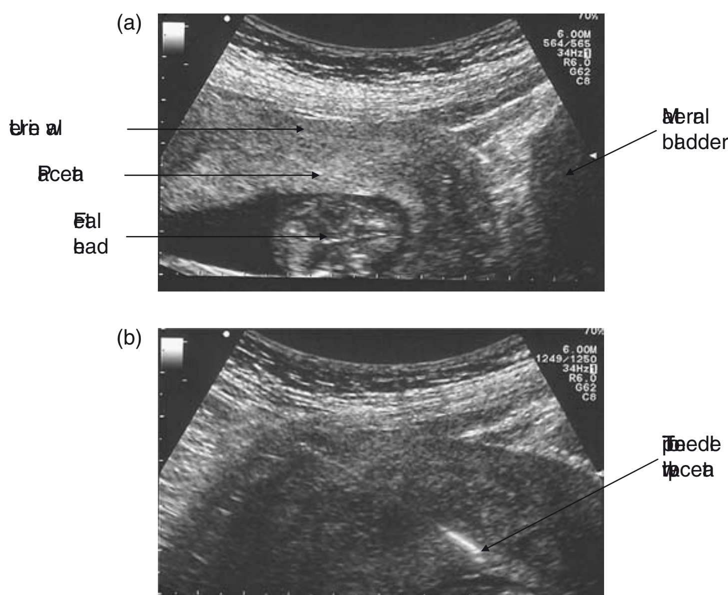

Figure 5.8 Ultrasound images of transabdominal (TA) chorionic villus sampling (CVS) using a double needle technique: (a) planning the CVS route; (b) taking the CVS – the tip of the needle may be seen at the chorionic plate in the placenta.

any gestation and is only limited by the accessibility of the placenta (Figure 5.8(a)). Transabdominal CVS is usually performed with a double needle system and under strict aseptic conditions with ultrasound guidance. After infiltration of the skin and subcutaneous tissues with local anesthetic, an 18 gauge needle is inserted through the uterine wall into the placenta (Figure 5.8(b)). A 20 gauge needle is then passed down to aspirate placental villi (Jauniaux *et al*., 2000). Confirmation of an adequate biopsy is, again, by visual inspection but may be more difficult as aspiration samples may be heavily contaminated by maternal blood. For TC CVS the mother is placed in the lithotomy position. Under strict aseptic conditions, fine biopsy forceps (Figure 5.9(b)) or a catheter are passed through the internal cervical os under transabdominal ultrasound guidance. The tip of the forceps is visualized in the longitudinal and transverse planes to confirm localization within the placenta, near the chorionic plate (Figure 5.9(a)). A biopsy of placental villi is then taken. Confirmation of an adequate biopsy is possible by naked eye examination (Figure 5.9(c)) and is achieved with a single passage of the forceps in 98 percent of cases. This is important as multiple passages will increase the risk of infection and of miscarriage. Slight vaginal bleeding may occur after TC sampling (1–4 percent) (Rhoads *et al*., 1989), but is unimportant as it is usually from the cervix. Intrauterine infection and chorioamnionitis are rare. Early reports associated CVS with a higher incidence of transverse limb reduction defects and oromandibular hypoplasia than the background frequency of limb reduction defects in the general population (between 0.03 percent and 0.06 percent). However, this is now recognized to be closely related to the gestation at the time of the CVS since a much higher incidence of limb reduction defects occurs in pregnancies when a CVS is performed before nine weeks of gestation, compared with sampling after nine and a half weeks (Brambati *et al*., 1992; Rodeck, 1993). Trauma to the placenta is probably also an important factor and it has been shown that TA single-needle CVS aspiration causes more placental disruption and feto–maternal hemorrhage than TC forceps (Rodeck *et al.*, 1993).

Fetal blood sampling

Fetal blood sampling (FBS) was performed for many indications such as hemoglobinopathies, coagulopathies, and immunodeficiencies, and to diagnose fetal infections. These are now mostly amenable to CVS and DNA-based diagnosis. FBS is still used to investigate fetal non-immune hydrops, and to confirm the level of fetal anemia or platelets prior to fetal transfusion. FBS is an outpatient procedure and is well tolerated by the mother. It is performed under ultrasound control and full aseptic precautions. Neither tocolysis, antibiotic prophylaxis, nor fetal neuromuscular blockade are used routinely. The site for sampling is determined by the fetal and placental position, with the umbilical vein at the placental insertion being the

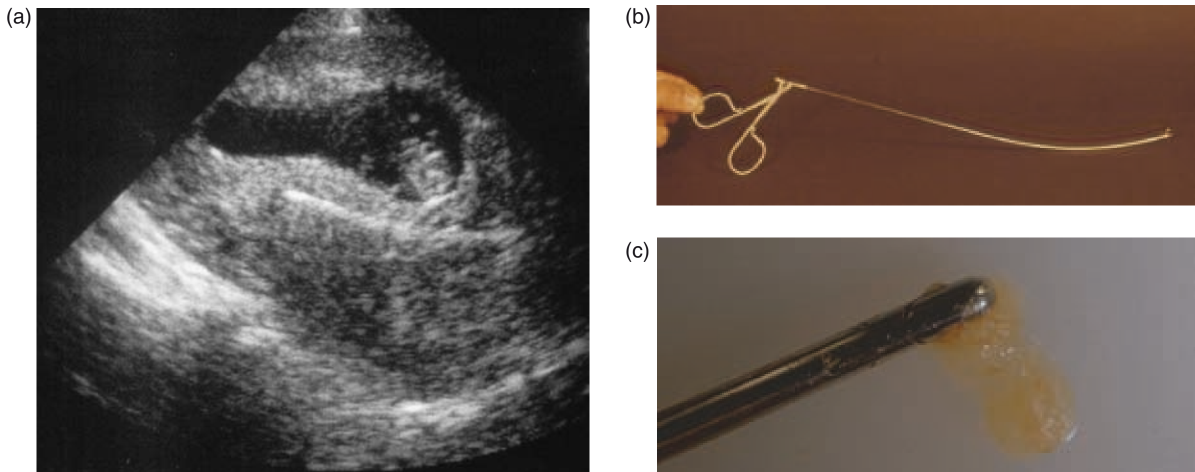

Figure 5.9 Transcervical chorionic villus sampling (CVS) using a biopsy forceps. (a) Ultrasound image of transcervical CVS. The biopsy forceps (b) is introduced through the cervical canal under transabdominal (TA) ultrasound visualization and a sample is taken (c) using a biopsy forceps.

most commonly used site (Vaughan & Rodeck, 2001). The intrahepatic portion of the umbilical vein is often a preferred site as it reliably yields a pure fetal venous sample, minimizing risks of arterial spasm, cord tamponade, and materno–fetal hemorrhage (Nicolini *et al.*, 1990). The fetal heart is an alternative sampling site, but it is rarely used apart from intracardiac delivery of potassium chloride to cause asystole/fetocide as part of a late termination of pregnancy procedure. Following sampling the mother is monitored for half an hour, to exclude active bleeding, preterm labor, or fetal distress.

Published fetal loss rates after FBS are generally up to 1.9 percent (Pielet *et al.*, 1988; Weiner & Okamura, 1996). However, fetal losses are related to the indication for the procedure, with losses of up to 12.7 percent in severe growth retardation (Antsaklis *et al.*, 1998) and up to 25 percent in the presence of fetal hydrops (Maxwell *et al.*, 1991). Other complications include feto–maternal alloimmunization, chorioamnionitis, preterm delivery, and placental abruption. The complication rate falls as both fetal gestation and operator experience increase.

Fetal tissue or body fluid sampling

Many disorders do not have a chromosomal abnormality or an enzyme defect expressed in chorion, fetal blood, or cultured amniocytes, or, alternatively, a DNA test may not be available. Prenatal diagnosis is then achieved by direct fetal tissue sampling. The risk of fetal loss secondary to ultrasound-guided invasive procedures is 1–2 percent, probably no higher than the risks associated with FBS.

For inherited skin disorders such as epidermolysis bullosa, histology, immunofluorescent, and ultrastructural studies on fetal skin can provide the diagnosis. Fetal skin is biopsied in the second trimester when the majority of fetal skin tissues are differentiated. Under ultrasound guidance a biopsy of the preferred fetal location is made. For example, the buttock or leg may be biopsied for the prenatal diagnosis of epidermolysis bullosa letalis (Rodeck *et al.*, 1980), or the hair follicles and sweat glands of the scalp and axilla can be sampled to detect hyperkeratosis in harlequin ichthyosis (Fassihi *et al.*, 2006).

In a few rare and lethal inborn errors of metabolism where protein expression is localized to hepatocytes, prenatal diagnosis is made by fetal liver biopsy. The exact enzyme defect from the previous pregnancy, for example ornithine carbamyl transferase deficiency (Rodeck *et al.*, 1982) or the maternal carrier status is identified. The procedure is delayed until 18 weeks of gestation when normal liver enzyme activity and mid-trimester reference ranges are established. The needle is guided to the right hypochondrium and introduced swiftly into the fetal flank, and liver tissue is aspirated.

Ultrasound-guided TA needle aspiration of the fetal bladder allows direct measurement of the components of fetal urine in cases of bladder outlet obstruction. This is most often caused by posterior urethral valves, but may also occur with urethral atresia, obstructing ureterocele, megacystis-microcolon, or cloacal dysgenesis.

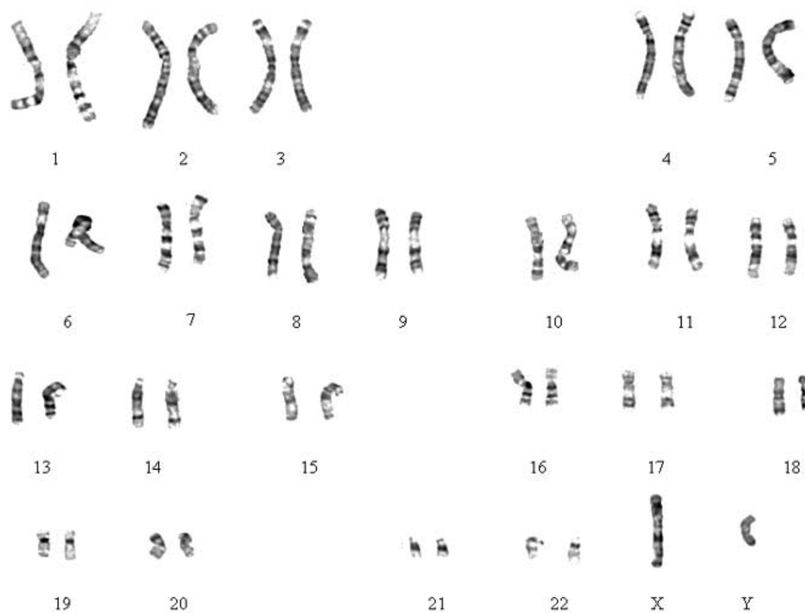

Figure 5.10 Karyotype of G-banded chromosomes, showing a normal male chromosome complement. Picture courtesy of Cytogenetics Department, Guy's and St Thomas' NHS Foundation Trust, London, UK.

Fetal urinalysis assesses the ability of the renal tubules to reabsorb a variety of compounds, such as sodium and beta 2 microglobulin (Nicolini *et al*., 1992) and their fetal urinary levels are a reasonably good predictor of postnatal serum creatinine at 1–2 years of age (Muller *et al*., 1996), although more long-term follow-up data are needed. The first tap usually contains the most concentrated urine but serial taps may show improvement in renal function and may be helpful in its evaluation (Nicolini *et al*., 1991). High levels of sodium, chloride, potassium, phosphate, and beta 2 microglobulin suggest renal failure and are associated with a poor prognosis (Lipitz *et al*., 1993).

Cytogenetics and prenatal diagnosis

The rapid development of cytogenetics over the last 30 years has increased the number of options available for prenatal diagnosis. Amniotic fluid contains a variety of fetal cells that require one to three weeks' culture to provide sufficient dividing cells (metaphase nuclei) for karyotype analysis. Between 15 and 20 weeks of gestation, the viable cell to amniotic fluid ratio optimizes culture yield. Chorionic villi from CVS consist of an inner mesenchymal core and an outer cytotrophoblast, the latter in particular containing dividing cells. The cytotrophoblast will yield metaphases suitable for direct analysis and short-term culture (48 hours) and results may be backed up by long-term culture of the mesenchymal core. Fetal blood provides lymphocytes which grow well. With any technique there is a risk of cell culture failure, a risk quoted as approximately 1 percent. Maternal cell contamination is a recognized complication, though rare, and particularly important in long-term culture of chorionic villi. Results from chorionic villi may be mosaic (1 percent risk), a recognized cause of failed diagnosis, though it may be confined to the placenta, and have arisen from non-disjunction in the actively dividing cytotrophoblast cells. Culture of the mesenchyme, which is less prone to mosaicism than the trophoblast and is embryologically closer to the fetus, may solve the problem but an amniocentesis may be required to determine fetal karyotype. The diagnostic accuracy of karyotyping cultured amniotic fluid cells has been found to be 99.4–99.8 percent (NICHD, 1976) and that of CVS 97.5–99.6 percent (Shaffer & Bui, 2007). A normal male fetal karyotype using G-banded chromosomes is shown in Figure 5.10. Fluorescence *in situ* hybridization (FISH) avoids the resolution limitations of conventional cytogenetic analysis, and its introduction provided a platform for rapid prenatal diagnosis for common aneuploidies in non-dividing cells (interphase nuclei) (Klinger *et al*., 1992). It accurately and rapidly excludes the major trisomies (13, 18, 21) and sex chromosome anomalies (XO, XXY, XYY) by using specific DNA probes, providing a result usually within 24–48 hours (Shaffer & Bui, 2007). Double-stranded

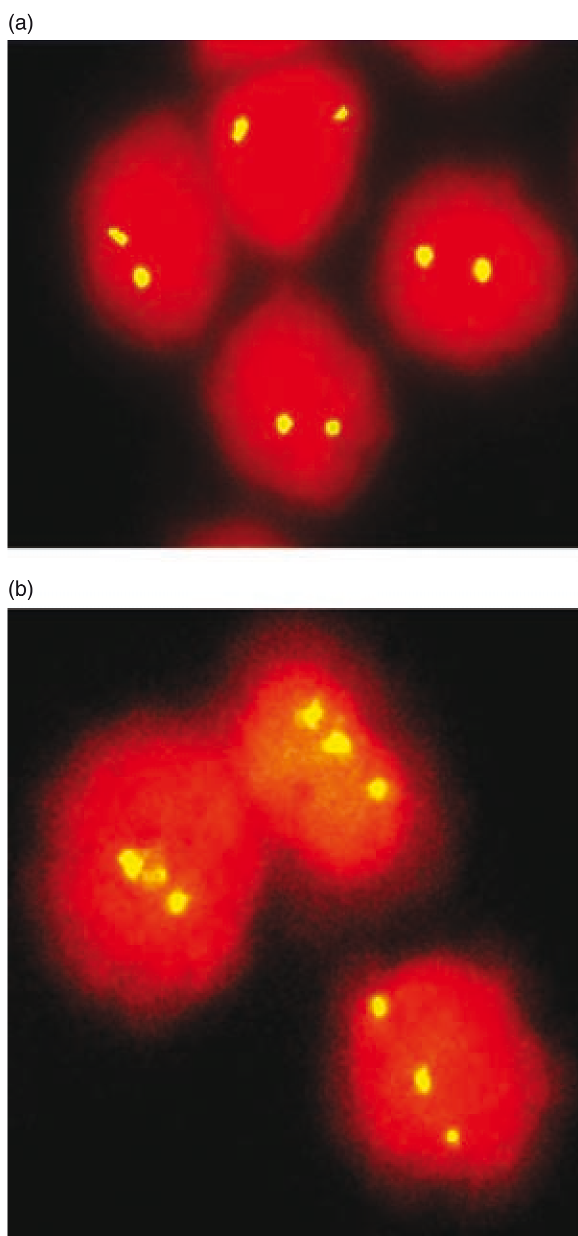

Figure 5.11 (a) *In situ* hybridization of uncultured amniocytes with a probe for the centromere of chromosome 18, showing a normal signal pattern, consistent with two copies of chromosome 18. (b) *In situ* hybridization of uncultured amniocytes with a probe for the centromere of chromosome 18, showing a signal pattern consistent with trisomy 18. Picture courtesy of Cytogenetics Department, Guy's and St Thomas' NHS Foundation Trust, London, UK.

DNA is treated to become single-stranded and mixed, to allow hybridization, with fluorescently labeled single-stranded probes. An *in situ* hybridization showing a normal complement of chromosome 18 (Figure 5.11(a)) and trisomy 18 (Figure 5.11(b)) is shown. FISH on uncultured amniocytes may be used in preference to direct preparations from CVS, with its inherent risk of placental confined mosacism. FISH has also become important in the evaluation of structurally abnormal chromosomes and marker chromosomes in prenatal diagnosis (Liehr *et al*., 2004).

In response to the increased demand by clinicians and patients for more rapid diagnostic methods that do not require cell culture, quantitative fluorescence polymerase chain reaction (QF-PCR) has been introduced to allow rapid detection (one or two days) of the common aneuploidies. In this technique, highly polymorphic short tandem repeats (STRs) on chromosomes 13, 18, 21, X, and Y are amplified using fluorescence primers and PCR in a multiplex assay (Cirigliano *et al*., 2004). The fluorescence intensity of the alleles is then detected using an automated genetic analyzer and generates reliable results even with very small samples (Mann *et al*., 2001). A QF-PCR trace showing a normal copy number of chromosomes 13, 18, and 21 is illustrated in Figure 5.12. Studies show that the accuracy of QF-PCR for common aneuploidies is similar to that of interphase FISH (Shaffer & Bui, 2007), although QF-PCR has the advantage of being less expensive and allowing the simultaneous processing of a much larger number of samples than FISH. For these reasons QF-PCR has replaced interphase FISH in an increasing number of genetic laboratories (Mann *et al*., 2004).

With the advent of rapid trisomy testing there has been discussion about the utility and cost implications of continuing to offer QF-PCR with full karyotyping for prenatal diagnosis. In 2004, the UK National Screening Committee (UKNSC) recommended that new screening programs for Down syndrome need not include karyotyping and could offer prenatal diagnosis for the syndrome with FISH or PCR as a rapid diagnostic test for trisomies 13, 18, and 21 only. One regional UK cytogenetics audit suggested that replacement of full karyotyping with rapid testing for trisomies 13, 18, and 21 after a positive screen for Down syndrome would result in one in 100 amniocenteses and one in 40 CVS samples having an undetected abnormal karyotype (Caine *et al*., 2005). Another study examined the fetal karyotypes that would not have been detected by rapid trisomy testing and characterized them into those with a good, a poor, or an uncertain prognosis (Ogilvie *et al*., 2005). They concluded that a change in policy would fail to detect chromosome abnormalities likely to have

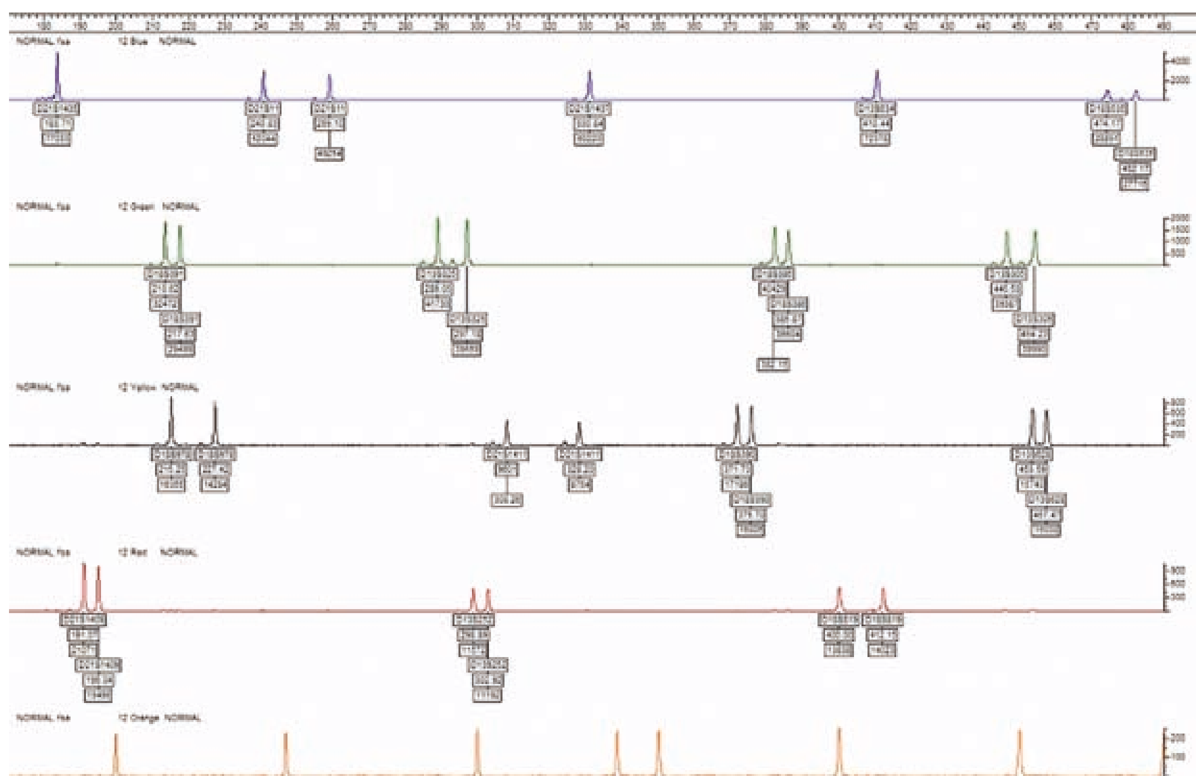

Figure 5.12 Quantitative fluorescence polymerase chain reaction (QF-PCR) trace from a single capillary showing STR markers amplified with primers labeled with four different fluorochromes. Peak area ratios of 1:1 for each marker indicate normal copy number for chromosomes 13, 18, and 21. Picture courtesy of Cytogenetics Department, Guy's and St Thomas' NHS Foundation Trust, London, UK.

serious clinical significance in approximately 0.06 percent (one in 1659) cases, and possibly in up to 0.12 percent (one in 833) cases if all those fetuses that were terminated were assumed to have a fetal abnormality. Prenatal diagnosis is likely to become more targeted in the future and QF-PCR will probably be the stand-alone test for pregnancies at increased risk of Down syndrome after screening.

Newer tests such as multiplex ligation-dependent probe amplification (MLPA), a technique that detects gene dosage abnormalities by the relative quantification of up to 45 different DNA sequences in one reaction, are now becoming available (Hochstenback *et al*., 2005). Micro-array technology allows the whole genome to be investigated for copy number changes, identifying abnormalities of the telomeric regions or microdeletions reliably (Bejjani *et al*., 2005). These could be used in prenatal diagnosis. However, they are subject to the same concerns as for full karyotyping since many copy number changes are of no clinical significance, which will lead to difficulties in counseling patients, and further work needs to be done using micro-arrays on postnatal samples before it can be applied prenatally.

Cell free fetal DNA

Cell-free fetal DNA was first detected in the maternal circulation a decade ago, and it has already proved its usefulness in prenatal diagnosis. The original report first described the identification of the SRY gene in the plasma of pregnant women carrying a male fetus (Lo *et al*., 1997). Shortly after this it was used to successfully type the Rh group of the fetus in pregnant women who were Rh-negative and at risk of alloimmunization (Lo *et al*., 1998) and is now offered as a clinical service (Finning, 2002). Blood is taken from the Rh-negative pregnant woman early in the first trimester (eight to nine weeks of gestation), and using a sensitive real-time PCR method, the *RHD* gene is amplified in a highly accurate and specific reaction. False-negative cases are usually attributed to either insensitive methods or a

lack of fetal DNA in an early-trimester sample; false-positive results can be due to genotypic variants seen in individuals of African descent. Overall, its lower associated costs and higher safety profile compared to traditional methods make noninvasive fetal *RHD* genotyping appropriate for universal clinical application (Bianchi *et al.*, 2005) and it is rapidly becoming part of the routine clinical practice in the UK and the Netherlands.

Early fetal gender determination is important for prenatal diagnosis of several X-linked disorders (Avent & Chitty, 2006). Ultrasound may be used to sex the fetus but is dependent on the operator and the image quality, and is therefore not completely reliable. Neither may it be used in the first trimester. Determining whether the fetus is female using analysis of the SRY gene in the maternal serum avoids the need to perform a CVS. Using noninvasive prenatal diagnosis of fetal sex in one unit reduced the number of invasive interventions by 46 percent (Hyett *et al.*, 2005).

Cell-free fetal DNA detection has been used in the prenatal diagnosis of several autosomal dominant single-gene disorders such as myotonic dystrophy (Amicucci *et al.*, 2000) and achondroplasia (Saito *et al.*, 2000) when the gene has been inherited from the father. Using new detection techniques, such as size fractionation of cell-free DNA in maternal plasma, paternally inherited DNA polymorphisms of β-thalassemia may also be identified (Li, 2005).

Early studies showed that levels of cell-free fetal DNA are higher in pregnancies where the fetus is aneuploid (Lo *et al.*, 1999), in particular trisomy 21. Adding second-trimester cell-free fetal DNA levels to the panel of markers used in the quadruple test increased the detection rate for Down syndrome from 81 percent to 86 percent at a false-positive rate of 5 percent (Farina *et al.*, 2003). More recently, attention has turned to the use of noninvasive prenatal diagnosis of aneuploidy, which requires better differentiation of cell-free fetal from maternal DNA. Identifying differential DNA methylation between the fetus or placenta and the mother has been used to detect maternally and paternally inherited fetal alleles (Poon *et al.*, 2002). Further work has identified a placental epigenetic marker, *maspin*, which is methylated in maternal leukocytes and hypomethylated in placenta and which is the first universal fetal DNA marker in maternal plasma (Chim *et al.*, 2005). To increase the level of cell-free fetal DNA obtained from the maternal circulation various techniques have been used, such as formaldehyde treatment (Dhallan *et al.*, 2004), although this effect has not been reproducible in other laboratories. Recently this method has been used to enrich samples in the noninvasive diagnosis of trisomy 21, using the ratio of multiple single nucleotide polymorphisms (SNPs) in fetal and maternal cell-free fetal DNA. Dhallan *et al.* (2007) correctly established the copy number of fetal chromosomes 13 and 21 in 58 out of 60 samples. Cell-free fetal nucleic acid detection is being increasingly applied to diagnose genetic diseases and it is very likely that the use of cell-free fetal DNA in noninvasive prenatal analysis will become an established part of prenatal diagnosis in the future.

Choice of invasive test in prenatal diagnosis

The choice of test for prenatal diagnosis involves balancing the risk to the fetus and the appropriate test for diagnosis. The potential parents desire rapid and early prenatal diagnosis if they know they are at high risk, for example from a genetic history or a previously affected child. However, both these stipulations appear to increase the fetal risk and enhance the potential for cytogenetic error. Further assessment of these risks will only be obtained from on-going multicenter trials and research. First-trimester diagnosis provides the opportunity for surgical abortion, but whether this lessens the psychological morbidity of prenatal diagnosis is still being debated. Many pregnancies complicated with serious abnormalities will abort spontaneously in the first or early second trimester and any benefit attributed to early first-trimester diagnosis must be carefully weighed against potential harm that may result from making parents choose to terminate a wanted pregnancy which might have been lost spontaneously (Statham *et al.*, 2007). A normal, low-risk population having screening for Down syndrome has, in our experience, a more relaxed approach than those needing prenatal diagnosis. For example, they are happy to have the integrated test, with a better detection rate and lower FPR but slightly later result, than the combined test, which gives an earlier result but a lower detection rate and higher FPR; that is, it is less accurate and less safe (Weisz *et al.*,2007b).

There is a decline in the use of FBS, particularly for rapid fetal karyotyping, which is attributed to advances in molecular techniques for genetic diagnosis, fetal genotyping, and fetal infection. The molecular cytogenetic techniques of FISH and QF-PCR, using

chromosome-specific probes, have allowed rapid investigation of the five most important chromosomes (13, 18, 21, X, and Y) from amniocytes and chorionic villi. Declining rates of FBS are also due to the use of noninvasive assessment of the fetus for Rh alloimmunization and intrauterine growth restriction.

Conclusion

Prenatal diagnosis and screening have been expanding rapidly over the last 30 years. They have become incorporated into routine antenatal care, in the guise of routine ultrasonography for major structural abnormalities and maternal serum screening for aneuploidy. As ultrasonographic equipment, cytogenetic technology, and molecular biology have improved, it has become possible to perform prenatal diagnosis for an increasing number of fetal conditions and earlier in gestation. In the future there is the very real possibility that many invasive diagnostic tests may become redundant with the advent of noninvasive prenatal diagnosis.

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Chapter 6

Preimplantation embryo development

Kay Elder

Key points

- The successful development of embryos prior to implantation is dependent initially upon the interaction between healthy mature male and female gametes to undergo a process of normal fertilization that results in the creation of a zygote with their combined chromosome complement.
- The early stages of embryo development are largely dependent upon maternal reserves, driven by translation of maternal mRNA.
- All factors that influence in vivo oocyte development and maturation therefore influence embryo quality.
- Similarly, the process of embryo development may also be influenced by the contribution of the male gamete, and sperm with abnormal chromosomes or DNA damage may successfully fertilize the oocyte, but later developmental stages of the embryo may be compromised
- Preimplantation development follows a programmed timeline, during which a carefully orchestrated series of critical events must take place.
- The procedures of clinical in vitro fertilization (IVF) allow this timeline of events to be studied in detail, from early cleavage stages, activation of the new zygote genome at the four- to eight-cell stage, polarization and establishment of cell junctions, and interactions during the morula stage, with progression to blastocyst development and the formation of a blastocyst with clearly differentiated cell layers
- The evaluation of embryos in order to select those with optimal prognosis for implantation after embryo transfer has been traditionally based upon morphological assessment.
- Recent research, and especially accumulated data from preimplantation diagnostic procedures have revealed the limitations of assessment by morphological criteria alone. Current criteria for embryo selection will be reviewed and summarized in this chapter

Introduction

The development of the preimplantation embryo depends upon a myriad of synchronized interdependent mechanisms, choreographed so that each must function at the right time during embryogenesis. A series of elegantly programmed events begins at gametogenesis and continues through to parturition. Considering the complexity and diversity of this remarkable process, it is little wonder that our efforts to overcome infertility so often fail. Although the past decade has witnessed great advances in our ability to fertilize oocytes successfully and to develop embryos in vitro we still struggle to achieve implantation rates comparable with those seen in other species (see Chapter 2). This may reflect the level of aneuploidy present within human embryos (see Chapter 7), which is possibly even greater within the population who seek assisted conception, but is probably compounded by our inadequate attempts to mimic in vivo conditions with in vitro culture. Efforts to improve our understanding and identification of the features and events that contribute to the development of a viable embryo continue to be essential in order to improve the prognosis for infertility patients. This chapter considers the major elements that may determine embryo viability in the human. Owing to the complicated logistic and

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ethical problems involved in conducting scientific research on the human embryo, many of the underlying principles described here have been elucidated or extrapolated from research in other species, in particular the preimplantation mouse embryo.

Gametogenesis

Male and female gametes which will unite to form each embryo are derived from a unique population of primordial cells that are designated to become the gametes: they develop into highly specialized gametes by the processes of spermatogenesis in the male and oogenesis in the female. In both cases, the primordial germ cells originate outside the gonad, and are first seen in the embryonic yolk sac at around four weeks post-conception. Primordial germ cells (PGC) have unique properties in terms of their morphology, behavior, and gene expression, including erasure of epigenetic information from the previous generation. The cells are motile and invasive, and migrate through the dorsal mesentery of the hindgut to arrive at the gonadal ridges where they colonize the indifferent gonad, a mass of mesoderm on the dorsal body wall. When the primordial germ cells have completed their migration, they lose their motile characteristics and proliferate rapidly, dividing by mitosis to increase their number. This proliferation is followed by a period of cell growth, which is much more significant in the female gamete than in the male gamete.

Meiosis

The key event of gametogenesis, in both sexes, is the halving of the number of chromosomes during meiosis (Figure 6.1). Meiosis is a specialized cell cycle consisting of two successive rounds of chromosome segregation following a single round of DNA replication, producing progeny cells with half as many chromosomes as their parents. Thus in man, where the chromosome number of somatic cells is 46, each oocyte and each spermatozoon has only 23 chromosomes. However, the similarity between oogenesis and spermatogenesis ends at this point. As illustrated in Figure 6.2, in the male, each primary spermatocyte divides meiotically to produce four spermatids, each destined to become a functional spermatozoon, and the process of spermatogenesis continues throughout adult life. In the female, primary oocytes are produced only during early fetal life, and each primary oocyte leads to the development of a single mature pre-ovulatory oocyte. An unequal distribution of cytoplasm at division results in the production

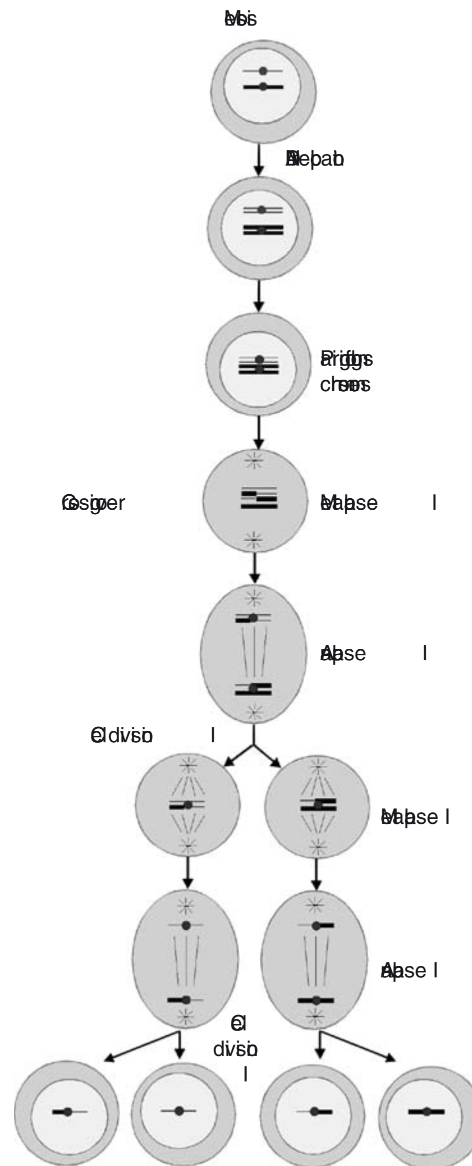


Figure 6.1 Meiosis. Four chromosomally unique haploid cells are generated from each diploid cell. (From: Elder & Dale, 2000; with permission).

of two small cells, the polar bodies, which eventually degenerate. These may be used for polar body biopsy and diagnosis (see Chapter 9).

A major difference between mitotic and meiotic cell cycles lies in the fact that during meiosis the oocyte can be blocked at precise phases of the cell cycle, until a specific stimulus (e.g. hormone or sperm) removes the block. In somatic cells, a state of quiescence, or cell-cycle block, in response to a specific physiological state

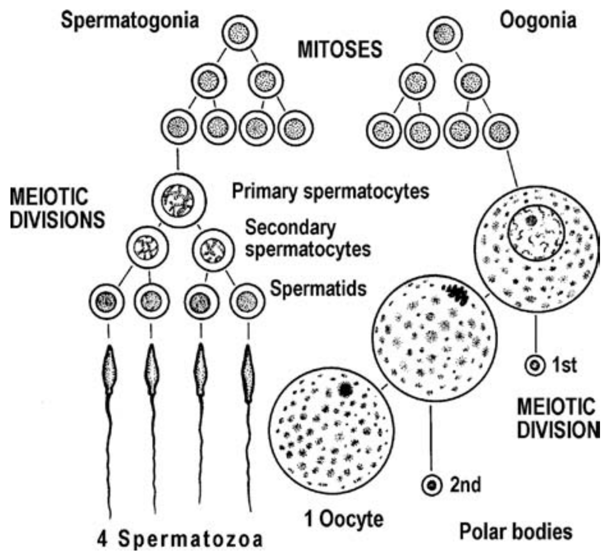


Figure 6.2 Gametogenesis in the male gives rise to four haploid spermatozoa; in the female, only one of the four daughter cells becomes a functional oocyte. (From Elder & Dale, 2000. Originally adapted from Dale B, *Fertilization in Animals*. London: Edward Arnold, 1983; with permission from Hodder).

of the cell is described as the G0 phase of the cell cycle. However, G0 differs from meiotic blocks in terms of cell-cycle regulation and the activity of the key kinases that maintain the arrest; in other words, meiosis has specific cell-cycle checkpoint controls which differ from those that control mitosis.

Spermatogenesis

The process of spermatogenesis takes place over three phases: proliferation, reduction division (meiosis), and differentiation. These are associated with specific germ cell types – spermatogonia, spermatocytes, and spermatids, respectively (Figure 6.3). In the male, interphase germ cells start to proliferate by mitosis at puberty. This is followed by meiosis and a gradual reorganization of cellular components, characterized by a loss of cytoplasm. The stem cells, or A0 spermatogonia, are located in the intratubular compartment, at the base of the seminiferous epithelium. At intervals, A-l spermatogonia emerge from this population and undergo a fixed number of mitotic divisions to form a clone of daughter cells. After the final mitotic division (B spermatogonia) the primary spermatocytes move into the adluminal compartment and enter into meiosis. In this compartment they undergo two meiotic divisions to form, first, two daughter secondary spermatocytes, and eventually four early spermatids, which then undergo a process of terminal differentiation and structural remodeling known as "spermiogenesis." During spermiogenesis spermatid DNA histones are replaced with protamines, and the sperm tail, mid-piece, and acrosomal cap are formed; round spermatids transform into elongating and elongated spermatids before evolving into mature spermatozoa.

Mammalian spermatozoa leaving the testis are not normally capable of fertilizing oocytes (this is not the case when oocytes are injected with sperm by intracytoplasmic sperm injection (ICSI)). They gain this ability while passing down the epididymis, a process known as "epididymal maturation."

A number of different etiologies can disrupt the orderly pattern of spermatogenesis, and immature forms, especially spermatocytes, slough into the lumina of the tubules in the presence of testicular pathology. Less frequently, maturation may proceed to the spermatid stage and arrest at this point. The epithelium of the tubules is very sensitive to toxins and to ischemia, and any lesion that arrests maturation at a stage preceding spermiogenesis will result in azoospermia. A number of studies in animal models have examined the effect of radiation or toxic chemicals on spermatozoa and subsequent effects on reproductive outcome, and these suggest that perturbations of biological mechanisms that control spermatogenesis can interfere with the sperm's capacity for successful fertilization and embryo growth (Hales & Robaire, 2001; Robaire & Hales, 2003; Hales *et al*., 2005).

Oogenesis

The mitotic phase of germ cell proliferation in the human female terminates before birth, and by the fifth month of fetal life all oogonia have entered their first meiotic

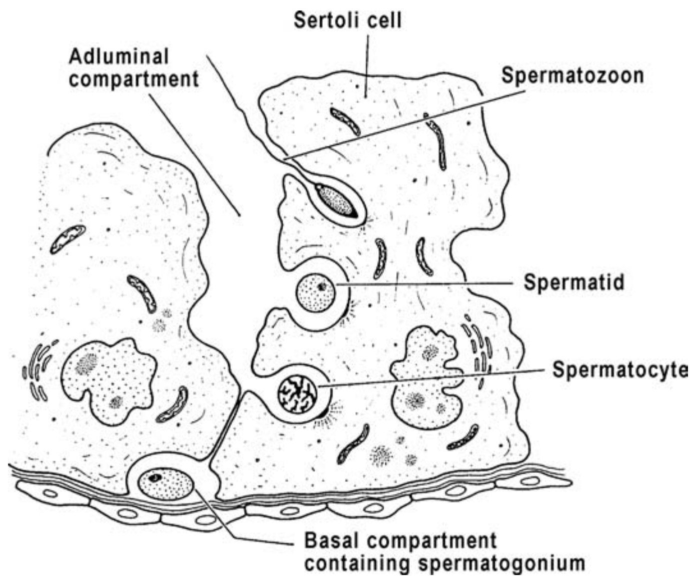


Figure 6.3 Spermatogenesis: maturation and modeling of the male gamete is regulated by the Sertoli cell. (From Elder & Dale, 2000. Originally adapted from Johnson M and Everitt B. *Essential Reproduction*. Oxford, Blackwell Science, 1990; with permission).

division to become primary oocytes. Around week nine of gestation, the oogonia are in the first meiotic prophase, and a layer of mesenchymal pregranulosa cells starts to secrete a proteinaceous layer that surrounds the primordial oocyte, forming a basal lamina that delineates each primordial follicle within the ovary. The oocyte nucleus (germinal vesicle) is arrested in diplotene (the last stage of Prophase I), after chromatid exchange and crossing-over have taken place. The primordial follicles remain quiescent, in meiotic arrest, for many years, and resume their growth and development to produce mature oocytes capable of fertilization only after puberty. A total of about 200 000 germ cells are available for the reproductive lifespan at puberty, when recruitment of some of these primordial follicles begins. Over 99 percent of follicles are destined to die rather than ovulate; the degenerative process by which these cells are irrevocably committed to undergo cell death is termed "atresia."

Oocyte growth

Following puberty, when a primordial follicle is recruited into a developmental pathway that will lead to ovulation, the oocyte enters a growth phase which involves a significant increase in size: the recruited follicle grows from 20 to several hundred microns (μm), and the oocyte itself grows from l0 to about 100 μm (Figure 6.4). This phase may last for several months, during which the nucleus (germinal vesicle) remains in meiotic arrest. There is an intense synthesis of RNA and, to a lesser extent, of proteins as cellular machinery makes and stores the material required to support early post-fertilization development of the embryo. The surrounding granulosa cells divide mitotically and the zona pellucida (ZP) is secreted between the oocyte and the granulosa cells (Heikinheimo & Gibbons, 1998). Gap junctions allow transfer of substrates and developmental information between the oocyte and the cytoplasmic projections of the accessory cells that penetrate the ZP. A second layer of specialized cells, the cumulus oophorus, plays an essential role in the maturation of the oocyte, contributing to its intrafollicular environment during development. The layer of cumulus cells in contact with the oocyte is radially striated: the "zona radiata." The follicle cells transfer materials used in oocyte growth and also provide signals to trigger the oocyte into maturation.

Maturation of the oocyte is associated with polarization of the cumulus cells and secretion of a hyaluronic acid extracellular matrix (ECM). Cumulus cells express complement-binding proteins in vitro, which might help to protect the embryo from complement in tubal and uterine fluid. Their significant steroidogenic activity may contribute to local steroid levels in the luteal phase and early pregnancy. When the oocyte has completed its growth phase, the follicle is "pre-antral," and is able to undergo rapid development in response to follicle stimulating hormone (FSH) secreted from the pituitary gland. FSH induces a rapid proliferation of the granulosa cells, and fluid accumulates within the follicle to create an antrum that separates the cell layers into mural granulosa lining the follicle wall, and cumulus cells surrounding the oocyte. The fully expanded antral follicle is now

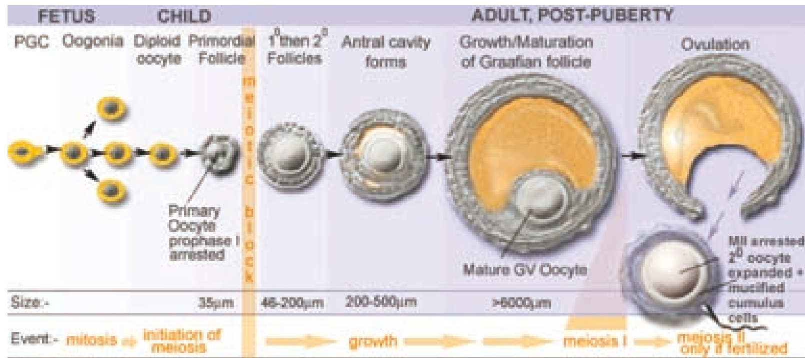


Figure 6.4 Follicle development, oocyte maturation and ovulation. With permission from J. Huntriss, University of Leeds. (Adapted from Picton *et al.*, 1998; with permission from Elsevier.)

responsive to luteinizing hormone (LH) secreted from the pituitary gland, which is released rapidly (LH surge) in response to feedback signals from the ovary. At the pre-ovulatory stage, gap junctions between the cumulus cells create a compact tissue mass around the oocyte. The LH surge induces an expansion of the cumulus oophorus by almost 40-fold, with the accumulation of a voluminous mucified ECM. Under natural conditions, the endogenous LH surge will induce final nuclear maturation of the oocyte within the next 24–36 hours (Heikinheimo & Gibbons, 1998). In this final maturation stage of oogenesis the nuclear membrane breaks down (germinal vesical breakdown, GVBD), meiosis is re-initiated, and the first polar body is extruded. The female cell is now in the stage of second metaphase (M-II). This surge of LH causes a rapid further accumulation of fluid in the pre-ovulatory oocyte over a period of approximately 36 hours, leading to a follicle of around 25 mm diameter that then ovulates; follicle rupture causes a small amount of bleeding, and a small scar (stigma) forms on the surface of the ovary. Steroidogenesis rapidly switches to progesterone synthesis after the LH surge. It is believed that oviductal fimbria sweep the ovulated oocyte into the oviductal ampulla the site where fertilization may take place.

Storing information

The oocyte accumulates specific RNA macromolecules during growth, which are required later in the control of embryogenesis: localized short- and long-lived maternal mRNAs regulate the initial stages of development and differentiation in the early embryo. The storage of information is controlled by a number of molecular processes that affect mRNA stability, including the formation of secondary structures, association with cytoplasmic factors, and polyadenylation. Messages with short polyA tails are repressed during storage, until the appropriate signals at the time of oocyte maturation or fertilization, when they become functional via a mechanism involving a cytoplasmic polyadenylation element (CPE) and CPE-binding proteins (Bachvarova, 1992; Huarte *et al.*, 1992).

New RNA synthesis is required at the critical stage of maternal-to-zygotic transition, when the new zygotic genome is activated in order to direct the further development of the embryo, but the transition takes place gradually, and some maternal transcripts are still required at the stage of blastocyst development (Nothias *et al.*, 1995; Schultz, 1995, 2002).

Oocyte polarity

Although the oocyte itself appears to have a homogeneous structure under the light microscope, many cytoplasmic organelles become segregated to specific regions of the oocyte. This regional organization determines some of the basic properties of the embryo, and the oocyte cortex, including the oolemma, may possibly be a determining factor. The intracellular location of mRNAs and protein translation machinery may play a part in regulating the cell cytoskeleton. Several lines of evidence suggest that mammalian ooplasm redistributes after sperm entry during fertilization (Edwards & Beard, 1997). Unlike mouse oocytes, which have a microvillus-free area, human oocytes show no polarity in the distribution and length of surface microvilli. Studies with fluorescent lectins reveal no signs of polarization in membrane sugar distribution. However, Antczak and Van Blerkom (1997) found that two regulatory proteins involved in signal transduction and transcription activation (leptin and STAT3) are polarized in mouse and human oocytes and in

preimplantation embryos. Edwards and Hansis (2005) also reported that transcripts of beta-human chorionic gonadotrophin (β-hCG) were asymmetrically distributed in human four-cell embryos: individual cells expressed either hCG or Oct-4. It has been suggested that a subpopulation of follicle cells may be partly responsible for the polarized distribution of these transcripts in the oocyte, and that they may be involved in determining its animal pole, and in the establishment of the inner cell mass (ICM) and trophoblast in the preimplantation embryo (Eppig, 2001; Grammont & Irvine, 2002; Rossant, 2004). Any disruption of spatial organization or concentration gradients within the oocyte cytoplasm (such as may occur during in vitro manipulation and ICSI in particular) could have an adverse effect on its future development (Eichenlaub-Ritter, 2002).

Oocyte maturation

Oocytes normally arrest twice during meiosis:

1. Throughout the growth period the diffused chromosomes are surrounded by an intact nuclear membrane which encloses them into the large oocyte nucleus known as the germinal vesicle (GV). The cell cycle is blocked at prophase of the first meiotic division, and can remain viable in this arrested state for up to 50 years. Re-initiation of meiosis in fully grown oocytes (after puberty) is the first indication of oocyte maturation. This transition from prophase to metaphase involves condensation of interphase chromatin and breakdown of the nuclear membrane (GVBD), resulting in a mixing of nucleoplasm and cytoplasm, spindle formation, and eventual chromosome segregation. The semicontracted chromosomes, now in the cytoplasm, migrate to the periphery of the oocyte where they become arranged on the spindle. The oocytes complete the first meiotic division by extruding half of their chromosomes in the first polar body.
2. Hormones trigger the release from this first meiotic block, driving the oocyte to a second arrest at metaphase II. The spermatozoon triggers release from the second meiotic block. Progression from the first to the second meiotic arrest is usually referred to as "oocyte maturation," and the oocyte is now ready to be ovulated and subsequently fertilized. The removal of the second meiotic block at fertilization is called "oocyte activation," a process that will be described below.

Mammalian oocyte maturation and ovulation are under the control of pituitary hormones, in particular FSH and LH; the situation is complex, and involves the additional interplay of the ovarian hormones, estrogen and progesterone. Before the LH surge the oocytes are "on standby," in meiotic arrest, which is maintained by the diffusion of cyclic adenosine monophosphate (cAMP) from the cumulus to the oocyte. Although the exact substrates have not been identified, it is generally accepted that progression through the meiotic cell cycle is regulated by a series of protein kinases and phosphatases (Cho *et al*., 1974; Dekel, 1996; Coticchio & Fleming, 1998; Mattioli & Barboni, 2000). LH alters gap junctions, and the flux of inhibitory signals to the oocyte is interrupted. Within a few seconds, a calcium rise reduces membrane conductance to K^+ and causes cumulus-corona cells to selectively undergo progressive depolarization of their membrane potential. A rapid increase in intracellular calcium is transmitted to the oocyte, diffusing from the cortical region to the center of the cell. Calcium elevations are transient in the cumulus cells, and long lasting in the oocyte. Two protein complexes, maturation promoting factor (MPF) and cytostatic factor (CSF) are involved in regulating progression through meiosis. It is generally accepted that the main components of CSF include the product of the proto-oncogene c-mos, mitogen associated protein kinase (MAPk), and possibly cdk2 kinase (Masui, 2001).

The ability to resume meiosis in vitro is not shared by all isolated oocytes, and in assisted conception treatment cycles the processes of nuclear and cytoplasmic maturation may be uncoupled by ovarian stimulation or by attempts to mature oocytes in vitro. In addition, activation competence in oocytes is continually changing in vitro, and is not a stable, prolonged feature of ovulated eggs; therefore timing is critical in the handling of in vitro manipulations. The rate of follicular development may be accelerated by external endocrine manipulation, but this does not necessarily ensure equivalent acceleration of oocyte development and maturation, and may compromise the quality or developmental competence of the eggs. Oocyte competence is related to the storage of protein factors and stable mRNA, and meiotic incompetence may be related to deficiencies in the MPF subunits, or of some of the regulatory elements responsible for MPF kinase activation. Changes in microtubular morphology or activity may also play an important role in the acquisition of meiotic competence, and

these may be regulated in part by MAPk (Fulka *et al*., 1998; Lee *et al*., 2000).

A failure in MAPk activity may in turn be related to a deficiency in Mos protein. In summary, the production of a viable egg depends on three key processes:

1. The fully grown egg must recognize regulatory signals generated by follicular cells.
2. Extensive molecular reprogramming within the egg must be induced – this involves activation of appropriate signal transduction mechanisms.
3. Individual molecular changes must be integrated to drive the two parallel but distinct processes involved in meiotic progression and the acquisition of developmental competence.

Fertilization

In nature, fertilization is a highly specialized example of cell-to-cell interaction, where each gamete activates its partner. The introduction of ICSI in assisted-conception treatment bypasses the majority of events which were previously thought to be essential for the fertilization process, opening a whole new area of questions and molecular events that are yet to be elucidated (Hewitson *et al*., 2000). The discussion here is limited to "natural" fertilization without reference to ICSI.

In order to trigger metabolic activation of the oocyte, the spermatozoon itself must encounter and respond to signals originating from the oocyte and its investments. Sperm–oocyte interaction is a complex multistep process that starts with the specific recognition of complementary receptors on the surfaces of the two gametes and terminates with syngamy, the union of the maternal and paternal chromosomes. In addition to delivering the paternal genome, the spermatozoon triggers the quiescent female gamete into metabolic activity via a cascade of cell signaling events, releasing the meiotic block so that early embryogenesis may be sustained.

The central event of the normal fertilization process is fusion of the plasma membranes of the two cells in order to create a new zygote. Both activation of the spermatozoon and activation of the oocyte are regulated by changes in intracellular messengers such as Ca^{2+}, H^+, cAMP, cyclic adenosine diphosphate ribose (cADPr) and inositide triphosphate (IP_3). The initial stages of fertilization depend principally on two structures: the acrosome of the spermatozoon and the ZP of the oocyte. There are three major events in sperm–oocyte interaction:

1. Attachment of the spermatozoon to the ZP.
2. The spermatozoon undergoes the acrosome reaction, as a result of which digestive enzymes are released and the inner acrosomal membrane is exposed.
3. This highly fusogenic sperm membrane makes contact with the oolemma and the two membranes fuse together.

Sperm activation and the acrosome reaction

In order for successful fertilization to take place, the spermatozoon must first be activated. Activation of the male gamete involves several behavioral, physiologic, and structural changes, some of which are induced by exposure to environmental signals, and others by the interaction of the spermatozoon with the oocyte and its extracellular investments. All of these changes are essential for successful fertilization, including changes in motility, capacitation, acrosome reaction, penetration, binding, and fusion. Capacitation involves molecular modifications in the outer layers of the sperm plasma membrane, a prerequisite for developing the ability to undergo the acrosome reaction; this is characterized by a change in the sperm's pattern of motility ("hyperactivated motility"). In vivo, sperm capacitation takes place in the female genital tract. Before interacting with the oocyte ZP the spermatozoa must traverse and interact with the outer oocyte investments, the cumulus cells. These drastically reduce the number of spermatozoa that reach the underlying ZP. The spermatozoa that succeed in traversing the cumulus layer can bind to and then penetrate the ZP. It appears that not all of the bound sperm are able to do this, and many are not triggered into an acrosome reaction (Henkel *et al*., 1993; Payne *et al*., 1994). This may be attributed to the heterogeneity of the glycoproteins in the ZP. Three major glycoproteins were originally identified, known as ZP_1, ZP_2, and ZP_3, and a fourth ZP protein was recently discovered in humans. This protein was originally named ZPB, but has more recently been renamed ZP_4. The existence of ZP_4 in human oocytes has been shown both at the mRNA level by use of the reverse-transcription polymerase chain reaction (RT-PCR) and at the protein level via tandem mass spectrometry (Lefièvre *et al*., 2004).

Complementary molecules are found on the surface of the spermatozoal head and on the ZP. According to Wassarman (1987, 1990) and Wassarman *et al*. (1985), sperm-receptor activity resides in the O-linked oligosaccharides of ZP_3. The complementary molecule of

the spermatozoon may be a lectin-like protein, present on the acrosome-intact sperm head. The membrane-bound acrosomal granule, which contains lytic agents such as proteases, sulphatases, and glycosidases, is bound within the plasmalemma of the spermatozoon. When the spermatozoon attaches to ZP_3 the permeability of the sperm plasmalemma is altered, causing a transient change in the concentration of several intracellular ions and alkalinization of the cytoplasm. The influx of calcium triggers the fusion of acrosomal membranes and the exocytosis of acrosomal contents, which are then lost during penetration of the zona so that only the inner acrosomal membrane is in direct contact with the zona, perhaps with ZP_2.

Oocyte activation

Intracellular calcium release

The universal messenger for the trigger of meiotic resumption in oocytes at fertilization is an increase in intracellular Ca^{2+}, released from intracellular stores in periodic waves or transients (Tosti, 2006). Calcium release with similar increases may be induced parthenogenetically by a variety of physical and chemical stimuli, but the kinetics of the calcium transients are different and do not sustain development (Tosti, 2006).

The active fraction in spermatozoa that induces calcium release is not species-specific, or specific to gametes. Until a common activation pathway for the various calcium-release mechanisms is identified, the possibility remains that "sperm factor" represents a collection of second messengers found in many cell types, but packaged and delivered differently in spermatozoa. Oocytes in turn possess several calcium-release mechanisms, and the mode of calcium release at fertilization varies from species to species.

Maturation-promoting factor (MPF) activity in oocytes is maximal at M-I and M-II, and decreases at exit from meiosis I and II (Gosden & Bownes, 1995; Dekel, 1996; Picton *et al.*, 1998). The fertilizing spermatozoon fuses to the plasma membrane and releases sperm factors into the oocyte. These factors stimulate the production of intracellular messengers that gate the release of intracellular calcium, required for the inactivation of MPF and the completion of meiosis II.

The cortical reaction

The first morphological indication of activation in oocytes is the exocytosis of cortical granules, which are small spherical membrane-bound organelles containing enzymes and mucopolysaccharides that originate as vesicles in the Golgi complex. The cortical reaction in the mammalian oocyte elicits the zona reaction, changing the characteristics of the ZP. A second result of the cortical reaction is that the oolemma now becomes a mosaic of cortical granule membrane and the original plasma membrane (Dulcibella, 1996). The process of sperm–egg fusion is mediated by guanosine triphosphate- (GTP-)- binding protein, and the cortical reaction is probably mediated by activation of the inositol phosphate (PIP-2) cascade, which induces calcium release from intracellular stores and protein kinase C (PKC) activation, leading to cortical granule exocytosis. The contents of the cortical granules cause zona hardening, and induce a modification in the sperm receptor, which then blocks penetration by further sperm. A modification of the oolemma following sperm–egg fusion and the cortical reaction also contribute to the polyspermy block (Sun, 2003).

Cortical reorganization is a common feature of oocyte activation. By piecing together all of the information from animal systems, some general conclusions may be drawn about the role of cortical reorganization in embryogenesis. First and foremost, the zona provides protection for the developing embryo. The products of cortical granule release catalyze hardening of the oocyte's relatively thin extracellular coat that hardens after activation, and the embryo remains in its protective coat until hatching. A second extracellular structure, the hyaline layer, is also produced as a result of cortical granule exocytosis, and this serves to keep the dividing blastomeres of the embryo in close contact. The early embryo is a compact mass of continually dividing cells, and the embryo is therefore continually changing shape. Such movement would be hindered if the cells were attached to a rigid structure, so possibly for this reason the embryo is surrounded by the fluid-filled perivitelline space. This gap may also provide a micro-environment to buffer the embryo from changes in the external environment.

Reorganization of the plasma membrane is dramatic and rapid, and appears to be related to the metabolic de-repression of the oocyte, without the participation of the cells' synthetic apparatus.

Sperm–oocyte fusion

The process of membrane fusion between gametes is not understood, but Ca^{2+} and a close approximation of the two membranes is essential. Fusion of gametes seems to be facilitated by the presence of numerous microvilli on the oocyte surface; these have a low

radius of curvature that may help to overcome opposing electrostatic charges. During fusion the ooplasm rises up in a protuberance around the spermatozoal nucleus to form the fertilization cone (Sathananthan *et al*., 1990).

The fertilizing spermatozoon continues flagellar movement for some 20 seconds after attachment to the oocyte surface, and there then follows a sudden cessation of flagellar motion that may occur simultaneously with the process of gamete fusion. Sperm motility, although necessary for penetration of the zona, is not required for gamete fusion. Fusion is temperature, pH, and Ca^{2+} dependent, and it seems that the terminal glycoprotein saccharides are not directly involved in the process (Marangos *et al*., 2003).

In some mammals (including humans), the sperm tail is incorporated by the progressive fusion of the oocyte and spermatozoal plasma membranes (Chen & Sathananthan, 1986; Sathananthan *et al*., 1990).

After incorporation, the mid-piece mitochondria and the axial filament of the tail appear to disintegrate and the sperm centriole is exposed to ooplasm. The microtubule-based sperm aster then forms, initiating the union of male and female pronuclei. The disassembly of the sperm tail occurs as a series of precisely orchestrated events, involving the destruction and transformation of particular sperm structures into zygotic and embryonic components. The spermatozoal plasmalemma, however, is integrated into the oocyte plasma membrane and may play a role in development. Following gamete fusion the sperm plasmalemma remains in the oolemma and indicates the point of fusion. Experimental results in animal systems showed that complete incorporation of the sperm depends upon the integrity of oocyte microfilaments, and is inhibited by the microfilament disrupter cytochalasin B (Sathananthan & Chen, 1986; Simerly *et al*., 1995).

Centrosomes

The centrosome represents a fundamental paternal contribution to embryogenesis, providing a "division center" for the zygote. Although it is generally accepted that in humans the centrosome is of paternal origin, in the mouse and hamster the centrosome is apparently of maternal origin, lending support to the observation that these rodents are poor model systems for human fertilization (Simerly *et al*., 1995; Hewitson *et al*., 1997).

In somatic cells the centrosome is composed of two structures called "centrioles," placed at right angles to each other and surrounded by dense pericentriolar material. Each centriole is made up of nine triplets of microtubules arranged in a pinwheel array. With the exception of rodents, during spermatogenesis mammalian sperm reduce their two centrioles to a single, inactive proximal centriole (Schatten *et al*., 1991; Schatten,1999; Simerly & Navara, 2007). The majority of the pericentriolar proteins are extruded into the cytoplasmic droplet, which is shed during the process of sperm maturation. In contrast, during oogenesis the centrosomes degenerate after meiosis, leaving the oocyte without a "division center," which is then contributed by the sperm during fertilization. The oocyte, however, does retain a significant pool of pericentriolar proteins, which may contribute to the microtubule organizing center (MTOC) (Simerly & Navara, 2007). After the sperm enters the oocyte a small "aster" of microtubules grows from the sperm centriole, which directs the migration of the sperm pronucleus to the center of the oocyte to make contact with the decondensing maternal pronucleus, initiating its migration toward the forming male pronucleus. The new zygotic centrosome then duplicates and splits apart during late interphase, as microtubules extend from in between the eccentrically positioned, juxtaposed male and female pronuclei. After duplication the centrosomes migrate to opposite poles during mitotic prophase to set up the first mitotic spindle of the zygote, completing the process of fertilization. Although the centrosomes are the main organelles associated with cell division, it is now thought that the principal MTOC may be provided by maternal pericentriolar material (Schatten, 1994).

In cases of polyspermy, human oocytes develop multiple sperm asters, each associated with a sperm. During parthenogenesis, where there is no paternal centrosomal contribution, no sperm astral microtubules are nucleated, and cytoplasmic microtubules are instead found throughout the oocyte (Kola & Trounson, 1989; Schatten *et al*., 1991). In this case, the female centrosome becomes fully functional, duplicating and forming the mitotic spindle poles. Microtubules are present in metaphase-arrested second meiotic spindles in unfertilized oocytes. Although cattle and human oocytes can organize microtubules without sperm entry, this happens later, and less completely, than it does after sperm entry. Defective centrosome function may result in fertilization failure (Simerly *et al*., 1995). Six hours post-insemination, a small microtubule sperm aster extends from the

sperm centrosome, and the activated egg extrudes the second polar body.

Formation of pronuclei and syngamy

Transformation of the sperm nucleus into the male pronucleus involves disassembly of sperm nuclear lamina, chromatin dispersion, enlargement, the disintegration of the nuclear envelope, and the formation of a new pronuclear envelope.

During spermatogenesis gene expression is completely repressed, DNA replication ceases, and sperm chromatin is tightly packed into a nuclear envelope that lacks pores. The mature sperm nucleus is packed with distinct protamines. The association of nuclear DNA with these highly charged basic amino acids is thought to cause condensation and repression of DNA activity (Eddy, 1998). The rigidity of the mammalian sperm head, necessary for penetration of the zona, is attributed to extensive disulphide linkage in these protamines. In the human spermatozoon, Zn^{2+} from the prostate gland regulates protamine cross-linking (Wolffe, 1998). In mammals, the first step of nuclear transformation is the reduction of disulphide bonds in nuclear protamines. Once inside the ooplasm the male nucleus undergoes a reverse process of morphological and biochemical transformation; the sperm nuclear envelope breaks down, the protamines are lost, and pronuclear decondensation occurs, while the oocyte transits from M-II to telophase-II: during telophase-II the sperm chromatin decondenses as the female pronucleus develops. Spermatozoal protamines are replaced by histones, and the male and female pronuclear envelopes develop synchronously.

The next step after decondensation is the formation of a new nuclear membrane around the decondensed male and female chromatin to produce the pronuclei. During pronuclear development, a limited quantity of sperm pronucleus development factors is found within the cytoplasm. Membrane vesicles present in the cytosol bind to the decondensed chromatin and subsequently fuse together to form a continuous membrane. The nucleus now swells by fusion of additional membrane vesicles, and soluble nuclear lamins are imported through functional pores. This last process of swelling is dependent on calcium, ATP, and GTP. Specific chromatin proteins are replaced during this structural modification, and the chromatin regains its capacity for DNA replication and transcription (Tesarik & Kopecny, 1989a, 1989b; Bouniol-Baly *et al.*, 1997).

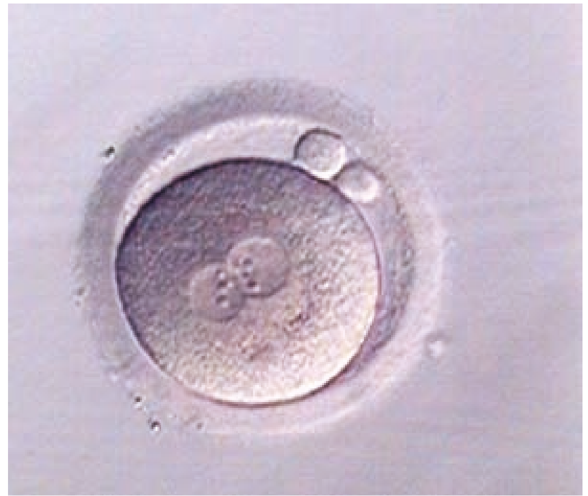


Figure 6.5 Zygote with normal fertilization, showing two juxtaposed pronuclei with nucleoli aligned centrally, and two polar bodies within the perivitelline space.

The newly formed male and female pronuclei migrate toward each other and subsequently move towards the center of the oocyte (Figure 6.5); the sperm aster is involved in this movement (in the mouse, in addition to the spindle microtubules there are 16 cytoplasmic MTOCs, or foci, and each centrosomal focus organizes an aster). The chromosomes in each pronucleus condense and concomitantly the pronuclear envelopes break down without fusing together. The male and female chromosomes then intermix in the cytoplasm and form the metaphase of the first mitotic spindle, with the duplicated centrosome forming the two poles of the spindle. Chromatid separation is then followed by a new cascade of events leading to the initiation of cleavage and early development (Tesarik & Kopecny, 1989c; Tesarik & Greco, 1999).

Imprinting

The term "imprinting" refers to the process whereby the two parental alleles of a single gene are differentially expressed. The molecular mechanism responsible for genome imprinting involves differential DNA methylation of CpG islands close to or embedded within gene promoters; this takes place during gametogenesis itself or before formation of the zygote nucleus during embryogenesis (Sapienza *et al.*, 1987). During the process of both male and female gametogenesis, the sperm and the oocyte acquire a genomic "imprint," which dictates which gamete is destined to express a particular

gene; dynamic reprogramming of DNA methylation takes place during gametogenesis and continues throughout preimplantation development.

After fertilization, chromosomes in the male pronucleus are actively demethylated whilst those of the female pronucleus are passively demethylated (Reik *et al*., 2001; Xu *et al*., 2005). However, the nucleus of a newly formed zygote inherits an imprint memory that is retained throughout prenatal and postnatal development; this controls the timing of gene expression. Functional differences between parental chromosomes are heritable, and they survive activation of the embryonic genome. Data derived from both pronuclear transplantation experiments and classical genetic experiments indicate that the maternal and paternal genetic contributions to the mammalian zygote nucleus do not function equivalently during subsequent development (Surani *et al*., 1986). These observations suggest that there is differential "genome imprinting" during male and female gametogenesis. Genomic imprinting must be distinguished from Y-linkage and cytoplasmic and other maternal effects, as processes that influence genetic inheritance. The molecular bases for these critical genetic events require further studies into the initiation and maintenance of imprinting mechanisms.

Cleavage and development to the blastocyst stage

After fertilization the zygote divides by mitosis into a number of smaller cells called blastomeres. Although cleavage may be considered a mitotic process, as found in adult somatic tissues, there is one important difference: in adult tissue the daughter cells grow following each division and are not able to divide again until they have achieved the original size of the parent cell. The cells in a somatic population thus maintain an average size. During cleavage this is not the case: with each division the resulting blastomeres are approximately half the size of the parent blastomere – it is a period of intense DNA synthesis and replication, without growth. Differences arise between the blastomeres that may result from the unequal distribution of cytoplasmic components as already laid down in the oocyte during oogenesis, or from changes occurring in the blastomeres during development. Each blastomere nucleus will be subjected to a different cytoplasmic environment that in turn may differentially influence the genome activity and subsequent potential for cell differentiation.

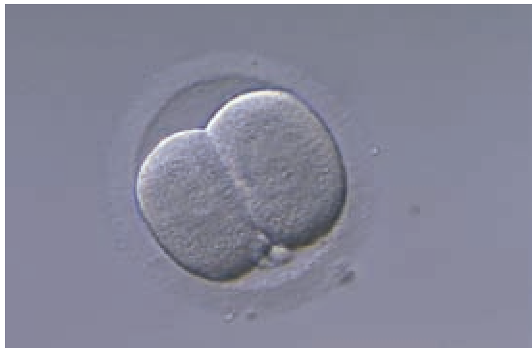
Zygote genome activation

As mentioned previously, the developing oocyte accumulates reserves of mRNA, proteins, organelles, and so forth, which are required to support and direct early development. The main function of the zygote is to ensure that the new embryonic cell cycle is correctly timed and regulated, with the correct timing of DNA synthesis during S-phase, after the long period of gene suppression in both gametes. The zygote initially is dependent on stored maternal mRNA to direct the first two cleavage divisions, and then activation of the new embryonic genome provides novel transcripts and reprograms the pattern of gene expression to direct further development. Without appropriate zygote genome activation (ZGA), the mammalian embryo fails to develop further. This critical transition takes place during the early cleavage stages of the embryo, at the four- to eight-cell stage in humans, and maternal mRNA rapidly disappears whilst the zygote genome gradually increases its expression (Braude *et al*., 1988; Tesarik *et al*., 1988; Schultz, 2002; Levy *et al*., 2004). However, the transition is not absolute, and a small amount of maternal message is needed almost until the blastocyst stage (Nothias *et al*., 1995), therefore previous failures at any stage of oocyte development, maturation, and handling can affect development even after ZGA. The timing and coordination of gene expression required for the ZGA transition may be regulated at the level of maternal mRNA translation. Gene expression involves conformational changes in nucleosome organization (like uncoiling a spring), regulated by interactions between DNA methylation, histone acetylation, and messenger RNA polyadenylation patterns (Schultz, 1995; Levy *et al*., 2004).

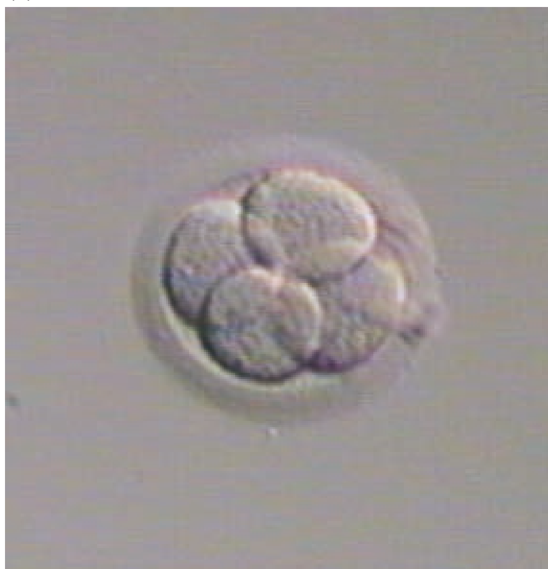
The process of embryogenesis is complex, involving cell growth and differentiation. In order to coordinate these cellular activities, the embryonic cells must be in communication. Communicative devices arise early in development and may serve additional roles in the synchronization of early divisions and the determination of the future planes of mitotic spindles. Two types of intercellular junction have been described:

1. Structural tight junctions and desmosomes which serve to anchor the cells together and also form permeability seals between cells. Tight junctions are composed of several integral and peripheral proteins, including occludin and cingulin (ZO-1) (Sathananthan *et al*., 1982; Campbell *et al*., 1995).
2. Low-resistance junctions, such as gap junctions, that allow the flow of electrical current and the

(a)



(b)



(c)

Figure 6.6 Early cleavage-stage embryos: (a) two-cell embryo; (b) four-cell embryo; (c) eight-cell embryo.

direct transfer of small molecules, including metabolites and second messengers (cAMP), between blastomeres (Goodall & Johnson, 1984; Lee *et al.*, 1987; Warner, 1992; Houghton, 2005).

Compaction

The newly fertilized zygote lies in the oviduct, where it undergoes the first few cleavage divisions to reach the four- to eight-cell stages; the individual blastomeres of the developing embryo may be clearly seen (Figure 6.6(a–c)). The next stage of embryo development involves compaction, where the blastomeres flatten against each other and begin to form junctions between them, so that the boundaries between blastomeres can no longer be distinguished (Figure 6.7). The cells of the compacted embryo become highly polarized, and are tightly associated and communicating. This process has been extensively studied in the mouse: surface polarity may be seen by the appearance of dense microvillar and amicrovillar regions, and cytoplasmic polarity can be seen in the distribution of endocytotic vesicles, actin filaments, and the location of the cell nucleus. In the mouse, polarity is maintained in isolated blastomeres following experimental decompaction, and requires neither the prior round of DNA replication nor protein synthesis (Kidder & McLachlin, 1985). Therefore, the four-cell embryo probably contains some of the proteins required for compaction. Although the factors that trigger the timing of its onset are not known, experimental evidence suggests that this may be regulated

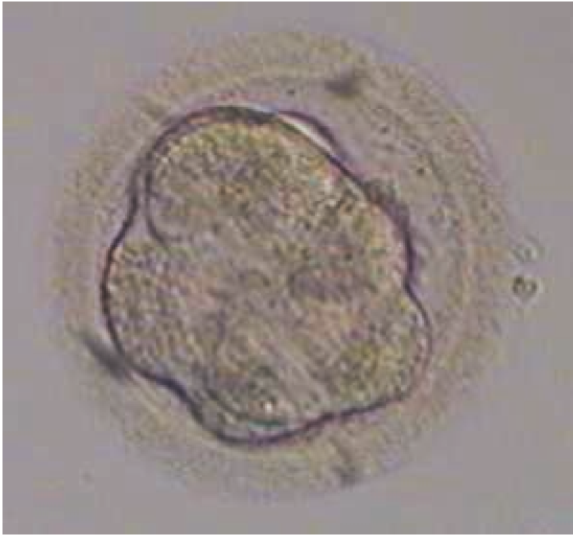


Figure 6.7 Compacting embryo, Day 4.

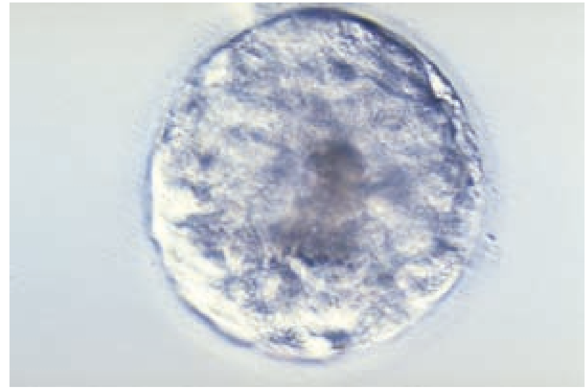


Figure 6.8 Early blastocyst with signs of central cavitation.

by post-translational modification of specific proteins such as E-cadherin (Clayton *et al*., 1995; Machell *et al*., 2000). The protein E-cadherin (uvomorulin) is expressed in the oocyte, and during all stages of preimplantation development (Campbell *et al*., 1995).

E-cadherin is uniformly distributed on the surface of blastomeres and accumulates in the regions of intercellular contact during compaction; E-cadherin phosphorylation may be observed in the mouse eight-cell embryo. Culturing embryos in calcium-free medium prevents compaction, and this also inhibits E-cadherin phosphorylation, but the situation is complex, and the precise mechanisms behind the molecular basis for compaction and its timing remain unclear.

In human embryos, tight junctions begin to appear on day 3, at the 6–10-cell stage, heralding the onset of compaction. Scanning electron microscopy studies showed evidence of compaction on day 4, when the majority of embryos with 10 or more cells revealed dense microvilli with a polarized distribution over the free surface of the compacted blastomeres (Nikas *et al*., 1996). In the mouse, gap junctions are expressed at the eight-cell stage, and their *de novo* assembly during compaction is a time-dependent event. In human embryos, gap junctions are not apparently well developed until the early blastocyst stage, when intercellular communication is clearly seen between ICM cells (Tesarik, 1989; Dale *et al*., 1991).

Following compaction, the developing embryo is described as a morula, seen in the human normally four days after fertilization. Whereas cleavage planes up to this stage are apparently random, in the mouse the cleavage planes are no longer random after the 16-cell stage, and subsequent cleavage divisions allocate cells to the interior of the morula (Edwards & Beard, 1997; Rossant, 2004). The embryo now shows a significant increase and change of pattern in RNA, protein, and phospholipid synthesis, and this results in a process of differentiation so that cells are now allocated to the ICM, with outer cells forming an epithelial layer of trophectoderm. These morphological transitions are thought to be brought about by differential gene expression with corresponding protein expression profiles, but these have not yet been clearly defined at the molecular level (see Posillico *et al*. (2007) and Steuerwald (2007), for reviews). ICM cells preferentially communicate with each other and not with trophectoderm cells via gap junctions, whereas trophectoderm cells communicate with each other and not with ICM cells.

Cavitation

Between the 16- and 32-cell stages, a second morphological change occurs, known as "cavitation" (Figure 6.8). Activation of Na^+, K^+ ATP-ase systems results in an energy-dependent active transport of sodium pumped into the central area of the embryo, followed by osmotically driven passive movement of water to form a fluid-filled cavity, the blastocele. The movement of other ions such as chloride and bicarbonate also contributes to blastocele formation. Tight junctions form a continuous belt

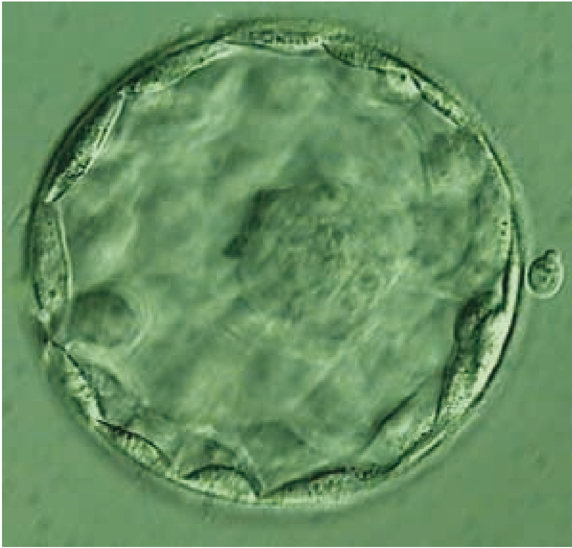


Figure 6.9 Fully expanded blastocyst with clear inner cell mass centrally and trophectoderm cells around the periphery.

between trophectoderm cells, and prevent leakage of small ions present in the blastocele.

Blastocele formation and expansion are critical for further development, as they are essential for further differentiation of the ICM. This is now bathed in a specific fluid medium, which may contain factors and proteins that will influence cell proliferation and differentiation. The position of cells within the ICM in relation to the fluid cavity might also contribute to the differentiation of the outer cells into primitive endodermal cells. The trophectoderm cells will eventually form the placenta and extra-embryonic tissue.

Blastocyst expansion and hatching

The early blastocyst (days 4/5) initially shows no increase in size, but it subsequently expands over the next one or two days (days 5/6) by active accumulation of fluid in the central blastocelic cavity (Figure 6.9). Throughout these early stages the embryo is enclosed in the ZP, which keeps the cells together prior to compaction and prevents two embryos fusing and forming a chimera. If the ICM divides at this early stage monozygotic (identical) twins may develop.

During the transition from morula to blastocyst the embryo enters the uterus, where it derives oxygen and metabolic substrates. At the site of implantation the trophectoderm cells produce proteolytic enzymes which digest a passage through the ZP, as the blastocyst "hatches" free of the zona. The uterine environment may also contain proteolytic enzymes, but very little is known about the molecular basis for hatching. The exposed cell layers of the hatched blastocyst make firm physical contact and implantation starts. In the human embryo the first 14–18 days of development are concerned mainly with the differentiation of various extra-embryonic tissues, and separate tissues are identifiable only after this time.

Growth factors have a key role in growth and differentiation from the time of morula to blastocyst transition. However, in in-vitro culture systems, defining their precise role and potential for improving preimplantation development is complicated by mechanisms such as gene expression, both of the growth factors and their receptors. There is also the potential for ascribing positive effects to specific growth factors when the result may in fact be attributed to a combination of multiple other causes. The mammalian blastocyst expresses ligands and receptors for several growth factors, many of which can cross-react, making it difficult to interpret the effects of single entities added to the medium. Insulin, leukemia inhibitory factor (LIF), epidermal growth factor (EGF), transforming growth factor alpha and beta (TGFα, TGFß), platelet-derived growth factor (PDGF), and heparin-binding EGF-like growth factor (HB-EGF) have all been studied in in vitro fertilization (IVF) culture (for a review, see Kane *et al.*, 1997). Although it is clear that these and other growth factors may have an influence on in-vitro blastocyst development and hatching, further assessment remains an area of research. It has been suggested that the mechanism whereby serum induces abnormalities in domestic animal systems may involve the overexpression of certain growth factors – there is no doubt that complex and delicate regulatory systems are involved (Spanos *et al.*, 2000; Glabowski *et al.*, 2005; Kawamura *et al.*, 2005; Torres-Padilla & Zernicka-Goetz, 2006). The culture of embryos in "groups" rather than singly has been found to improve viability and implantation in some systems: therefore, it is possible that autocrine or paracrine effects or "trophic" factors exist between embryos (Lightman *et al.*, 1997).

Metabolic requirements of the early mammalian embryo in vitro

The metabolic requirements of the preimplantation embryo vary through the preimplantation period, and clear differences in metabolism are seen between pre- and post-compaction stages. Prior to genome

activation, embryos have a relatively low level of biosynthesis, low respiratory rates, and their ability to metabolize glucose is limited; high levels of glucose at this stage have been found to be toxic, possibly through the formation of free radicals, which may inhibit embryo development by inappropriate interaction with metabolic pathways. At this stage pyruvate and lactate are the primary energy sources (Lane & Gardner, 2000; Orsi & Leese, 2004; Jansen *et al.*, 2006). After activation of the embryonic genome, metabolism shifts to glucose-dependent pathways. Post-compaction, the formation of tight junctions between the outer cells creates two different micro-environments for the inner and outer cells, and the embryos have an exponential increase in their energy requirements (Gopichandran & Leese, 2003; Houghton, 2006). Sequential media for use in IVF culture have been developed that complement this shift in metabolic requirements, adapted for the shifts in metabolic pathways and the consequences of new gene expression that accompany the embryo's development through compaction to blastocyst (Devreker, 2007; Ménézo & Guerin, 2007).

The ratio of different amino acids is also thought to be important for preimplantation embryo development. The precise amino acid requirements of the preimplantation embryo in vitro remain to be elucidated, but the requirements before and after genome activation probably vary (Gardner, 1998; Houghton *et al.*, 2002).

Causes of embryo arrest

Cleaved embryos frequently arrest their development in culture, and a great deal of research has been carried out in animal systems to elucidate possible causes and mechanisms. Embryonic arrest is often a result of events surrounding maturation, but can be a result of any metabolic problem. The longest cell division cycle during development is that during which genome activation takes place, when there is degradation of maternal transcripts and massive synthesis of embryonic transcripts. Accumulation of delays due to epigenetic effects of defective spermatozoa will normally result in arrested development: maternal reserves are sufficient until transcription begins. Anti-sperm antibodies can have deleterious effects at this stage, by immunoneutralization of proteins that signal division (CS-1) or regulation (Oct-3). After genome activation, the transition from morula to blastocyst is a sensitive phase. Complex remodeling takes place, and poor sperm quality also negatively influences this transition (Ménézo & Janny, 1997).

Paternal factors

Increasing paternal age is thought to have an influence on fertility, possibly through increased non-disjunction in the sperm. Damage during spermatogenesis may be induced by reactive oxygen species and defective oxidative phosphorylation, or via inherited dysfunctional mitochondrial DNA (Cummins *et al.*, 1994). Fertilization by a sperm which is diploid, with incomplete decondensation and DNA activation, or inadequate chromatin packaging may cause aneuploidy or a lack of genome competence in the embryo. The quality of condensation and packaging of sperm DNA are important factors for the initiation of human embryo development, even after ICSI (Sakkas *et al.*, 1998).

The centrosome, involved in microtubular organization, is the first epigenetic contribution of the sperm. A correct and harmonious microtubule arrangement is necessary for chromosome segregation and pronuclear migration. An imperfect centrosome carried by an abnormal sperm can disrupt mitosis, provoking problems at the beginning of embryogenesis with the formation of fragments, abnormal chromosome distribution, and early cleavage arrest. Of apparently unfertilized eggs, 25 percent have been shown to be fertilized, but with anomalies of cell division (Munné *et al.*, 1995). In the human, paternal Y-linked genes are transcribed as early as the zygote stage (Ao *et al.*, 1994), and compromised paternal genetic material transcribed at even this early stage might be responsible for fertilization failure or embryonic arrest.

Fragmented embryos

"Fragments," anucleate membrane-bound sacs of cytoplasm, are frequently seen in human preimplantation embryos, and have long been associated with diminished viability for embryos that exhibit a high degree of fragmentation (Puissant *et al.*, 1987). Different types and distinct patterns of fragmentation have been identified, based on the number, size and position of the fragments in relation to the size and position of nucleated blastomeres (Alikani *et al.*, 1999). Some embryos, in particular those with extensive fragmentation, show a non-distinct combination of patterns. The most common pattern that is observed in human embryos on day 3 of development is the presence of small, scattered fragments associated with more than one blastomere (Type III) (Figure 6.10) (Alikani, 2007).

Attempts have been made to improve the implantation potential of human embryos produced in vitro by creating a hole in the zona and then excising the

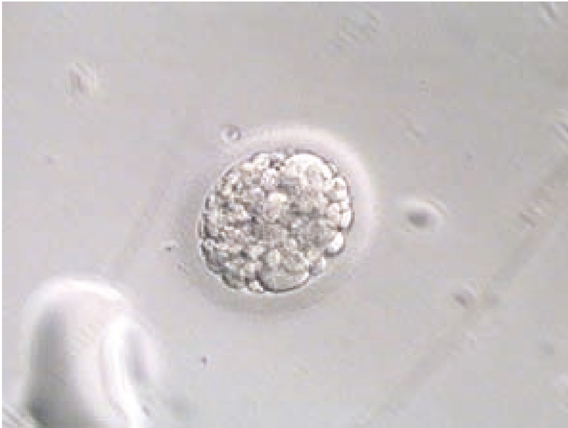


Figure 6.10 Day 3 embryo with Type III fragmentation.

cytoplasmic fragments. Removal of all fragments from highly fragmented surplus embryos, carefully matched with other embryos with similar morphology and development rate that were only zona drilled, resulted more frequently in the formation of single-cavity blastocysts in the fragment-free embryos, indicating that some intracellular fragments may inhibit cell–cell contact (Alikani *et al*., 1999; Alikani, 2001).

Surprisingly, fragmented embryos, repaired or not, do implant and often come to term. Sophisticated time-lapse photography technology has enabled *in situ* imaging of cleaving embryos, with imaging amplification to minimize light exposure that might be potentially harmful to them. This technique has clearly demonstrated that an individual embryo may radically change its morphological appearance in a short period of time: fragments which are apparent at a particular moment in time can be subsequently absorbed with no evidence of their prior existence (Hamberger *et al*., 1998). This demonstrates the highly regulative nature of the early embryo.

Apoptosis

Apoptosis, or programmed cell death, may be seen in ICM cells of the mouse blastocyst (Brison & Schultz, 1997), and has been suggested as a mechanism related to the formation of fragments in cleavage-stage embryos. The cellular machinery that is required for apoptosis is present in cleavage-stage embryos, but the relationship between apoptosis and fragmentation remains unclear. Brenner and colleagues were the first to report a study of human embryonic transcription of specific genes that regulate apoptosis during the preimplantation period (for a review, see Warner *et al*., 1998). Two genes are involved in apoptosis: bax & bcl-2. Bax is a regulatory gene that promotes cell death. bcl-2 functions to enhance cell survival in various cell types. Bax mRNA is expressed at all stages of preimplantation human embryonic development – transcripts are both maternal and embryonic, and varying levels are expressed at different stages of oocyte maturation. bcl-2 mRNA can be found at two-cell, eight-cell, 16-cell stages, and in morulae – but in fewer embryos. The ratio of bcl-2 to bax expression is the critical determinant of cell fate, so that increased bcl-2 leads to further survival, and increased levels of bax accelerate cell death in an incremental fashion. Activation of bcl-2 transcription upon fertilization may play a unique role in protection of the preimplantation embryo from apoptosis. Apoptosis in the blastocyst may fulfill the function of eliminating inappropriate or abnormal cells from the ICM, and its regulation may be critical for further embryonic development. In mouse embryos, there is evidence that apoptosis is influenced by peptide growth factors such as TGF-alpha acting as survival factors. However, knock-out mouse experiments indicate that other factors may compensate for the absence of a specific factor (Brison & Schultz, 1998) – a salutary reminder that in vitro embryo development involves complex molecular interrelationships that are currently beyond our understanding. Further elucidation of genetic mechanisms involved in preimplantation death and survival may have implications relevant for successful IVF and preimplantation genetic diagnosis (PGD) (Adams & Cory, 1998; Hardy, 1999; Jurisicova & Acton, 2004; Kawamura *et al*., 2005).

Conclusion

The recent application of PGD by fluorescent *in situ* hybridization (FISH) analysis of biopsied blastomeres has shown a surprising discrepancy between gross morphology and genetic normality of embryos. Even the most "beautiful" top-grade embryos may have numerical chromosomal anomalies, whilst those judged to be of "poorer" quality, with uneven blastomeres and fragments, may have a more normal chromosome complement (Delhanty *et al*., 1997). Multinucleated blastomeres have been reported, from karyotyping and FISH analysis; the presence of such blastomeres may be more common in arrested embryos, and may occur more readily in some patients. Also, non-mosaic aneuploidy in normally developing embryos increases with maternal age (Munné *et al*., 1995).

The application of extended culture using sequential media has enabled us to better understand embryogenesis and achieve viable pregnancies from embryos that have undergone their entire course of preimplantation development in vitro. Clearly, many factors of both maternal and paternal origin may determine the outcome of this development. However, since "embryogenesis begins in oogenesis" maternal age and oocyte quality remain the main determinants.

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Chapter

7

Preimplantation genetics

Joy Delhanty and Dagan Wells

Key points

- Preimplantation genetics covers events during gametogenesis and early embryogenesis and as such is closely tied to the causes of infertility.
- In males meiosis begins before puberty but spermatogenesis continues throughout life whereas in females meiosis up to prophase I is completed during fetal life and meiosis is only completed during fertilization.
- Infertility is a common problem in the human population, affecting 10 percent of couples, and overall fecundity is low compared with other studied mammalian species.
- The advent of in vitro fertilization (IVF) and preimplantation genetic diagnosis (PGD) has facilitated molecular genetic studies of human gametes and embryos, and provided insight into mechanisms that lead to the high rate of genetic abnormality at this stage of life.
- Methods used to study the chromosomes of gametes and embryos include karyotyping, fluorescent *in situ* hybridization (FISH), metaphase- comparative genomic hybridization (metaphase-CGH), and array-CGH.
- Males carrying chromosome abnormalities and those with normal karyotypes but various sperm pathologies are at increased risk of producing aneuploid sperm.
- Oocytes are vulnerable to nondisjunction, which may include predivision of chromatids.
- Embryos that have been identified as abnormal after either PGD for a specific disorder or after aneuploidy screening have provided a wealth of material for research. Information gained from this source has increased understanding of the underlying reasons why only one in five cleavage-stage embryos has the potential to implant and lead to an ongoing pregnancy.
- Chromosomes in human embryos show high levels of mosaicism.
- However, it is clear from further in-depth analysis of embryos from couples affected by repeated miscarriage or failed IVF cycles that for these couples particular factors are relevant to their poor reproductive history.

Introduction

An essential prerequisite for the formation of a euploid zygote is that the intricate meiotic process should be completed successfully in both parents. The first requirement is for synapse of homologous chromosomes, through formation of the synaptonemal complex, followed by recombination between non-sister chromatids. A third factor is that chromatid cohesion must be maintained until chromosomes are arranged on the first meiotic spindle; with the onset of anaphase I it is released along the arms but maintained at the centromeres of each chromosome until anaphase of meiosis II. Recombination plays a crucial role in maintaining the association of homologs until bivalents are arranged correctly on the metaphase I spindle to ensure proper chromosome segregation. Reduced levels of recombination have been shown to be associated with all trisomies in humans, and in most cases evidence has been found for complete failure of crossing over in a proportion of gametes (reviewed in Lynn *et al*., 2004). XY bivalents without crossovers (achiasmate) are the most frequent cause of paternally derived Klinefelter syndrome (47, XXY), and achiasmate bivalents are involved in over a third of trisomy 21 cases that are derived from the mother. However, clear evidence for non-crossover bivalents has not been found for cases of trisomy 16. Recombination at unusual sites, frequently more distal, is also associated

with autosomal trisomies in human females (Lynn *et al.*, 2004).

The occurrence of autosomal trisomy is, however, most closely associated with increased maternal age, whereas recombination frequencies have not been shown to decrease with age. To resolve this apparent paradox, a "two-hit" model has been proposed (Orr-Weaver, 1996). The first "hit" occurs in the fetal ovary when a proportion of bivalents are formed which are achiasmate or have exchanges in less than optimal positions. In younger women, the fully functioning meiotic apparatus would have little difficulty in ensuring proper segregation of chromosomes in most cases, with the possible exception of genetically susceptible individuals. In older women, the combination of a susceptible configuration and age-dependent degradation of meiotic proteins would increase the likelihood of mis-segregation and the formation of aneuploid oocytes.

For such an essential process, recombination shows a wide degree of variation. Indirect genetic studies have shown that genome-wide variation in recombination is an individual trait that is likely to have a genetic basis (Savage Brown *et al.*, 2000). The use of immunofluorescence techniques to detect foci of the DNA mismatch repair protein MLH1 that are known to correspond to positions of chiasmata allows direct determination of recombination in prophase I of meiosis. Studying autosomal bivalents from 1100 pachytene-stage cells from 11 normal males with this approach, Sun *et al.* (2005) showed that the mean frequencies of foci between individuals ranged from 42.5 to 55.0 per cell. Bivalents without recombination were rare with a frequency of 0.3% (62 of 24 200 synaptonemal complexes). In a further study, the same group analyzed all non-crossover bivalents in 886 pachytene cells from 10 of the normal men (Sun *et al.*, 2006). Of the 60 cases of autosomes without recombination, the highest frequencies were 2.1 percent and 1.7 percent for chromosomes 21 and 22, respectively, but in 27 percent of the 886 cells the sex chromosomes were lacking a focus of recombination. It was observed that these increased frequencies of achiasmate bivalents correlate with the incidence of disomy in sperm after karyotyping or fluorescent *in situ* hybridization (FISH) analysis, which shows that aneuploidy of the sex chromosomes and chromosomes 21 and 22 is significantly more frequent than that affecting the other autosomes. Interestingly, one of the donors was aged 81 and a strong association of non-crossovers with increasing age was observed in this study. With regard to females, direct study of recombination foci is very much more difficult since fetal oocytes must be used. An in-depth investigation of ovarian tissue from a 19-week fetus gave information from 95 pachytene nuclei and found a mean of 70 crossovers per oocyte, but with considerable intercell variability (Tease *et al.*, 2002). Chromosome bivalents 13,18, 21, and X were specifically identified and interesting differences were observed; in some cells bivalents 21 and 18 did not have any foci, but this was only true for chromosome pairs 13 and X if there were pairing errors. A recent direct study indicates that up to a third of human oocytes may fall below the optimal threshold of one or two crossovers per bivalent, putting them at increased risk of nondisjunction in meiosis I (Lenzi *et al.*, 2005). A large population-based genetic study found that recombination rate was linked to reproductive success in humans, so that mothers with a high oocyte recombination rate tend to have more children (Kong *et al.*, 2004).

Male infertility and meiotic anomalies

Although the incidence of constitutional chromosome abnormalities is about tenfold higher in the infertile male population than in normal men, 95 percent of infertile men have a normal karyotype. Abnormalities of chromosomal synapse in meiosis are one type of anomaly affecting fertility that cannot be detected by standard karyotyping (reviewed by Egozcue *et al.*, 2005). Anomalies of meiotic synapse may affect the rate of recombination of all bivalents and may lead to the formation of achiasmate small bivalents, leading to the production of abnormal sperm, or it may trigger meiotic arrest. Synaptic anomalies can be studied by immunofluorescence staining of the synaptonemal complex but also by M (multifluor)-FISH and multiprobe FISH analysis of meiotic preparations at metaphase I, obtained after testicular biopsy. The incidence of synaptic anomalies identified by classical meiotic studies at metaphase I in infertile males in general has been found to be 6–8 percent, rising to 17.6 percent in a group of males with severe oligoasthenozoospermia. One, as yet unconfirmed, study of 60 normozoospermic males with a long history of sterility or failure to conceive after IVF revealed an incidence of 27 percent of synaptic anomalies (Egozcue *et al.*, 2004, cited in Egozcue *et al.*, 2005). Aneuploidy in sperm from males with synaptic anomalies is not readily detected, but this may be due to selective elimination of the aneuploid

cells during maturation. In contrast, the incidence of diploidy was double that of control subjects. Based on two studies after preimplantation genetic screening (PGS) of embryos produced by males with known synaptic anomalies, 42 percent of the embryos were abnormal (said to be increased compared with controls), and of these 17 percent had complex anomalies (Egozcue *et al*., 2005).

Males with constitutional chromosomal rearrangements or other anomalies are clearly at increased risk of producing aneuploid sperm, but extensive investigation by several groups over a number of years has shown that men with a normal somatic karyotype but with various sperm pathologies are also at increased risk. An inverse correlation between sperm aneuploidy (detected by FISH analysis) and sperm concentration has been well documented. In severe oligospermia a significant increase in sex chromosome aneuploidy has been seen, culminating in a corresponding increase in the birth of infants with anomalies in these chromosomes after intracytoplasmic sperm injection (ICSI) (reviewed by Miharu, 2005). Patients with severe morphological abnormalities affecting their sperm and those with severe motility problems are also prone to an increase in sperm aneuploidy (Machev *et al*., 2005; Rives, 2005), while for those with nonobstructive azoospermia (NOA) or obstructive azoospermia (OA) sperm retrieved from the testes have an elevated aneuploidy rate, particularly the NOA group (Burrello *et al*., 2005).

Differences between males and females in the meiotic process

There are obvious differences between males and females in the timing of meiosis; in the female, the early stages of oogenesis, up to prophase I, are completed during fetal life after which the oocytes arrest at dictyotene until the onset of puberty. In contrast, male meiosis begins before puberty but spermatogenesis continues throughout adult life; in addition, the meiotic process is subject to more rigorous levels of checkpoint control in males than in females. Most detailed information is available so far only from studies in the mouse. In males, mutations causing failure of synapse or recombination often lead to meiotic arrest, apoptosis, and infertility. In females, identical mutations may also give rise to abnormalities of pairing and recombination but will not necessarily cause infertility; in some cases there may be subfertility and embryo death, often due to increased frequency of aneuploidy (reviewed in Morelli & Cohen, 2005). During meiosis, as in mitosis, the spindle checkpoint delays anaphase until all the chromosomes are correctly positioned on the spindle at metaphase. However, in the female the checkpoint operates at a lower stringency than in males. Studying oogenesis in XO mice, Le-Maire-Adkins and colleagues showed that oocytes proceed to anaphase I without delay despite anomalies in alignment of the X and other chromosomes, in contrast to the situation in males (LeMaire-Adkins *et al*., 1997). These differences in the stringency of the checkpoints between the sexes are reflected in the varying frequency with which aneuploid gametes are produced in humans. Reviewing the frequency of numerical and structural chromosomal abnormalities in spermatozoa from normal men, Templado *et al*. (2005) found an overall incidence of aneuploidy close to 2 percent, an order of magnitude lower than that found in human oocytes (Pellestor *et al*., 2002; Fragouli *et al*., 2006).

Aneuploidy in male and female gametes

In spermatozoa, data regarding aneuploidy are derived from both karyotyping (after penetration of hamster oocytes) and multicolor interphase FISH analysis on decondensed sperm nuclei; the latter technique generally gives a somewhat higher frequency (Templado *et al*., 2005). In both types of investigation, an increased incidence of disomy was seen for chromosome 21 and the sex chromosomes, and for chromosome 22 in the FISH studies, a reflection of the low number of chiasma typically seen in the corresponding bivalents and a concomitant increased risk of nondisjunction.

Similarly, in oocytes, data are available from different approaches; since the oocyte is released or retrieved at metaphase II, direct karyotyping is possible. However, the spreading of a single cell for analysis risks the loss of one or more chromosomes and the contracted nature of the chromosomes precludes the use of G-banding techniques for exact identification by direct karyotyping. These difficulties have led to the application of molecular cytogenetic methods, initially multiprobe FISH analysis and latterly analysis via comparative genomic hybridization (CGH). Obtaining unfertilized oocytes from natural cycles is very difficult in most countries so most studies of aneuploidy are carried out in association with IVF, using either eggs that have failed to fertilize or those that were immature when retrieved. The evidence to date

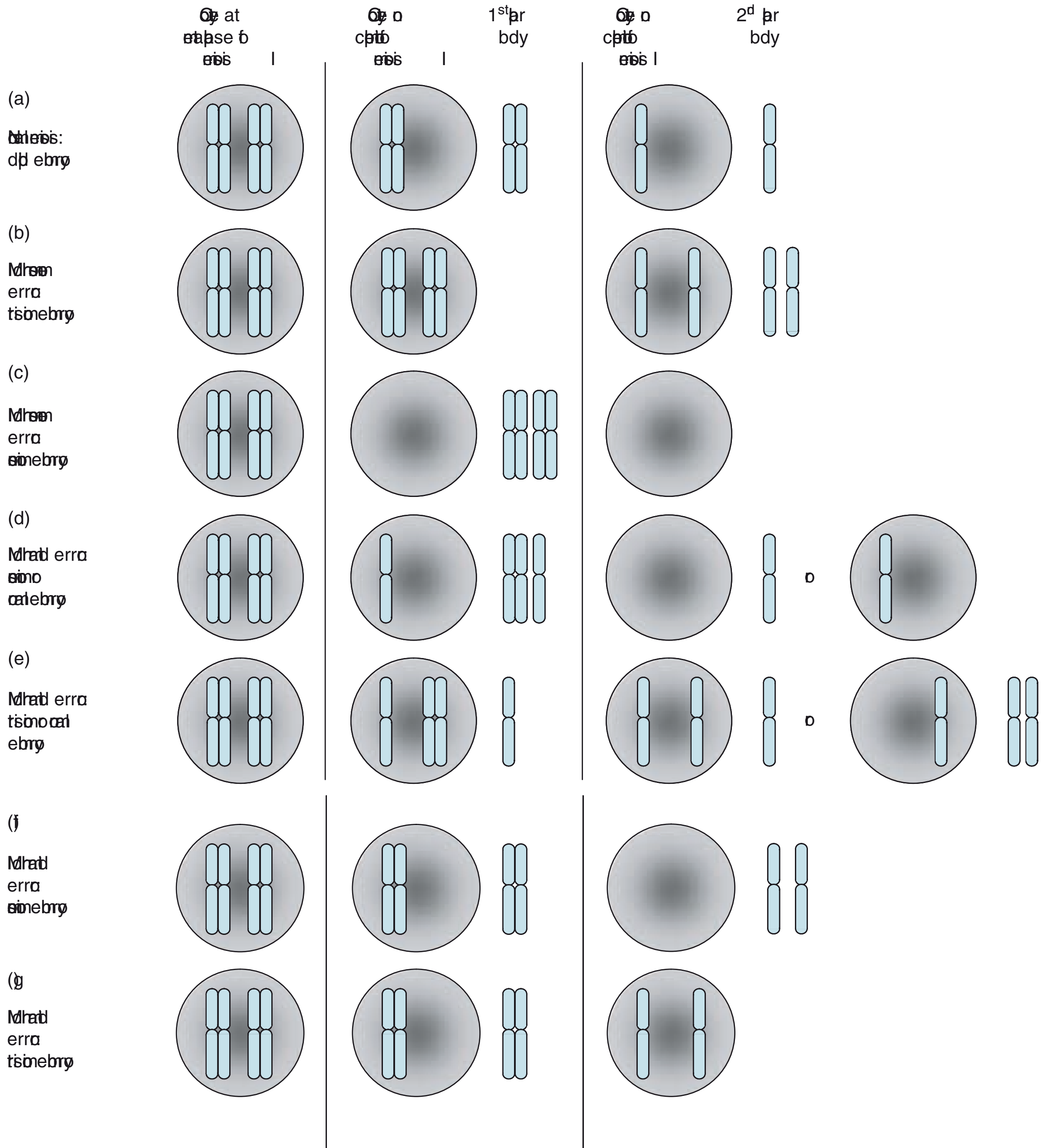

Figure 7.1 Diagram to show the effects of errors in disjunction at meiosis I (MI) and meiosis II (MII). A chromosome error at MI will always lead to aneuploid gametes, whereas the effect of a chromatid error at MI will depend upon events at MII. In contrast, a chromatid error at MII will always lead to aneuploidy in the mature gamete. (a) Normal chromosome segregation. During meiosis I (MI) homologous chromosomes exist in pairs (bivalents), each composed of two chromatids. Under normal circumstances one chromosome passes into the first polar body (PB) at anaphase of MI with the other remaining in the oocyte. At meiosis II (MII) the two chromatids separate with one passing into the second PB, leaving the oocyte haploid. (b) Meiosis I chromosome error. Both chromosomes remain in the oocyte during MI, predisposing the oocyte to the production of a trisomic embryo. (c) Meiosis I chromosome error. Both chromosomes pass into the first PB during MI, predisposing the oocyte to the production of a monosomic embryo. (d) Meiosis I chromatid error. One chromosome segregates appropriately at MI passing into the first PB. However, the second chromosome suffers premature separation of chromatids, one of which enters the first PB. At meiosis II the last remaining chromatid will segregate at random. If it remains in the oocyte a normal haploid chromosome complement is created, but

suggests that the various follicular stimulation regimes necessary for IVF do not affect the chromosome constitution of the oocyte itself since those obtained from natural cycles show similar frequencies of anomalies, and that eggs that remain unfertilized after exposure to sperm are not cytogenetically different from those that are fertilized (Gras *et al*.,1992; Almeida & Bolton, 1994; Hassold & Hunt, 2001).

Until 1991 all investigators had been scoring solely gains or losses of whole chromosomes due to nondisjunction, but in that year an important paper was published describing predivision in human oocytes at meiosis I (Angell, 1991). Precocious or premature centromere division of one homolog of a paired bivalent had previously been described in two studies on mouse oocytes (Polani & Jagiello, 1976; Hansmann & El-Nahass, 1979) and the term "predivison" was used in the first of these studies. Essentially, the observations made are of single unpaired chromatids at metaphase II. Either they may be additional to the haploid set (i.e. 23 + 1/2 in humans) or the cell can be missing a chromatid (i.e. 22 + 1/2). Balanced predivision occurs when both separated chromatids are present; however, these will segregate randomly at anaphase II, which occurs after fertilization. Predivision may predispose to second meiotic division nondisjunction, provided that the premature separation of centromeres is delayed until after anaphase I. However, unlike nondisjunction of whole chromosomes at anaphase I that will always give rise to disomic and nullisomic gametes, predivision, whether balanced or not, will be expected to lead to imbalance in only 50 percent of the mature gametes (Figure 7.1 and Figure 7.2).

The most recent, large-scale karyotyping study, of 1397 oocytes from 792 patients of mean age 33.7 (± 4.7) years, found aneuploidy in 10.8 percent of samples (Pellestor *et al*., 2002). Overall, 5.4 percent were *hypo*haploid, 4.1 percent *hyper*haploid, and 0.8 percent complex aneuploidies with 0.5 percent described as "extreme" aneuploidies (less than 18 chromosomes); these could be further subdivided into 1.9 percent with loss of whole chromosomes, 3.4 percent with loss of a chromatid, 1.6 percent with extra whole chromosomes, and 2.5 percent attributed to an extra chromatid. Twelve (0.86 percent) oocytes were found to have both whole chromosome and single chromatid anomalies. An important observation was that no significant difference was found in the incidence of chromosome anomalies with regard to the indications for IVF (e.g. male, female, or unknown factor). Although aneuploidy was found in chromosome groups of all sizes, the distribution was skewed toward a significantly higher frequency in the smallest groups (chromosomes 19–22), a consistent finding in all the larger series of investigations irrespective of the method used.

The application of FISH to metaphase II oocyte preparations allowed the specific identification of certain chromosomes; an additional advantage of using FISH was that precise information could also be obtained from the chromatin of the first polar body, even after the oocyte had been 48 hours in culture (as required by some regulatory authorities). A study by Dyban *et al*. (1996) was designed to determine whether FISH analysis of the first polar body could predict the chromosome constitution of the oocyte by using probes specific for chromosomes X and 18; reciprocal losses and gains with respect to the oocyte and the first polar body were observed in the majority of abnormal specimens. This approach has since been exploited on a large, diagnostic, scale with the aim of avoiding the use of oocytes that have been predicted to be aneuploid based upon polar body analysis of five autosomes by FISH (Kuliev *et al*., 2005).

Other fluorescent methods that allow specific identification of each and every chromosome are spectral karyotyping and multifluor-FISH (M-FISH). These have both been applied to human oocytes with interesting results. Material that may be used for spectral karyotyping or M-FISH is more limited as an absolute requirement is that the chromosomes must be well spread (and therefore particularly subject to artifactual loss of chromosomes), whereas with the basic FISH technique results may be obtained on material of quite poor quality. One spectral karyotyping study was

Caption for Figure 7.1 (cont.) if it passes into the second PB the oocyte will be at risk of producing a monosomic embryo. (e) Meiosis I chromatid error. One chromosome segregates appropriately at MI remaining in the oocyte. However, the second chromosome suffers premature separation of chromatids, one of which enters the first PB. At meiosis II the chromosome separates into chromatids, one of which passes into the second PB. However, the lone chromatid created during MI will segregate at random. If it remains in the oocyte, any resulting embryo will be at risk of trisomy, but if it passes into the second PB a normal haploid chromosome complement is created. (f) MII chromatid error. Normal segregation during MI is followed by nondisjunction of chromatids at MII, both passing into the 2nd PB. The oocyte is at risk of producing a monosomic embryo after fertilization. (g) MII chromatid error. Normal segregation during MI is followed by nondisjunction of chromatids at MII, both remaining in the oocyte. The oocyte is at risk of producing a trisomic embryo after fertilization.

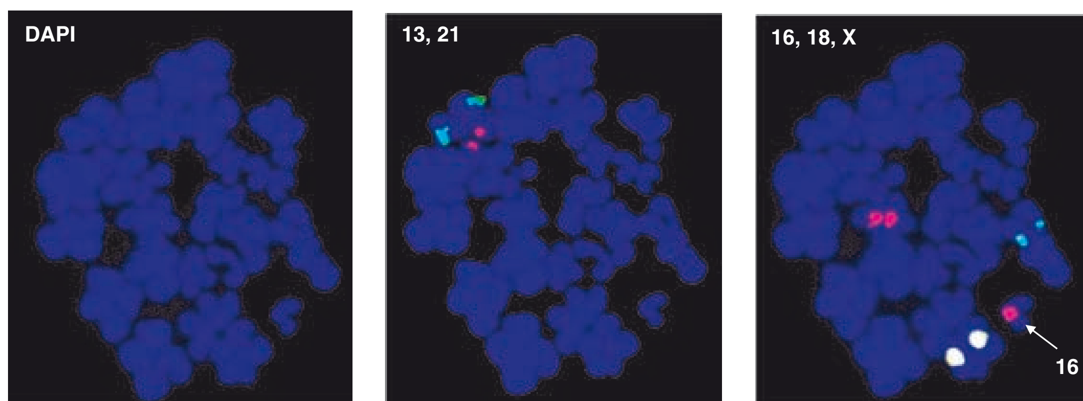

Figure 7.2 Human oocyte at metaphase of meiosis II after fluorescence *in situ* hybridization (FISH) with DNA probes for chromosomes 13, 16, 18, 21, and X. The chromosomes are represented by paired chromatids each of which gives a hybridization signal. Chromsomes 13, 18, 21, and X have the normal two signals each. However, chromosome 16 has an extra signal from an additional unpaired chromatid (arrowed). Reproduced from Mahmood *et al.* (2000), with permission from Springer Science and Business Media © Springer-Verlag 2000.

carried out, exceptionally, on fresh, non-inseminated oocytes obtained from 16 donors (Sandalinas *et al.*, 2002). Of the 131 donated eggs analysis was achieved for 47; nine anomalies of whole chromosomes were seen in eight metaphase II spreads, with gains of chromosomes 14 and 21, and losses of chromosomes 9 (twice), 11, 12, 18, 20, and 22. There were seven examples of extra chromatids and four of missing ones, in 10 oocytes. Importantly, 12 oocytes showed balanced predivision proving that this anomaly is not solely due to prolonged time in culture. Balanced predivision was also shown to increase significantly with maternal age and with decreasing chromosome size in this study.

The M-FISH technique was also used in a case study of oocytes from a 35-year-old woman with polycystic ovary syndrome (Clyde *et al.*, 2001). Two immature oocytes were matured in vitro to metaphase II. M-FISH analysis of one metaphase II and the corresponding first polar body revealed reciprocal chromatid anomalies; the oocyte was hyperhaploid (23, X+ 15cht + 19cht + 22cht) while the polar body was hypohaploid (23, X −15cht −19cht, −22cht) thus confirming the mechanism of chromatid nondisjunction leading to aneuploidy. The second oocyte had a hypohaploid karyotype of 21, X, −10, −20, but no polar body was available. Chromatid anomalies are clearly very commonly found in metaphase II human oocytes, but their contribution to aneuploidy in the resulting embryo depends upon events at anaphase II after fertilization.

A more wide-ranging application of FISH to the analysis of metaphase II oocytes and associated first polar bodies has been pursued by several groups (Mahmood *et al.*, 2000; Cupisti *et al.*, 2003; Pujol *et al.*, 2003). Two features of interest with regard to the mechanisms involved emerged from the first two of these studies. First, in broad agreement with karyotyping studies, anomalies were not randomly distributed between the chromosomes. The smaller chromosomes, 13, 16, 18, 21, and the X, were preferentially involved to a significant degree, with no anomalies detected for chromosomes 1, 9, or 12.

Second, obtaining results from an oocyte and its corresponding first polar body uncovered another unusual mechanism leading to aneuploid gametes. In two of the fourteen hyperploidies an extra chromosome was found in the polar body with no accompanying loss in the oocyte, and, in a third case, extra chromatids were found in both oocyte and polar body. All three of these mature eggs must have been derived from cells that were already trisomic for the chromosome involved (21 in two cases and 13 in the other). Without further investigation it is not possible to determine whether this preexisting aneuploidy arose during the pre-meiotic divisions (germinal mosaicism) or if it was present initially in the embryonic gonad (gonadal mosaicism). Cytologically proven gonadal mosaicism was reported by Cozzi *et al.* (1999) in a woman with three previous conceptions with trisomy 21 and for whom preimplantation diagnosis was performed. FISH analysis of unfertilized oocytes and corresponding first polar bodies proved that gonadal mosaicism was the cause of the repeatedly abnormal conceptions. In this respect, a study using nine chromosome probes in first polar bodies and metaphase II oocytes (with the aim of validating polar body analysis for preconceptual diagnosis) reported by Pujol *et al.* (2003), is of interest. Nine of

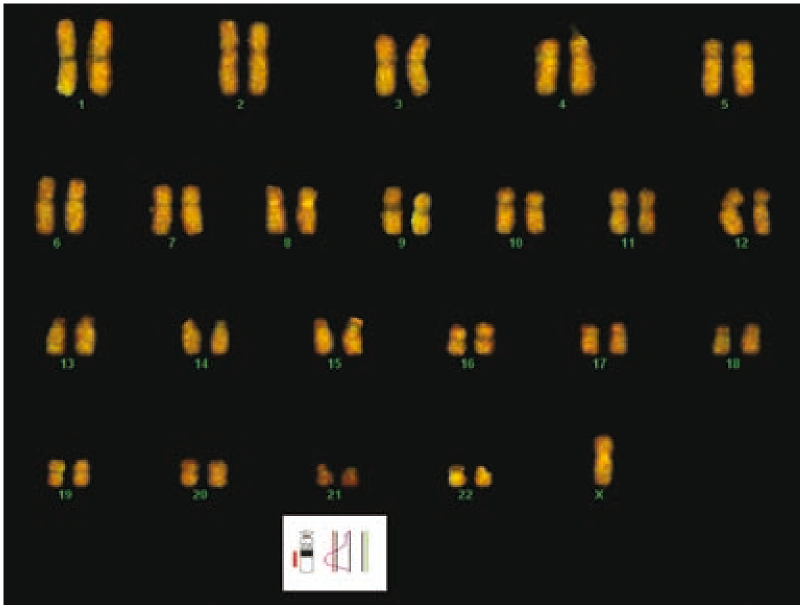

Figure 7.3 The image shows a set of normal metaphase chromosomes simultaneously hybridised with two differentially labeled DNA samples: a chromosomally normal DNA (reference) labeled with a red fluorescent molecule; DNA derived from a polar body biopsied from an oocyte, amplified and labeled with a green fluorochrome (test). Chromosomes present in equal copy number in the test and reference DNA samples hybridize an equal quantity of red and green DNA fragments and therefore appear orange/yellow. Chromosomes present in excess in the test DNA (e.g. a polar body with an additional chromosome or chromatid) have a green colouration, while chromosomes that have fewer copies in the test DNA (e.g. a polar body with loss of a chromosome or chromatid) adopt a red colouration. In this example, all chromosomes have a normal number of copies with the exception of chromosome 21. Computer analysis of the red:green fluorescence ratio along the length of chromosome 21, confirms a significant deviation towards red colouration (see inset), indicating loss of chromosome 21 material. Consequently, the corresponding oocyte is predicted to have an excess of chromosome 21 material, leading to a high risk of trisomy 21 following fertilisation.

the 35 patients where corresponding pairs (polar body and metaphase II oocytes) were studied presented with abnormalities in the form of extra chromosome material (with no reciprocal loss) that were considered most likely to have originated in early oogenesis.

In order to obtain a reliable and comprehensive estimate of aneuploidy in human oocytes, a method is needed that does not rely on the spreading of chromosomes on a slide. A DNA-based method such as CGH is appropriate but for single-cell analysis the DNA must first be amplified faithfully from the 6–10 picograms initially present to the 200 nanograms that are required. DNA from a small number of cells from a chromosomally normal control source is similarly amplified, and test and control DNAs are differentially labeled in red or green and hybridized to prepared slides of normal male metaphase chromosomes which act as indicators. The two DNAs compete for hybridization sites allowing differences in the number of copies of any of the chromosomes to be detected by analysis of the green:red ratio along each indicator chromosome on the slide (Figure 7.3). The validity of using single-cell CGH analysis to acquire data on aneuploidy in human oocytes has been proved by several recent studies (Wells *et al.*, 2002; Gutiérrez-Mateo *et al.*, 2004a, 2004b; Fragouli *et al.*, 2006a, 2006b, 2006c). It has been shown, as expected, that, since the first polar body and the metaphase II oocyte are (haploid) products of the first meiotic division, analysis of one of these cells will, with a few rare exceptions, predict the chromosome constitution of the other, so that reciprocal loss and gain should be expected. The rare exceptions are in cases of germinal or gonadal mosaicism where there is preexisiting aneuploidy in the oocyte. This advance has paved the way for both clinical and research applications of this technology.

The aneuploidy rate in human oocytes

Recent karyotyping data that include chromatid anomalies and the more specific FISH analyses indicated that the overall rate of chromosome and chromatid *imbalance* in human oocytes is about 11 percent for women of maternal age 32–34 years (Mahmood *et al.*, 2000; Pellestor *et al.*, 2002; Anahory *et al.*, 2003; Cupisti *et al.*, 2003). However, there are now several independent studies of aneuploidy in metaphase II oocytes and corresponding first polar bodies that have used the CGH

approach. Owing to the labor-intensive nature of the investigations, the earliest of these studies reported on relatively small numbers of samples (Gutiérrez-Mateo *et al.*, 2004a, 2004b; Fragouli *et al.*, 2006a, 2006b). Gutiérrez-Mateo *et al.* (2004a) reported an investigation of 30 metaphase II oocyte–polar body pairs that gave a 48 percent aneuploidy rate, and an analysis of 23 oocytes from a single center by our own group similarly yielded a rate of 43.5 percent (Fragouli *et al.*, 2006b). The reason for these very high levels seems to be that the results of small studies are prone to bias by the inclusion of a few exceptional patients with an increased predisposition to aneuploidy (Fragouli *et al.*, 2006b).

New data on CGH analysis of human oocytes

We have now examined a total of 221 metaphase II oocyte–polar body complexes, donated from 82 patients (Fragouli *et al.*, 2006a, 2006b, 2006c, and unpublished data). These data confirm that CGH is able to detect chromatid losses and gains, as well as whole-chromosome changes, and partial aneuploidies thought to be caused by chromosome breakage.

The total aneuploidy rate for our sample set was calculated to be 20.8 percent, close to the estimate of 20–25 percent from sources such as the spontaneous abortion data (Hassold, 1986). Chromosome loss and gain occurred almost equally in the abnormal samples, in accordance with the theoretical expectations of the outcome of nondisjunction. The mechanism of gonadal/germinal mosaicism leading to aneuploid oocytes was confirmed both in our studies and in those of Gutiérrez-Mateo by the observation of oocytes or polar bodies with an additional or missing chromosome, without the reciprocal anomaly being scored in the corresponding polar body or oocyte.

Abnormalities affected all chromosomes except 7 and 14, but most frequently the X, and then chromosomes 21, 22, followed by 8, 12, and 20. Three structural anomalies were detected. Mechanisms involved whole-chromosome nondisjunction, unbalanced chromatid predivision, chromosome breakage, and, rarely, germinal mosaicism. The larger autosomes, numbers 1–12, were affected solely by whole-chromosome nondisjunction and were unaffected by chromatid anomalies. This finding is thought to reflect the increased cohesion of larger bivalents as well as the role of crossing over in holding paired homologs together, since larger chromosomes have a greater number of chiasmata. Smaller chromosomes with few or no crossovers are more likely to separate early, in turn predisposing them to nondisjunction as well as to chromatid anomalies. However, the X chromosome stands out as being a special case; eight of the sixteen X chromosome anomalies were seen in just three patients, age range 18–42 years. At least half of these were chromatid anomalies. From both the X chromosome and the autosome data it is clear that age-independent mechanisms are operating in some of the younger women, and there may be a causal link to their infertility. These mechanisms would include germinal or gonadal mosaicism as well as a predisposition to the production of aneuploid gametes owing to reduced recombination in meiosis I.

CGH analysis of polar bodies provides an indirect means of assessing oocytes for aneuploidy involving any chromosome. This is potentially useful in the context of IVF treatment, allowing the identification and transfer of embryos derived from chromosomally normal oocytes. Such embryos are likely to have the greatest probability of forming a viable pregnancy and their preferential transfer is expected to lead to increased success rates for IVF treatment.

Although CGH analysis of polar bodies has been accomplished in several research studies, the application of this technology in a clinical context (i.e. PGS) is problematic because of the length of time the method requires (Wells *et al.*, 2002). Standard CGH requires 3–4 days and analysis of data is labor intensive, precluding analysis of large numbers of cells. An increasing proportion of IVF cycles involve blastocyst transfer, usually on day 5 post-fertilization, providing sufficient time for polar body CGH analysis to be completed. However, blastocyst transfer is not appropriate for all patients undergoing IVF and it is important to retain transfer at earlier stages as a clinical option. Consequently, the optimal strategy for applying polar body CGH clinically is probably to cryopreserve zygotes immediately after biopsy of the first and second polar bodies. Modern cryopreservation protocols provide extremely high survival rates for frozen oocytes and embryos, and permit as much time as necessary for CGH analysis to be completed. A clinical trial to assess the efficacy of this approach is currently under way.

Aneuploidy in early human embryos

Almost all recent information on the chromosomal status of the early human embryo has been derived from the outcome of PGD and follow-up studies of non-transferred embryos. Reliable karyotyping of single embryonic cells is virtually impossible, but the advent of interphase FISH analysis, using fluorescently labeled chromosome-specific probes, allowed its application to determine the copy number of individual

chromosomes in nuclei of single cells obtained from preimplantation embryos. The data from the follow-up studies after PGD, in particular those cycles carried out for the purpose of aneuploidy screening, complement those obtained from research studies on human oocytes since the majority of aneuploid conceptions are of maternal origin.

Four groups of chromosome patterns in human embryos have been described (Delhanty *et al*., 1997).

Constitutional aneuploidy

Munné *et al*. (2004) reported on the analysis of single cells from over 2000 embryos by FISH using probes for up to 14 chromosomes. Cells were tested for a minimum of four chromosomes from the set: X, Y, 1, 4, 6, 7, 13, 14, 15, 16, 17, 18, 21, or 22. Those most frequently involved in aneuploidy overall were chromosomes 22, 16, 21, and 15; those least involved were 14, X and Y, and 6. However, since full follow-up analysis was not carried out on all the non-transferred embryos it was not possible to distinguish errors arising during meiosis from those arising post-zygotically.

Aneuploidy screening (PGS) in embryos from couples considered to be at increased risk was licensed for treatment in the UK in 2003 by the Human Fertilisation and Embryology Authority (HFEA). At University College London Centre for PGD we began aneuploidy screening in 2004, selecting couples who were having particular difficulties conceiving via IVF (repeated implantation failure, RIF), or were aged over 40 (advanced maternal age, AMA), or who were experiencing repeated miscarriage (RM), usually after natural pregnancies. Following treatment, we have been carrying out full follow-up studies to determine the mechanisms leading to aneuploidy, and therefore implantation/pregnancy failure, in this cohort of patients. To date, embryos from 75 couples undergoing PGS have been investigated for chromosomes 13, 15, 16, 18, 21, and 22 using FISH with individual probes in two rounds of hybridization (Mantzouratou *et al*., 2007; unpublished data). The study included pretreatment lymphocyte investigations, the screening of single blastomeres on day 3, and full follow-up analysis on days 5/6 of embryos that were not transferred. Ninety-four PGS cycles are included in the study, in which 847 embryos were biopsied, with results obtained for 91 percent. Of these 19 percent were found to be diploid for the chromosomes tested at diagnosis on day 3 and 81 percent showed an abnormal result. The pregnancy rate per cycle that progressed to embryo biopsy was 29.5 percent, 32.9 percent per cycle in which embryos were suitable to be transferred. Satisfactory follow-up was obtained from 536 embryos. All those diagnosed as chromosomally abnormal were confirmed as abnormal on follow-up. Of these 94 percent were mosaics, with mixed cell types, the majority with highly abnormal "chaotic" chromosome complements in a high proportion of cells, and 5.3 percent were uniformly abnormal. Although mosaicism in general is common in human embryos generated by IVF (see below) this almost universal mosaicism is beyond what is normally seen in embryos from routine IVF patients.

Meiotic errors were identified in 14.8 percent of embryos, most frequently for chromosomes 21, 18, and 22. Errors in mitosis were detected mostly for chromosomes 15, 16, and 13. There was a significant difference in the distribution of embryos that were uniformly abnormal ($p<0.005$) and of those with meiotic errors ($p<0.005$) between the referral groups. The rates for meiotic errors were 24 percent for the RM group, 20 percent for the AMA group, and 8.9 percent for RIF patients. Striking similarities were seen in the abnormalities affecting embryos from the couples with RM and AMA. Couples with RIF appeared to be different owing to the low frequency of identifiable meiotic abnormalities in their embryos, suggesting that for the vast majority of embryos from this patient group post-zygotic abnormalities may be the main factor leading to implantation failure. Alternatively, it is possible that patients with recurrent RIF may be predisposed to the production of gametes with unusual forms of meiotic abnormality (e.g. aneuploidies involving chromosomes not generally tested during PGS). This is a possibility that warrants further investigation, using comprehensive chromosome screening methods such as CGH. As a group, the RM patients resemble those of advanced maternal age, although the average age (36 years) in the RM group is much lower than in the AMA group, which had an average age of 42 years in our study.

Mosaic aneuploidy

In general, for women undergoing IVF who are younger than 37 years of age, their embryos are chiefly at risk of mosaic aneuploidy rather than the full constitutional type. Interphase FISH detection of the X and Y chromosomes first indicated that mosaicism is a common feature of human preimplantation embryos (Delhanty *et al*., 1993). Subsequent studies using autosomal probes for testing between three and nine chromosomes have detected mosaic aneuploidy in more than half of the embryos investigated (Munné *et al*., 1993; Munné *et al*., 1998; Harper *et al*.,1995;

Delhanty *et al.*,1997). The development of single-cell CGH analysis, allowing the copy number of every chromosome to be determined, and its application to a total of 24 good-quality three-day-old embryos clearly showed that 62 percent were mosaic (Voullaire *et al.*, 2000; Wells & Delhanty, 2000). The extent of mosaic abnormality varied between the presence of a single abnormal cell to every cell being abnormal, but with the chromosomal constitution varying randomly from cell to cell, a condition classed as "chaotic." Importantly, the CGH analysis also showed that a quarter of the embryos were totally euploid, with no chromosome imbalance. Clearly, it is these euploid embryos that have the greatest potential to implant.

The realization of the extent of chromosomal mosaicism in the early human embryo led to the hypothesis that this extraordinary state of affairs could be due to reduced expression of certain of the cell cycle checkpoint genes at this stage in development (Delhanty & Handyside, 1995), thus allowing mitotic errors to arise and persist.

The fate of chromosomally mosaic embryos

Evidence gleaned from detailed FISH analysis of early (5–8.5 weeks' gestation) spontaneous abortions (Lebedev *et al.*, 2004) suggests that preimplantation embryos containing lethal chromosome anomalies in more than half of their cells or with chaotic mosaicism have a minimal chance of survival beyond implantation or early pregnancy. Those that have few abnormal cells may implant successfully, since a low number of cells will be involved in forming the inner cell mass from which the embryo proper is derived. If the abnormal cells remain in the trophectoderm, from which placental tissue is derived, a pregnancy may be established but it may later miscarry or lead to confined placental mosaicism. A seminal study by Lebedev *et al.* (2004), using interphase FISH, demonstrated intra-tissue mosaicism in 94 percent of non-cultured samples of cytotrophoblast and extra-embryonic mesoderm from 60 first-trimester spontaneous abortions that had failed to grow in culture. The observations included six cases of mosaic monosomy for chromosomes 7, 15, 21, and 22; a pertinent finding since complete monosomies for these chromosomes are almost never found in spontaneous-abortion material. Since probes were used for the complete set of chromosomes there is no evidence for the involvement of other meiotic errors in these cases of mosaic monosomy.

At the other end of the spectrum, it is clear that some conceptions that appear to be fully trisomic are of mitotic origin; this is usually presumed to be postzygotic but could arise during the pre-meiotic divisions of the germ cells. About 10 percent of trisomy 18 and trisomy 15 cases, and almost 20 percent of triple X cases, come into this category (Fisher *et al.*, 1995; Robinson *et al.*, 1998; Hassold & Hunt, 2001).

Mechanisms leading to aneuploidy in early embryogenesis

Interphase FISH analysis of human preimplantation embryos that has led to the acquisition of so much information is not without technical limitations. Compared with hybridization to metaphase chromosomes, it is measurably less efficient (Ruangvutilert *et al.*, 2000). For this reason, in order to investigate mechanisms leading to mosaic aneuploidy, some studies have used two probes that map to different locations on one chromosome. Early data to emerge from this approach were obtained during the development of PGD for carriers of chromosomal rearrangements (Conn & Delhanty, 1995, unpublished observations). Two different probes were used for each of chromosomes 13, 18, and 21 in separate experiments on a total of 37 very good quality day-3 embryos, consisting of 6–8 cells. In all, eight embryos were mosaic for these chromosomes. In four cases this was due to chromosome loss, in one case to chromosome gain, and in the remaining three it was caused by mitotic nondisjunction (MND). These observations led to further studies using two probes for each of the three chromosome pairs 1, 11, and 18 together with single probes for the X and Y in a series of sequential hybridizations (Daphnis *et al.*, 2004). The material for this study comprised 47 embryos that had reached day 5 of development, of which many had progressed to the blastocyst stage. In total, of the 69 postzygotic events that led to mosaic aneuploidy, 31 were due to chromosome loss, 27 were due to chromosome gain, and there were 11 instances of MND. Thus in both studies chromosome loss was the most common mechanism leading to mosaicism, with gain of a chromosome being slightly less frequent, and MND being the least frequent overall. It is of interest that the frequency of MND has been shown to be related to maternal age, although embryonic mosaicism in general appears largely independent of maternal age. A study involving a large number of cleavage-stage embryos from women in an older age group showed a significant association of mitotic aneuploidy with advanced maternal age (Munné *et al.*, 2002). The association was particularly marked for the

MND category. In that study chromosome loss was only a third as frequent as MND and consequently mitotic anaphase lag failed to show a significant increase with maternal age. The increased frequency of MND may be due to the age of the group studied but since only a single probe per chromosome was used a cut-off point of 10 percent was used to avoid "FISH error." That is, embryos with fewer than 10 percent abnormal cells were considered "normal," almost certainly leading to an underestimate of anomalies affecting a single cell. Possibly a study of a large number of embryos using two probes per chromosome might show a significant association of mitotic anaphase lag with increasing maternal age.

Coonen and colleagues (2004) also concluded in their study on a large number of blastocysts that anaphase lagging is the major cause of chromosomal mosaicism. However, since they too were using a single probe for each chromosome they were only able to count as valid abnormalities affecting at least two cells.

The stage in embryonic development when errors leading to aneuploidy arise

The predominant type of mosaicism affecting most human preimplantation embryos from routine IVF patients is that of the diploid/aneuploid type, where the embryo begins life as normal diploid and an aneuploid line develops during the cleavage stage. However, in many of these embryos other cell lines are also present, including polyploid, haploid, and "chaotic types" with multiple anomalies. The available evidence suggests that aneuploid mosaicism detected at the cleavage stage is generated during one of the first three divisions, typically at the first or second division (Delhanty *et al.*, 1997; Munné *et al.*, 2002; Katz-Jaffe *et al.*, 2004). Several cell-cycle checkpoint genes, essential for maintaining accurate chromosome segregation, show extremely low levels of expression during the first two cell divisions of the embryo (i.e. prior to the initiation of embryonic gene expression) (Wells *et al.*, 2005). Reduced numbers of transcripts from certain cell-cycle checkpoint genes could allow errors to arise. In the mouse evidence suggests that the window of susceptibility to chromosome malsegregation is narrower, affecting primarily the first embryonic division (Bean *et al.*, 2002). This is interesting given that embryonic gene expression begins earlier in the mouse than the human (two-cell stage).

Data from the five-day-old embryos in the study by Daphnis *et al.* (2004) suggest that if the aneuploid cells arose during cleavage they are at a selective disadvantage compared with the diploid cells and are outgrown as the embryo develops.

Other mechanisms leading to mosaic aneuploidy in embryos

Chromosomal fragments generated by breakage are not easily detectable by FISH analysis since this relies mainly on centromeric probes. However, the first data produced by CGH analysis of single blastomeres, showing reciprocal loss and gain of chromosome fragments in sister cells, clearly proved that partial aneuploidy was not uncommon in embryos at the cleavage stage (Voullaire *et al.*, 2000; Wells & Delhanty, 2000). Subsequent application of CGH technology to single blastomeres taken from three-day-old embryos to allow full aneuploidy screening in cases of repeated implantation failure demonstrated partial aneuploidy in 10 percent of samples (Voullaire *et al.*, 2002). Further evidence for chromosome breakage came from the outcome of preimplantation diagnosis for carriers of chromosomal rearrangements. The use of probes flanking the breakpoint of one of the involved chromosomes in interphase FISH analysis of embryos showed that breakage had occurred in several cases (Simopoulou *et al.*, 2003).

The definition of a "chaotic" embryo is that the chromosome constitution of individual cells varies widely, affecting several chromosomes, and that the mechanisms involved cannot be determined. There are two types, fully chaotic where no diploid cells are present, and partially chaotic with a core of diploid cells, indicating that the embryo began life with a balanced complement of chromosomes. A possible mechanism leading to the fully chaotic type would be a multi-polar spindle at the first mitotic division. This could arise due to centrosome anomalies; interestingly, in humans it is the sperm that contributes the centrosome. It was clear from early data obtained following embryo sexing by FISH analysis that certain couples are predisposed to develop chaotic embryos in repeated cycles of treatment, whereas others have none (Delhanty *et al.*, 1997). A high proportion (75 percent) of mosaic embryos, particularly chaotics, has been widely reported by most groups treating patients with a poor reproductive history who are carriers of chromosomal rearrangements (Conn *et al.*, 1998; Munné *et al.*, 1998; Van Assche *et al.*, 1999; Iwarsson *et al.*, 2000; Simopoulou *et al.*, 2003). High frequencies of chaotic embryos have also been described in couples with repetitive implantation failure (Bielanska *et al.*, 2002; Voullaire *et al.*, 2002; Mantzouratou *et al.*, 2007) providing evidence that it may be a factor in these

cases. The data accumulating from molecular cytogenetic investigation of embryos derived from both patient groups add weight to the earlier suggestion that carriers of chromosomal rearrangements who are unable to achieve a normal pregnancy outcome are the victims of two pathologies, namely the increased risk of an unfavorable meiotic outcome together with a predisposition to the production of embryos with the most extreme form of mosaicism (Conn *et al*., 1998).

Conclusion

A wide array of research and clinical studies has confirmed a high frequency of cytogenetic abnormalities in human oocytes and embryos. Chromosomal imbalance in oocytes can result from losses or gains of individual chromatids or whole chromosomes. The underlying causes remain to be fully elucidated, but important modifying factors include the number and location of chiasmata and maternal age.

Naturally, embryos inherit meiotic errors from the oocyte and sperm from which they are derived. Additionally, embryos may display a variety of chromosomal anomalies that occur after fertilization (i.e. post-zygotic errors). These include chromosomal breakage and most prevalent of all mosaicism, which arises as a consequence of mitotic nondisjunction or anaphase lag. Again, the underlying mechanisms producing these abnormalities are not fully understood, but inappropriate functioning of cell-cycle checkpoints, or the centriole, is likely to be involved.

Aneuploidy is a major cause of congenital abnormalities, mental retardation, and miscarriage. However, most of the chromosome abnormalities detected in human embryos are likely to be lethal at very early embryonic stages, and are probably incompatible with the formation of a clinical pregnancy. Many fertility clinics now screen the embryos produced during an IVF cycle in order to identify those that are chromosomally normal. This is the basis of preimplantation genetic screening (PGS), a method aiming to improve IVF outcome by minimizing the probability that the embryo(s) transferred harbor lethal genetic flaws (see Chapter 13). It is clear that a subset of patients undergoing IVF are predisposed to the production of aneuploid oocytes or embryos, either owing to advanced reproductive age, gonadal mosaicism, or a variety of other factors. Screening the oocytes/embryos from these patients for aneuploidy using PGS may be particularly beneficial in terms of IVF outcome.

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Section 2 Chapter 8

Procedures used in preimplantation genetic diagnosis

Clinical aspects of preimplantation genetic diagnosis

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Key points

- Most preimplantation genetic diagnosis (PGD) patients will be fertile but they are referred as they are carrying a genetic disorder or may have the disease themselves. Many have affected children or have terminated an affected pregnancy.
- PGD patients need to have had a full genetic work-up before attending the in vitro fertilization (IVF) or PGD clinic, including chromosomal/DNA testing, pedigree analysis, and assessment of risk. This should be followed by genetic counseling.
- During their IVF/PGD consultation information will be obtained about the male and female reproductive history Gynecology and andrology assessments are required. What is involved in PGD should be explained, including biopsy, single-cell diagnosis, mosaicism, transfer of undiagnosed or abnormal embryos, misdiagnosis, and prenatal diagnosis.
- Patients require a full IVF work-up to see if they are suitable for IVF, including hormone tests, ovarian response tests, semen analysis – and they need to know about the risks of IVF.
- Patients should be given information leaflets and consent forms specifically for PGD.
- PGD work-up includes confirming the chromosomal or genetic abnormality, which may include analysis of family members
- There have been 22 adverse misdiagnoses out of 12 397 cycles to date but this is probably an underestimate. It is important that PGD clinics follow up pregnancies and deliveries

Introduction

Preimplantation genetic diagnosis (PGD) may be divided into two categories. "High-risk" PGD for couples at high risk because of a serious genetic disorder and "low-risk" PGD (also referred to as preimplantation genetic screening, PGS) carried out for couples undergoing regular in vitro fertilization (IVF) with the aim of increasing the IVF pregnancy rate. Roughly two-thirds of more than several thousands PGD procedures in the USA are PGS for aneuploidy, as are well over half in Europe (Goldman, 2007; Harper *et al.*, 2008). Current indications for PGS include advanced maternal age, repeated implantation failure, repeated miscarriages, and male infertility. Further details are given in Chapter 13. The present chapter mainly focuses on "high-risk" PGD. PGD, followed by implantation of disease-free embryos, offers couples at high risk of transmitting a serious genetic disorder a possibility of seriously diminishing or avoiding the risk of disease in their offspring. Also, couples at high risk for (repeated) spontaneous pregnancy loss because of a structural chromosome abnormality in one of the spouses may benefit from PGD. Finally, many couples opt for PGD because they experience a combined genetic and fertility problem and require medically assisted reproduction anyway.

Close collaboration between clinical genetic departments and IVF centers is necessary (Geraedts *et al.*, 2001). The gynecologist, in collaboration with the IVF laboratory, is responsible for evaluation of the suitability of the couple for undergoing IVF, for the IVF or intracytoplasmic sperm injection (ICSI) treatment, i.e. ovarian stimulation, ovum pick up, embryo transfer, and for the embryo biopsy. Clinical geneticists or counselors, and molecular geneticists are mainly responsible for evaluation of the genetic indication for PGD, pre-test counseling and the genetic testing of the embryo. A proper selection of candidates and a collaborative approach to each couple undergoing PGD optimize their chance of

becoming pregnant. International guidelines, such as those developed by the European Society for Human Reproduction and Embryology (ESHRE) (Thornhill *et al*., 2004) and by the Preimplantation Genetic Diagnosis International Society (PGDIS) (2004) guarantee best-quality practice.

Referrals and indications for PGD

Patients referred for PGD show a number of different histories, but basically they are referred because they are carrying an inherited disorder which they could pass on to their future child. Many couples have already had an affected child and are bringing up a diseased child, have suffered the tragedy of losing a child, or have experienced recurrent miscarriages of genetic origin or (repeated) terminations of pregnancy. Others, like carriers of Huntington disease, are in the presymptomatic phase of their diseases. Moral or ethical objection to prenatal diagnosis and abortion may be another reason for PGD. Combined in- or subfertility is relatively frequent; in the ESHRE PGD Consortium data collection V, in- or subfertility (Harper *et al*., 2006) accounted for 51.5 percent of referrals for PGD, and in the ESHRE PGD Consortium data collection VI (Sermon *et al*., 2007), nearly half of the PGD cycles resulting in oocyte retrieval were performed in couples with combined genetic and fertility problems.

The total number of referrals for preimplantation genetic testing has increased exponentially in the past 10 years, after the introduction of PGS and with enhancing technical possibilities of diagnosing structural chromosome abnormalities and monogenic disorders. PGD is technically possible for most monogenic disorders in which a gene defect is known. A comparison of referrals in the first ESHRE PGD Consortium data collection (spanning 1997–1998) (ESHRE PGD Consortium, 1999) to data collection V (spanning 2002) (Harper *et al*., 2006) is given in Table 8.1. Referrals were left out of the ESHRE data collections thereafter. In parallel with this, the total number of PGD tests has shown a jump from 131 cycles in 1999 to 1984 cycles in 2003 (Sermon *et al*., 2007).

Controversial indications for PGD remain the relatively "mild" disorders, such as (late-onset) inherited deafness, blindness, and skin diseases, or disorders with incomplete penetrance. Different countries have different political acceptance for these indications (de Wert, 2005). PGD is also used for social sexing: that is, sex selection for non-medical reasons. Since 2002 several clinics were reported to perform gender selection for non-medical reasons (ESHRE PGD Consortium, 2002). It is mainly asked for family balancing, i.e. the couple already have at least one child. Its moral and ethical acceptability is still under discussion (Pennings & de Wert, 2003).

Chromosome abnormalities

Excluding aneuploidy screening, the number of referrals for chromosomal abnormalities has increased tremendously and in the last few years over 1000 cycles were started for structural chromosome abnormalities (Sermon *et al*., 2007). Couples in whom one of the partners carries a chromosomal abnormality, such as a balanced Robertsonian or reciprocal translocation, may be at high risk of repeated miscarriage and/or viable offspring with an unbalanced chromosome pattern, leading to multiple congenital anomalies and mental retardation in the child. Also, future parents with a structural chromosome abnormality, such as an interstitial deletion of chromosome 22q11.2, leading to the clinical signs and symptoms of velo-cardio-facial (VCF) syndrome, may opt for PGD (Iwarsson *et al*., 1998). The risk of an abnormal chromosome pattern in offspring depends on the exact nature of the chromosomal abnormality as well as on the family history. It is of great importance to determine this risk in advance (Stephenson & Sierra, 2006). Infertile males are also at increased risk of carrying a translocation or other structural chromosome abnormality and previously would not have been able to reproduce (Simopoulou *et al*., 2003). With the use of ICSI they are now able to father children. However, such pregnancies are still at risk for being chromosomally abnormal, and it is appropriate to combine ICSI treatment with PGD in such cases.

Currently, in most centers cleavage-stage embryos are analyzed with two or more fluorescence *in situ* hybridization (FISH) probes. In this way it may be determined if an embryo is unbalanced or not, but it is not possible to make a distinction between balanced carrier embryos and chromosomally normal embryos, and this should be explained to the couple. In PGD for chromosomal abnormalities the number of unbalanced embryos is often remarkably high (70–80 percent), leading to a low pregnancy rate per cycle (Simopoulou *et al*., 2003; Ogilvie & Scriven, 2004; Stephenson & Sierra, 2006). Pregnancy rate per transfer is better, as once a normal embryo is diagnosed, the "major issue" has been solved.

To offer PGD to female carriers of balanced translocations, Munné and colleagues (Munné *et al*., 2000) analyzed the first polar body of their eggs. If biopsied within a couple of hours after oocyte retrieval, first polar bodies are still in metaphase and may be assessed

Table 8.1 Referrals for preimplantation genetic diagnosis (PGD): comparison of data collections I and V

	Data collection I (1997 to September 1998)	Data collection V (January to December 2002)
Chromosomal	**83**	**1150**
Structural chromosome abnormality	40	208
Aneuploidy screening	27	882
Other reasons for numerical screening	16	60
Monogenic disorders	**233**	**453**
Autosomal dominant	47	111
Autosomal recessive	88	102
X-linked (sexing)	95	109
Others or unknown mode of inheritance	3	131

ESHRE PGD Consortium (1999); Harper *et al.* (2006), with permission from Oxford University Press.

by chromosome painting. A major drawback of this method is that only maternal translocations can be analyzed (Sermon *et al.*, 2004).

A point of concern regarding PGD for chromosomal abnormalities is that for an individual couple the benefit of PGD, in terms of ongoing pregnancy rate of a healthy child, compared to a spontaneous conception, eventually followed by prenatal diagnosis has not yet been proven (Sugiura-Ogasawara & Suzumori, 2005; Stephenson & Sierra, 2006). This would require a randomized study of spontaneous pregnancy followed by prenatal testing versus PGD, which is ethically full of concerns. Sugiura-Ogasawara and colleagues (Sugiura-Ogasawara *et al.*, 2004; Sugiura-Ogasawara & Suzumori, 2005) calculated a cumulative rate for a successful pregnancy of 68 percent for PGD and of 68.1 percent for a natural pregnancy. However, for translocation carriers who have experienced repeated miscarriages, or unbalanced offspring, PGD may be the only way to achieve a normal pregnancy.

Monogenic disorders

The first PGD diagnosis for a monogenic disorder was for cystic fibrosis in 1992 (Handyside *et al.*, 1992). The number of referrals and the number of treatments for monogenic disorders has shown a gradual increase over the years (Table 8.1) (Sermon *et al.*, 2007). Nowadays, PGD is feasible for the most frequent autosomal heritable disorders (Renwick & Ogilvie, 2007).

Autosomal dominant disorders

PGD for autosomal dominant disorders is applied on a routine basis for several trinucleotide repeat disorders such as Huntington disease, myotonic dystrophy, several spinocerebellar atrophy (SCA) types (Renwick & Ogilvie, 2007), and also for other disorders such as Charcot–Marie–Tooth disease, Marfan syndrome (Sermon *et al.*, 2007) and achondroplasia (Moutou *et al.*, 2003).

In case of an autosomal dominant late-onset disorder couples who are reluctant to terminate an established pregnancy often show a deep interest in PGD to prevent passing their own disease to their offspring. This may also be true for the inherited cancer syndromes, such as autosomal dominant hereditary breast and ovarian cancer (BRCA1 and BRCA2 mutations), familial adenomatous polyposis coli (FAP), hereditary nonpolyposis colon cancer (HNPCC), and also several syndromes complicated by an increased cancer risk, such as neurofibromatosis 1/2 (NF1/2), von Hippel–Lindau disease, and tuberous sclerosis complex (TSC) (Olsen *et al.*, 2003; Friedmann & Kramer, 2005; Offit *et al.*, 2006; Menon *et al.*, 2007; Moutou *et al.*, 2007; Renwick & Ogilvie, 2007; Spits *et al.*, 2007). Because of an increasing uptake of cancer genetic testing and the improved survival of young patients with cancer, reproductive options to prevent the disease in offspring will increasingly be requested. Prenatal diagnosis is emotionally difficult and in some cases socially not well accepted or even prohibited by law. In 2006 the UK Human Fertilisation and Embryology Authority (HFEA) published its decision to license PGD for BRCA1 and BRCA2 mutations and HNPCC (HFEA, 2006). These conditions are distinct from those previously licensed for testing in the UK because of a combination of three factors: they have a later age of onset; they have a lower penetrance; they are potentially treatable. Before this decision the HFEA had licensed PGD only for conditions for which one, or at most two, of these three factors applied.

For some autosomal dominant disorders future parents may ask for PGD without being tested themselves, mostly in the situation where they have a 50 percent risk of being a gene carrier themselves because one of their own parents is affected. The paradigm for this situation is Huntington disease (Braude *et al*., 1999). Two options are available. The first is so-called "non-disclosure testing," which involves direct mutation testing of the embryos without knowing the carrier status of the parent at risk. Several moral and ethical questions arise, one of them being that after the first treatment, the PGD center might well know the status of the parent at risk but is obliged to keep this information secret and continue with IVF/PGD without it being necessary. Therefore, nondisclosure PGD is applied only on a very limited scale and the ESHRE ethics task force discourages nondisclosure testing (Braude *et al*., 1999; Shenfield *et al*., 2003). The second possible option is exclusion testing, involving the discharge of all embryos at 50 percent risk of being affected, which is an acceptable method in prenatal testing. Such an exclusion test is usually performed with linked markers, and aims to exclude the transmission of the disease gene of the affected grandparent. Also, for this indication, one of the main disadvantages is that a complicated and potentially dangerous procedure such as IVF may in fact not be necessary if the patient is not a carrier of the mutation (Sermon *et al*., 2002).

Autosomal recessive disorders

Among the autosomal recessive disorders, cystic fibrosis (CF), the inherited hemoglobinopathies (beta thalassemia, sickle cell anemia) and spinal muscular atrophy (SMA) represent the largest group and this mainly reflects the high incidence of the respective disorders in different populations (Sermon *et al*., 2007). Males affected by CF usually have congenital bilateral absence of vas deferens (CBAVD), leading to obstructive azoospermia (Gillet *et al*., 2002; Ratbi *et al*., 2007). If by chance their partner is also a carrier of a CF mutation (and this chance is one in 25–30 in Western countries) their risk of CF-affected children is 50 percent. Obtaining sperm in these males requires a surgical procedure such as percutaneous epididymal sperm aspiration (PESA) followed by IVF or ICSI and may be combined with PGD in case of a high recurrence risk.

X-linked disorders

Embryo sexing for cases of X-linked disease were the first cycles of PGD to be performed (Handyside *et al*., 1990), and in the first years after introduction, in the early 1990s, sexing accounted for the majority of cycles worldwide (Table 8.1). Nowadays, the contribution of sexing for genetic diseases is relatively low compared with the total number of referrals and treatments. Disorders for which sexing has been performed include: Duchenne or Becker muscular dystrophy, hemophilia A/B, X-linked mental retardation, Wiskott–Aldrich syndrome, and many other more rare disorders (Braude *et al*., 2002).

The development of specific mutation testing by polymerase chain reaction (PCR) for X-linked disorders, with transfer of mutation-free male and female embryos, increasingly replaces sex selection. It is now applied routinely for Fragile X syndrome, and also for several cases of Duchenne muscular dystrophy and hemophilia (Sermon *et al*., 2007). The heterogeneity of the mutations in the latter disorders and the need to develop individual testing protocols remain technical obstacles, and the development of such a testing protocol takes time. However, in comparison with sex-selection this approach allows the transfer of healthy male embryos and the prevention of the birth of carrier daughters. Carrier females of X-linked diseases may show signs or symptoms of the disorder, albeit mostly milder and at an older age than affected males. Also, the risk of transmitting the disease to their future offspring is therefore excluded. Sexing remains a useful method, when the gene responsible for the disease is not known, when the search for a specific mutation is judged too difficult, or when the couple does not want to wait for a specific test to be developed (Sermon *et al*., 2004).

Mitochondrial disorders

Mitochondrial diseases are caused by defects in the oxidative phosphorylation pathway (OXPHOS) and manifest themselves in tissues with a high energy demand, such as muscle, brain, and heart. Mutations in both nuclear and mitochondrial (mt) genes can cause OXPHOS diseases. PGD for mitochondrial diseases coded by nuclear genes is essentially the same as for other Mendelian disorders. However, PGD for mutations in mitochondrial genes has its own complexity (Jacobs *et al*., 2006). The expression of mt-encoded diseases is very variable and, although severity is influenced by the mutation load, this is not the only determining factor. Mitochondrial mutations may be heteroplasmic (mixture of wild-type and mutant) or homoplasmic. Transmission of such mt mutations is exclusively maternal, as sperm as a rule does not

contribute mitochondria to the embryo. Prerequisites for a reliable prenatal diagnosis have been formulated by Poulton and Turnbull (2000), and these criteria also apply for PGD (Poulton *et al.*, 2007). They include the following.

- There is a close correlation between the mutation load and clinical severity.
- There are no significant time-dependent changes in mutation load.
- There is a uniform distribution of mutation load in the different tissues.

Only few (heteroplasmic) mitochondrial mutations are known to fit these criteria properly, and accordingly reliable PGD may presumably be offered. PGD for neurogenic ataxia retinitis pigmentosa (NARP) syndrome (8993 T>G mutation) has once been reported (Steffann *et al.*, 2006). Even more debatable is PGD for mitochondrial disorders which do not follow the Poulton and Turnbull criteria. The mitochondrial encephalomyopathy lactate acidosis stroke-like episodes (MELAS) syndrome is the classical example. It is usually heteroplasmic, and clinical expression is very variable. Although a certain correlation between mutation load and clinical severity is the rule, many individual exceptions exist. We may argue that PGD for MELAS syndrome may lower the risk of a severely affected child by transfer of embryos with the lowest possible mutation load. However, it remains ethically debatable whether only the possible reduction of the risk of a severely affected child, may be a valid reason to perform PGD.

PGD with human leukocyte antigen typing

A landmark in the application of PGD has been the development of human leukocyte antigen (HLA) typing, in order to create a brother or sister without the disorder who is HLA identical to his or her affected sibling, and who may serve as a stem cell donor for the affected sibling. The first application of PGD combined with HLA typing was reported in 2001 for a couple at risk for offspring with Fanconi anemia. Of 30 embryos, in four IVF attempts, five were unaffected and also HLA-compatible. Transfer of one embryo resulted in a healthy child (Verlinsky *et al.*, 2001). Indications for PGD with HLA typing nowadays include heritable disorders complicated by bone marrow failure, hematological malignancies, immunodeficiencies, and metabolic disorders, such as X-linked adrenoleucodystrophy. PGD of the HLA genotype is also applied without testing for a causative gene for couples who have children with (malignant) bone marrow disorders at need of HLA-matched stem cell transplantation, such as leukemia (Boyle & Savulescu, 2001).

With this new application, the "designer baby" concept was born. The ethical debate concerning this indication focuses on the instrumentalization and on the welfare of the "created" sibling on the one hand, and the health interest of the affected sibling on the other (Pennings & de Wert, 2003). There are several limitations to this indication (Shenfield *et al.*, 2005). One of the main practical drawbacks of combined genetic and HLA testing is that the number of embryos that are unaffected and are HLA-identical, and thus suitable for transfer, is limited (3/16 for autosomal-recessive disorders, 1/8 for sex-linked disorders). This implies that a high number of embryos (and therefore several IVF cycles) is needed and also limits the selection of embryos for transfer on the basis of morphology after the genetic diagnosis has been made. This results in a lower overall pregnancy rate. Another difficulty is that the time needed for pre-test technical work-up, the PGD treatment(s), and the pregnancy itself may be too long considering the urgent need for transplantation of the affected sibling. This is especially true for malignant disorders and it is not uncommon that the affected sibling has already died before the donor sibling is born. Couples who may opt for PGD/HLA typing should also be referred to a center for stem cell transplantation for information about available alternative treatments, possible complications of stem cell or bone marrow transplantation, optimal timing of transplantation, and the chances of success for their particular situation.

Inclusion criteria and individual patient selection

Each referral for PGD should be accompanied by appropriate information to avoid inconsistencies or duplications of consultations. The patient file should include a genetic counseling report, copies of the original chromosomal or DNA testing reports, or other specific testing of affected child, future parent(s), or other family members. Also, a full pedigree and family history (at least three generations) should be present as well as data on health problems of female and male partners and specialist consultations which may affect genetic diagnosis or IVF success and pregnancy. Furthermore, a female reproductive history,

gynecologic, and fertility status, and a male reproductive history, andrological history, and results of sperm analysis are also needed (Thornhill *et al.*, 2004).

A team approach with geneticists and gynecologists, assisted by other (para) medical specialists, social workers, psychologist, and ethicists, is required for appropriate patient selection for PGD. General genetic inclusion criteria include an (almost) certain genetic diagnosis, a high recurrence risk for a specific genetic disorder (e.g. >10 percent for chromosomal rearrangements, 25–50 percent for monogenic disorders), or recurrent miscarriage related to parental structural chromosome abnormality (Thornhill *et al.*, 2004). In addition, for HLA typing, the presence of an affected child with a genetic disorder or a malignant disease which is likely to be cured or life expectancy substantially prolonged by stem cell transplantation with cord-blood from an HLA-identical sibling (after alternative clinical options have been exhausted). General exclusion criteria are an uncertain diagnosis or a low recurrence risk, or both. Another point for consideration may be whether PGD is appropriate if an affected spouse has serious mental, psychological, or psychiatric problems caused by the genetic disorder for which PGD is requested, such as Huntington disease or otherwise.

The intake procedure involves a complete history from or about the affected individual and the future parents, including their general profile and their motivation for and expectations of PGD. A team discussion of the acceptability of new referrals, especially with new indications and of the acceptability of individual cases with several complicating medical, gynecologic or genetic factors is necessary and may prevent couples from undergoing "useless," disappointing, or potentially harmful PGD cycles.

Reasons for couples to refrain from PGD may be inconvenience or the burden of IVF or ICSI, the low success rate, spontaneous pregnancy whilst waiting to start the first cycle, financial costs, or inaccuracy of the test.

Gynecologic and genetic work-up

All indications for PGD rely on the condition that the diagnosis is confirmed genetically, and this must be stressed. In appropriate cases, chromosome analysis or DNA studies, or both, should be carried out in the patient, the prospective parents, and other family members.

Gynecologists advise on which couples are suitable for IVF. It is important to consider the woman's age and her ovarian reserve before embarking on IVF. Baseline serum follicle-stimulating hormone (FSH) is widely used as a screening test to predict the ovarian reserve and thus the ovarian response to medications for ovarian stimulation, and thus is an estimate of fertility potential. Moreover, it might be used to tailor the gonadotropin dose for ovarian stimulation. The number of antral follicles or serum anti-Müllerian hormones may also be used according to local standards (Van Voorhis, 2007). We should especially bear in mind the reduced ovarian reserve in some genetic syndromes, such as Fragile X syndrome (de Die-Smulders *et al.*, 2001; Hundscheid *et al.*, 2001; Platteau *et al.*, 2002), and the need for large numbers of follicles in women who undergo PGD for translocations or for combined monogenic and HLA testing.

Basic gynecological evaluation does not differ significantly from that in routine IVF or ICSI, and includes vaginal ultrasound and pelvic examinations. Both the female and the male are tested for infectious diseases (HIV, hepatitis B/C, lues) (Van Voorhis, 2007).

Semen analysis is done in order to trace any problem prior to the IVF treatment. Male fertility factors, as may be present in males carrying a translocation or another structural chromosomal abnormality or males affected by myotonic dystrophy, need to be considered.

Also, maternal risk factors for hormonal stimulation, ovum pick-up, or pregnancy need to be evaluated. For example, women with Fragile X syndrome, in particular carriers of a premutation (50–200 CGG repeats) are at increased risk for premature ovarian failure, which is preceded by a diminished ovarian reserve, increased basal FSH levels, and a lower response to stimulation. Carriers of hemophilia A (factor VIII deficiency) or B (factor IX deficiency) may have a bleeding tendency owing to skewed X-inactivation. Pretreatment evaluation and administration of 1-deamino-8-D-arginine vasopressin (DDAVP) or synthetic clotting factor before ovum pick-up are necessary to avoid excessive bleeding of the ovaries. Bleeding in hemophilia carriers will not occur immediately after the treatment but may be delayed for several hours. Prolonged monitoring of the patient's condition is necessary (Russell *et al.*, 2005). Women with myotonic dystrophy are at increased risk for cardiac myopathy and for fatal cardiac arrhythmias and cardiac arrest, and should be evaluated by a cardiologist (de Die-Smulders *et al.*, 1998). During IVF treatment cardiologic monitoring is warranted. Women affected by other neuromuscular disorders or a genetic connective

tissue disorder, such as Ehlers–Danlos syndrome or Marfan syndrome, may also have an increased risk of cardiovascular and pulmonary complications (Rossiter *et al*., 1995; Pepin *et al*., 2000).

The genetic laboratory needs to work up the specific diagnosis for each family. For chromosomal abnormalities probe combinations need to be checked for efficiency and reliability in both spouses.

Most PGD centers offer PGD for the most frequent monogenic disorders on a routine basis and the approach may be straightforward. Blood from affected and unaffected family members is collected for confirmatory testing of the recurrent monogenic mutation, combined with marker analysis. Even when the mutation is known, the accuracy of PGD is highly enhanced by simultaneous analysis of closely linked markers (Fiorentino *et al*., 2006). These specific markers need to be checked for each couple to find out if they are informative and to determine the mutation-associated haplotype. For trinucleotide repeat mutations the couple should be tested for informativity of the normal repeats.

However, for several individual mutations a new PCR protocol needs to be set up. This requires testing of the affected person and other relatives. The strategy usually followed is an initial multiplex PCR, followed by mini-sequencing to identify the mutation(s) combined with multiplex PCR for closely linked informative markers to increase accuracy. Linkage analysis, using intragenic and/or extragenic polymorphic microsatellite markers, is also applied in cases where the disease-causing mutation(s) is unknown or undetectable (Fiorentino *et al*., 2006). In a childless couple referred to PGD, where the female carries a *de novo* autosomal-dominant mutation, determining the risk-haplotype is not possible, and we must rely solely on mutation detection. If the male carries a *de novo* mutation a single-sperm haplotype analysis may be used to establish the haplotype linked to the mutation. The set-up of a novel PCR protocol may take many months and may be unproductive if the couple do not decide to come through.

Counseling

Genetic counseling is the sharing of information and advice about inherited conditions. In many countries patients are referred for PGD by clinical genetic centers, which means that they have been counseled with respect to the genetic condition relevant to that particular family and their reproductive options (Geraedts *et al*., 2001). Once admitted to the PGD center the couples receive further extensive information on IVF and PGD: brochures or written information may be sent to the couple prior to their appointment. We should check the couple's awareness about the nature and the severity of the genetic disorder, its recurrence risk, and their reproductive options and alternatives to PGD. The benefits and limitations of PGD compared with the alternative options, such as prenatal diagnosis, gamete donation, remaining childless, accepting risk without further examination, and adoption, should be discussed.

Genetic aspects of counseling include the scope of the test, for example rejection of normal and affected males after sexing for X-linked disorders, testing only for genetic disorders previously characterized in the family and not for other disorders, although in some centers aneuploidy screening is combined with PGD diagnosis (Sermon *et al*., 2007), and the number of embryos expected to be affected according to Mendelian ratios or segregation pattern. With respect to PGD itself, attention is focused on the biopsy procedure, the fact that one or two cells are removed from the embryo, the diagnostic method(s) used, and their efficiency, accuracy, and reliability. Patients should also be informed about the disposition of affected or undiagnosed embryos and the chance of misdiagnosis or adverse outcome. Some centers prefer to inform the patients in detail about the different sources of misdiagnosis, such as contamination, allele drop-out (ADO) in PCR procedures, and absent, split, or superimposed signals in FISH procedures (Geraedts *et al*., 2001; Thornhill *et al*., 2004). The patients are involved in decision-making about transfer of carrier embryos (for recessive disorders), mutation detection or FISH (for X-linked disorders), and sometimes about the number of unaffected embryos to be transferred.

Aspects of the IVF and ICSI procedure are dealt with in detail: the ovarian stimulation, risk of ovarian hyperstimulation syndrome or other medical complications during ovarian stimulation or oocyte retrieval, ovum pick-up, pain relief during the latter procedure, oocyte collection, embryo culture, and transfer policy (Venn *et al*., 2001; Van Voorhis, 2007). In regular IVF follicular stimulation is induced by a long protocol of gonadotropins and gonadotropin-releasing hormone (GnRH) agonists or antagonists, and is monitored by ultrasound and sometimes serum estradiol levels. Human chorionic gonadotropin (hCG) is given when enough follicles have reached 18 mm in size

and the ova are collected 34–36 hours after the hCG injection. Vaginal oocyte retrieval is performed by transvaginal ultrasound-guided follicle puncture and needle aspiration. At the same time the male partner is asked to produce semen, which will be processed in the laboratory. In PCR-based PGD, ICSI is always used to eliminate contamination with other sperm cells and all of the cumulus should be removed to avoid maternal contamination. The specifics of PGD treatment compared with regular IVF or ICSI include the fact that the number of oocytes to be retrieved needs to be maximized with regard to safe medical practice, the possibility that some embryos are unsuitable for biopsy or are lost due to the biopsy, and the possibility of all embryos being affected (in particular, in testing for chromosomal translocations).

In PGD ovarian stimulation is "pushed" in order to obtain the maximum number of oocytes. Ovarian hyperstimulation syndrome is a real threat, requiring hospitalization in 1–2 percent of women undergoing IVF. The enlarged ovaries cause pain, abdominal distension, and, in severe cases, fluid accumulation in the abdominal cavity (ascites), pleural effusion, and organ failure (Bergh & Lundkvist, 1992).

The cycle cancelation policy if the minimum number of follicles is not reached may differ per indication and per center. Couples with preexisting fertility problems or requirement for IVF may ask for regular IVF or ICSI, with skipping of the PGD test if the minimum number of follicles is not reached, and clear agreements should be made in advance. Agreements should also be made with couples regarding the transfer policy. This is especially important for infertile couples, as they may request that undiagnosed or even embryos diagnosed as affected are transferred, in the absence of normal embryos after PGD diagnosis, as – for them – getting pregnant anyway is the most important thing more or less irrespective of the health of their future child. In many centers the transfer of (possibly) affected embryos is excluded in the informed consent procedure.

With respect to pregnancy the following items are important: chance of ongoing pregnancy or live birth per started cycle and per transfer; pregnancy testing; risk of multiple pregnancy and policy to reduce this risk; and the recommendation or offer for prenatal testing to confirm the PGD diagnosis. Patients are further informed about the maximum number of PGD cycles offered, if applicable, the timeline for initial treatment, and the financial costs (Thornhill *et al.*, 2004).

There should be special attention for the personal situation of patients and their medical and psychological condition, in particular if they have experienced reproductive problems, problems with their own health, or otherwise important life events or traumatic experiences. A psychologist or social worker should be easily available. Psychological support may also be needed in further decision-making and during treatment. In some centers psychological counseling is routinely offered or is "mandatory" in cases in which one of the future parents is a carrier of an autosomal-dominant disorder and has signs and symptoms of the disorder or if one of the members of the IVF/PGD team has doubts about the welfare of existing or future children or the psychological well-being or mental capacity of the future parents.

Although nondirective counseling is the standard in medical genetics, a model of shared decision-making is probably more appropriate in PGD (Katz *et al.*, 2002). The order in which, and by whom, information is given, may vary from center to center. Patients have to take in much information and a follow-up consultation is recommended. In all cases, patients should be given sufficient time to make an informed decision about whether to proceed or to have a natural conception with prenatal diagnosis or use other alternatives. Patients may feel that the stress of undergoing IVF, accompanied by the relatively low chance of success, is more than the psychological stress of trying to achieve a natural conception followed by prenatal testing and possibly termination of pregnancy.

Outcome

Pregnancy rates

Even in fertile couples, IVF is more likely to fail than succeed. In general, success rates depend on the woman's age (Van Voorhis, 2007). Large series on pregnancy outcome in PGD, according to the various techniques and indications, are rare. The largest data series result from the ESHRE PGD Consortium (Sermon *et al.*, 2007). The overall clinical pregnancy rate was 18 percent per oocyte retrieval cycle and 25 percent per embryo transfer (Table 8.2). These numbers are lower than would be expected in regular IVF. Cumulative data of European IVF registers reported clinical pregnancy rates of 26 percent and 29 percent per oocyte retrieval cycle and per transfer, respectively, for regular IVF and ICSI combined (Nyboe Andersen *et al.*, 2007). We might speculate about the causes for the observed

Table 8.2 Pregnancy rates per indication

	No. of oocyte retrievals	No. of embryo transfers	No. of clinical pregnancies (positive heartbeats)	Pregnancy rate (%) per oocyte retrieval cycle	Pregnancy rate (%) per embryo transfer
Sexing X-linked disorders	703	537	137	19	26
Monogenic disorders					
Autosomal recessive	685	572	146	21	26
Autosomal dominant	516	385	88	17	23
Specific X-linked	161	115	34	21	30
Others	217	172	52	24	30
Total monogenics	1579	1244	320	20	26
Structural chromosome abnormalities					
Reciprocal translocations	862	539	111	13	21
Robertsonian translocations	481	353	90	19	25
Total translocations	1343	892	201	15	23
Total all indications	3625	2673	658	18	25

Adapted from Sermon *et al.* (2007), with permission from Oxford University Press.

differences. Maternal age seems to be comparable in IVF or ICSI and PGD. It has been suggested that blastomere removal could be responsible as it might interfere with the process of early cell differentiation, through a reduction in the cellular mass and alteration of embryo polarization toward trophoectoderm and inner cell mass (Liu *et al.*, 1993; Donoso *et al.*, 2007). Another explanation might be that the number of transferable embryos per cycle is lower in PGD compared with regular IVF or ICSI, as many embryos are discarded because they are affected by the genetic disorder examined. This results in a smaller pool of embryos for morphological selection of those embryos suitable for transfer. However, the International Working Group on Preimplantation Genetics reported a pregnancy rate of 29 percent for PGD, which is comparable with that for IVF populations (Anonymous, 2001).

Clinical pregnancy rates per indication are also depicted in Table 8.2. Rates per oocyte retrieval cycle and per embryo transfer were 20 percent and 25 percent after FISH diagnosis for sexing, and 20 percent and 26 percent after PCR testing for monogenic diseases, respectively. Although in the monogenic group the pregnancy rate is higher for PCR testing in X-linked disorders compared with autosomal-recessive and with autosomal-dominant disorders, the observed differences between the categories are not statistically significant. In general, the pregnancy rate for translocations is lower than for monogenic disorders, although not significantly so. Only for the group of reciprocal translocations is the number of pregnancies per oocyte retrieval cycle and per transfer significantly lower than for the monogenic disorders. This lower pregnancy rate, especially for carriers of reciprocal translocations, has been associated with a reduced chance of finding a chromosomally "normal" embryo (chromosomally completely normal or balanced translocation). The chance of finding a transferable "chromosomally normal" embryo is around 20% for reciprocal translocation carriers who undergo PGD (Simopoulou *et al.*, 2003). In addition a high incidence of aneuploidy in chromosomes not involved in the translocation, due to an interchromosomal effect, has been proposed though the issue remains under controversy (Pujol *et al.*, 2006; Donoso *et al.*, 2007).

It is now known that as many as half of all human embryos exhibit "mosaicism," in which the embryo is a mixture of chromosomally normal and abnormal cells. This is especially true for numerical chromosome abnormalities. The cell selected for PGD may have a different chromosome tally from the rest. Recent studies have suggested that these mosaic embryos tend to self-correct, or eliminate cells with aberrant chromosomes. This implies that in some embryos that are abnormal on the third day of development, later testing reveals a normal pattern. So embryos identified and eliminated using PGD would possibly develop as normal. Taking this into account, it is doubtful whether it is beneficial

to combine PGD for structural chromosome abnormalities with screening for numerical chromosome anomalies, in particular since the number of structurally normal embryos available for transfer is usually very low in translocation carriers (Simopoulou *et al*., 2003; Goldman, 2007).

Multiple pregnancies

The most important cause of morbidity and mortality in children born after assisted reproductive technology (ART) is multiple pregnancies. The twin rate in IVF or ICSI is 20–25 percent of all pregnancies (Land & Evers, 2003; Fauser *et al*., 2005; Sermon *et al*., 2007). Dizygotic pregnancies are relatively frequent after transfer of two or more embryos. However, the risk of monozygotic twins is probably increased after ART (Derom *et al*., 1987). The risks for low birthweight and preterm birth both exceed 50 percent for twins. These risks are six to nine times greater than the comparable risks for singletons. Moreover, multiplets are at a greatly increased risk for perinatal and infant mortality and neurological impairment (Sutcliffe & Ludwig, 2007). Carrying and delivery of a multiplet gestation is also riskier than a singleton gestation for women. Maternal mortality, hemorrhage, and pregnancy-induced hypertension are all associated with twin pregnancies (ESHRE Capri Workshop, 2000; Schieve & Reynolds, 2004). Usually, the number of embryos to be transferred is determined according to the age of the patient, the embryo quality, and the number of previous IVF attempts. This strategy is not different for PGD in most centers. Hence, from 1999 onwards, elective single embryo transfer (e-SET) was introduced as the most rational approach to reduce the number of twin pregnancies (Vilska *et al*., 1999; Pandian *et al*., 2005; Sutcliffe & Ludwig, 2007). In a retrospective study, Donoso and coworkers (2007) demonstrated that there was no significant reduction in the delivery rate for single-embryo transfer compared with double-embryo transfer in PGD cycles for women aged <36 years. So the application of e-SET is also feasible for PGD cycles.

Misdiagnoses

In total 22 misdiagnoses have been reported until now: 11 after PCR and 11 after FISH diagnosis (Table 8.3). All of the misdiagnoses were reported by the ESHRE PGD Consortium (Harper *et al*., 2008). Of a total number of 12 397 cycles to oocyte retrieval (OR) the rate of known and reported misdiagnosis is less than 1 percent, both for FISH and PCR (Harper *et al*., 2008). However, we must keep in mind that the number of misdiagnoses may be an underrepresentatation as several misdiagnoses may well stay undetected in the absence of confirmatory testing of the embryo or of the fetus in case of a pregnancy and the lack of clinical signs after birth.

For clinical practice, these misdiagnoses may be classed as acceptable, that is, the child born after PGD is not affected with the disease that was tested for, e.g. a carrier instead of a homozygous normal result in autosomal-recessive diseases, or unacceptable, that is, embryos were transferred that were thought to be free from disease but the fetus or child is affected. According to this classification, two of the misdiagnoses reported earlier would be classed as acceptable: two CF carriers were born while the transferred embryos had been diagnosed as homozygous normal. The 18 unacceptable misdiagnoses are further summarized in Table 8.3.

There are several ways a misdiagnosis may result during FISH analysis. If cleavage-stage biopsy is carried out a possible explanation could be mosaicism of the embryo, with a normal cell being biopsied from an otherwise abnormal embryo. If more than three FISH probes are used the efficiency of the FISH procedure is decreased and the risk of overlapping signals or failure of hybridization is higher. Cumulus cell contamination is also possible during FISH, which would result in a normal female nucleus being analyzed. When using FISH for sexing, it is advisable to include the sex chromosomes in the first round of FISH, and to reduce the number of other probes in this round, to ensure an accurate diagnosis of the gender. For FISH, slides with spread blastomeres or polar bodies should be kept and re-probed if any discordance with a conceptus is identified.

The problems that lead to PCR misdiagnoses are contamination or allelle drop-out. In the early days of PGD, the reported misdiagnoses for sexing using PCR, and for CF, were probably caused by the low efficiency of single-cell PCR. It is to be expected that current technical developments, such as multiplex fluorescent PCR or even preimplantation genetic haplotyping (PGH), rule out the occurrence of such errors. For confirmation after single-cell PCR the same sample cannot be analyzed twice and, in the case that non-transferred embryos are not systematically checked, there is no internal check to rule out human error (including incorrect labeling of the embryos, transfer of the wrong embryo).

However, the real cause of the diagnostic failure remains uncertain in most cases; spontaneous pregnancy in potentially fertile PGD candidates cannot be

Table 8.3 Misdiagnoses in preimplantation genetic diagnosis

Indication	Method used	PND-postnatal	Outcome	Reference
Monogenic disorders				
Myotonic dystrophy type 1	PCR	PND	TOP	ESHRE PGD Consortium(1999)
Beta thalassemia	PCR	PND	TOP	ESHRE PGD Consortium (2000)
SMA	PCR	Post	Born	Harper *et al.* (2008)
Familial amyloid polyneuropathy	PCR	PND	Born	Sermon *et al.* (2005)
Cystic fibrosis	PCR	PND	Born	ESHRE PGD Consortium (2000)
Cystic fibrosis	PCR	PND	Born	Sermon *et al.* (2005)
Cystic fibrosis (one of twins)	PCR	Post	Born	Sermon *et al.* (2005)
Charcot–Marie–Tooth disease 1A*	PCR	PND	TOP	Harper *et al.* (2008)
Charcot–Marie–Tooth disease 1A*	PCR	PND	TOP of both twins	Harper *et al.* (2008)
46 XY in Duchenne muscular dystrophy in one of twins	PCR	PND	TOP of one twin	ESHRE PGD Consortium (2002)
46 XY in X-linked retinitis pigmentosa	PCR	PND	Born	Sermon *et al.* (2005)
Translocations				
47,XX, der(t11;22)	FISH	PND	TOP	ESHRE PGD Consortium (2002)
Trisomy 13 after 45,XY,der(13;14)(q10;q10)	FISH	Miscarried	Miscarried	Sermon *et al.* (2007)
46,XY,der(15)t(13;15) (q10;q10)	FISH	PND	TOP	Harper *et al.* (2008)
PGS				
Trisomy 16 after first polar body biopsy	FISH	Miscarried	Miscarried	Sermon et al. (2005)
Trisomy 16 after first polar body biopsy	FISH	Miscarried	Miscarried	Harper *et al.* (2006)
Trisomy 16	FISH	Miscarried	Miscarried	Sermon *et al.* (2007)
Trisomy 16	FISH	Miscarried	Miscarried	Sermon *et al.* (2007)
Trisomy 21	FISH	Postnatal	Born	ESHRE PGD Consortium (2002)
45, XO	FISH	PND	TOP	Sermon *et al.* (2005)
47, XXX	FISH	PND	Lost to follow-up	Harper *et al.* (2008)
Social sexing				
Requested male but female fetus	FISH	PND	TOP	ESHRE PGD Consortium (2002)

PND, prenatal diagnosis; TOP, termination of pregnancy. *Haplotype analysis prior to PGD was incorrect. As a result affected embryos had been selected for transfer.

ruled out. Counseling protocols should be amended accordingly. The accuracy and reliability of the PGD diagnosis may also be improved by analyzing two cells (Van de Velde *et al.*, 2000).

Prenatal diagnosis may be offered after PGD to confirm the diagnosis. In practice, most couples do not opt for a prenatal test after PGD (ESHRE PGD Consortium, 2002). Also, postnatal confirmation is possible; however, for autosomal-dominant disorders it is usually not performed because presymptomatic testing for these disorders in minors is ethically unacceptable.

Examination of spare embryos may also be applied to confirm, or incidentally reject, the PGD diagnosis. In a study by Dreesen *et al.* (2008), it was found that the rate of misdiagnosis was significantly higher in poor-quality spare embryos. If these data are confirmed it may be wise not to transfer embryos with the worst morphology.

Follow-up of PGD children

A longstanding question in PGD is if it is truly harmless. The outcomes of PGD pregnancies are generally believed to be comparable with IVF or ICSI pregnancies, and no pregnancy complications or malformations at

birth are occurring in particular in the PGD population (Sermon *et al*., 2007). A large series of prospective and retrospective studies of children born as a result of ART has shown that, in general, development is normal and malformation rates are similar to, or only slightly higher than, those in the general population (Van Steirteghem *et al*., 2002; Sutcliffe & Ludwig, 2007). However, neonatal data of children born after IVF or ICSI treatment show that these infants (even as singletons) are at increased risk of low or very low birthweight and preterm delivery. A slight increase in sex-chromosomal anomalies was also suggested (Sutcliffe & Ludwig, 2007). However, the question remains about whether these risks stem from the ART treatment itself or from the underlying infertility of the couples using these treatments.

Another point of concern about ART is the possibly increased risk of imprinting diseases, such as retinoblastoma, Angelman syndrome, and Beckwith–Wiedemann syndrome (Maher *et al*., 2003; Moll *et al*., 2003; Sutcliffe *et al*., 2006; Sutcliffe & Ludwig, 2007). Epigenetic disturbances, referring to phenomena whereby modifications of DNA methylation and/or chromatin remodeling structure underlie changes in gene expression, may occur during in vitro culture. The use of immature sperm may also be associated with epigenetic defects, as immature sperm cells are epigenetically quite different from mature sperm cells (De Rycke *et al*., 2002). Failure or incomplete reprogramming of the gamete or preimplantation embryo may disturb normal development. Whether these epigenetic defects have a substantial effect on IVF/ICSI children's health indeed has to be determined. Also, the impact on later life is not known. In conclusion, long-term follow-up studies, in larger series of children after ART, are still needed. A complete safety analysis may even require studies of more than one generation (De Rycke *et al*., 2002; Sutcliffe & Ludwig, 2007). This also applies to babies born after PGD (Mayor, 2006).

Thousands of PGD babies have been born without obvious major problems and PGD thus is regarded as a safe method of avoiding the birth of children with a serious genetic defect. On the other hand, the question is whether removing a cell during PGD leaves a mark on the embryo that affects its ability to develop or affects the child's long-term health (Goldman, 2007). The traditional view among embryologists is that the first few cells of mammalian embryos are essentially equivalent: remove one or two, and the rest can ably fill the gap. However, some recent studies have challenged that idea, and many now believe that even these early cells are predisposed to contribute in different ways to future tissue (Torres-Padilla *et al*., 2007).

Conclusion

Patients undergoing PGD differ from couples referred for IVF in that most are fertile. Their inherited disorders may have resulted in an affected child, termination of pregnancy, repeated pregnancy loss, or in the knowledge that they themselves will develop signs or symptoms of the disease in the near future. PGD patients may be unaware of the procedures required for IVF, which is a prerequisite for PGD. Careful explanation should include discussion of potential risks and realistic assessment of success, so that the couple can make an informed decision about whether to proceed or to have a natural conception. As for all couples undergoing IVF, they should be offered counseling, with special consideration of the extra stresses they may encounter.

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Chapter 9

Polar body biopsy

Markus Montag, Katrin van der Ven, and Hans van der Ven

Key points

- Biopsy of the first and second polar bodies allows investigation of the chromosomal and genetic constitution of the corresponding oocyte.
- Polar body biopsy may be performed using a beveled pipette, by three-dimensional partial zona dissection, or a non-contact diode laser.
- Acid Tyrodes is not suitable for zona drilling in polar body biopsy.
- Polar bodies may be biopsied simultaneously or sequentially.
- This method was first proposed for the analysis of numerical chromosome disorders in 1990 and was later adapted for the detection of single gene disorders, for translocation analysis, for the detection of X-linked disorders as well as for human leukocyte antigen (HLA) typing.
- Biopsy of the first and second polar bodies followed by diagnosis of aneuploidies by fluorescence *in situ* hybridization (FISH) can be accomplished prior to the fusion of the male and female pronucleus. Thus, this method may be considered as pre-conception analysis, and does not fall within the legal regulations of some countries, like Germany or Switzerland, where preimplantation genetic diagnosis (PGD) of embryonic blastomeres is forbidden.

Introduction

One approach to the prevention of inherited disease is the diagnosis of oocytes before fertilization (preconception diagnosis). The removal of the first or second polar body, or both (which are byproducts of meiotic division – see Chapter 6 and Chapter 7) is an indirect approach allowing the genetic status of the oocyte to be inferred from that of the polar body. The first polar body is not required for successful fertilization or normal embryonic development. The second polar body, although a product of fertilization, is similarly not required for subsequent embryo development (Kaplan *et al*., 1995). Thus removal of either the first or second polar body, or both, for the purposes of genetic diagnosis should have no deleterious effect on the developing embryo.

Polar body biopsy with subsequent analysis of numerical chromosome disorders was first proposed in 1990 by Verlinsky and collaborators (Verlinsky *et al*., 1990). Since then it has mainly been used by two groups to investigate chromosome abnormalities (Munné *et al*., 1995; Verlinsky *et al*.,1996; Verlinsky *et al*., 1998), monogenic disorders (Verlinsky *et al*., 1997; Strom *et al*., 1998; Kuliev *et al*., 1999), translocation analysis (Munné *et al*., 1998), and HLA typing (Verlinsky *et al*., 2001) as well as the detection of X-linked disorders (Verlinsky *et al*., 2002). However, to date, most cases of polar body diagnosis are performed for aneuploidy screening.

Technical aspects: methodology

The first and second polar bodies may be biopsied simultaneously or sequentially. Biopsy of the first polar body can take place immediately following oocyte retrieval as long as the oocyte has entered metaphase II and fully extruded the first polar body without any remnants of spindle fibers. The second polar body is already present three hours after fertilization; however, it is connected to the oocyte by a cytoplasmic bridge containing remnants of the spindle during the first hours of polar body extrusion (see below).

Simultaneous sampling of both first and second polar bodies seems to be a straightforward approach. However, simultaneous biopsy can only be done during a time window of approximately 6–14 hours after fertilization, as thereafter the first polar body most likely starts to degenerate, which may lead to subsequent diagnostic failures (Munné *et al*., 1995).

If sampled sequentially, two independent manipulations are required with the possibility of

intracytoplasmic sperm injection (ICSI) in between for polymerase chain reaction (PCR) based cases, making a total of three manipulations on the same oocyte.

Polar body removal

Removal of polar bodies requires access to the perivitelline space through the zona pellucida (ZP) and various methods have been proposed.

Acid Tyrodes solution

An opening in the ZP may be introduced by using acid Tyrode solution as a chemical means (Gordon & Talansky, 1987). These authors demonstrated that live offspring could result in mice after treatment of the ZP of oocytes with acidified Tyrode's solution. However, similar studies using human oocytes showed that, although fertilization could be established, there was an inhibitory effect on embryonic development when oocytes were exposed to acid Tyrodes (Malter & Cohen, 1989) owing to a direct effect of acid on the oocyte, possibly as a result of the difference in thickness of the human and mouse ZP.

Therefore, since both the oocyte and polar body are sensitive to the effects of acid most approaches to polar body biopsy have adopted mechanical means.

Another disadvantage of acid Tyrodes solution is that the method is difficult to reproduce; the size of the opening varies from one oocyte to another and depends on the sensitivity of the oolemma of unfertilized oocytes. Altogether, zona drilling by acid Tyrodes solution is unsuitable for polar body biopsy.

Biopsy using a beveled pipette

To perform polar body biopsy, a holding pipette and a beveled micropipette (12–15 μm in diameter) are used. The oocyte is held in place with the polar body at the 12 o'clock position. The beveled micropipette is passed through the zona and into the perivitelline space tangentially toward the polar body. The polar body may then be aspirated into the pipette (Figure 9.1). If the polar body is still attached to the ooplasm, further incubation may be required to permit completion of the meiotic cell cycle leading to complete polar body extrusion (see below and Montag *et al*., 2006).

Three-dimensional partial zona dissection

Another efficient biopsy technique was elaborated by Cieslak *et al*. (1999). This approach is based on three-dimensional zona dissections. The oocyte is affixed to the holding capillary and, using a sharp needle, a slit is made close to the area where the polar bodies are located. After turning the oocytes by 90 degrees, a second slit is made creating a cross-like incision in the zona which allows access to the polar bodies (Figure 9.2). Although this method may be performed with simple glass tools, multiple steps, including dissection, release, and rotation of the oocyte are needed. This procedure is technically difficult, requires extensive experience, and is time-consuming compared to laser-assisted biopsy, which will be the focus of this chapter. However, it is preferentially used by the Chicago group, which has a very busy polar body biopsy program (Cieslak *et al*., 1999).

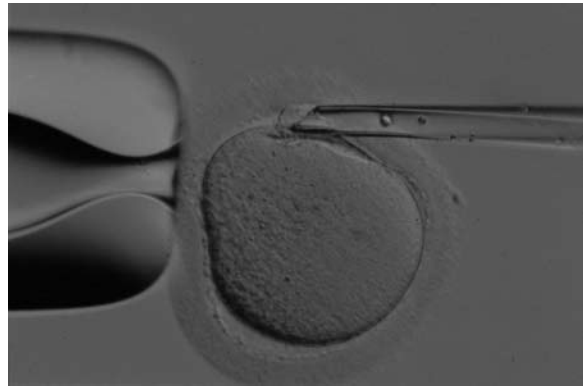

Figure 9.1 Polar body biopsy using a beveled pipette. A beveled micropipette may be passed through the zona and into the perivitelline space. However, the passage through the zona is not easy and the oocyte may suffer from a trauma if the pipette does not penetrate smoothly. Courtesy of Santiago Munné, New Jersey.

Laser-assisted biopsy

In assisted reproduction, lasers were initially used to assist fertilization in cases of severe male-factor infertility. One approach was to drill a small opening into the ZP, which eventually allowed a few sperm to gain access to the perivitelline space and to fuse with the oolemma (Liow *et al*., 1996). Another method favored the manipulation of spermatozoa by optical tweezers (Schutze *et al*., 1994). The aim was to guide laser-trapped sperm into the perivitelline space of the oocyte through artificial laser-drilled openings. These approaches have not succeeded, as the introduction of ICSI was far more successful (Palermo *et al*., 1992). However, this pioneering work showed that the laser is a good tool for creating openings in the ZP.

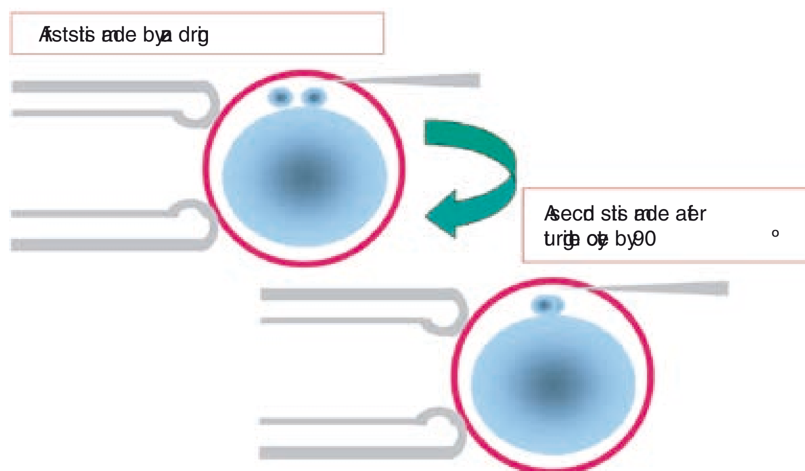

Figure 9.2 Schematic illustration of three-dimensional partial zona dissection.

Recently, the development of a 1.48 μm diode laser system has had a major impact on assisted reproductive techniques (Rink *et al.*, 1994, 1996; Montag *et al.*, 1999). The idea of using that laser system for polar body biopsy was proposed as early as 1997 (Montag *et al.*, 1997) and was clearly stimulated by the ease of laser-assisted hatching and the instantaneous opening of the zona within milliseconds. Based on animal experimentation, laser-assisted biopsy did not interfere with further development of mouse embryos (Montag *et al.*, 1998) as long as the laser was used in a proper way (Montag & van der Ven, 1999) in order to prevent inappropriate hatching of the blastocyst stage (Montag *et al.*, 2000). Since the very straightforward approach for laser-assisted polar body biopsy, proposed in 1998 (Montag *et al.*, 1998), the biopsy technique has not changed significantly.

In general, the size of the drilled opening is usually in the range of 18–25 μm but it can be easily adjusted to the diameter of the aspiration capillary. As the capillary may be introduced through the laser-drilled opening, there is no need for a sharp aspiration needle. This allows the use of flame-polished, blunt-ended aspiration needles and greatly reduces the risk of damaging the polar body or the remaining oocyte. The procedure is accurate, reproducible, and safe, and it also reduces the number of cells that cannot be reliably diagnosed as a result of technical problems during the biopsy procedure (Montag *et al.*, 2002). Another benefit is that laser drilling and subsequent biopsy may be performed without changing the culture dish or the capillaries in contrast to zona drilling using acid Tyrodes solution. This may help to prevent contamination of samples to be diagnosed by sensitive techniques such as PCR.

Laser-assisted polar body biopsy is best accomplished when the oocyte is affixed to the holding capillary with the first polar body at the 12 o'clock position and the second polar body located right of the first one but in the same focal plane. An opening of 18–25 μm is drilled at 2–3 o'clock and by pushing the biopsy capillary into the perivitelline space both polar bodies may be removed simultaneously (Figure 9.3). Usually the second polar body is affixed to the oocyte by a cytoplasmic bridge, which is very firm immediately after second polar body formation, but becomes less firm with time. The positioning of the second polar body next to the aspiration capillary allows pushing of the second polar body far to the left side, toward the holding capillary, and this stretching movement usually is sufficient to break the cytoplasmic bridge between the second polar body and the oocyte. Owing to the use of a blunt-ended capillary even manipulation in direct vicinity to the oolemma does not damage the oocyte.

Transfer of polar bodies onto glass slides for aneuploidy screening or translocation analysis

In contrast to embryonic blastomeres, polar bodies are rather small and do not require any special pretreatment like hypo-osmotic swelling or pronase digestion. Therefore it is advisable to transfer the first and second polar bodies from an individual oocyte with the biopsy capillary instead of with a hand-held pipette. Transfer may be done directly into a tiny drop (0.2 μL) of water placed on a clean glass slide. The small volume of the droplet guarantees that the polar bodies will attach to

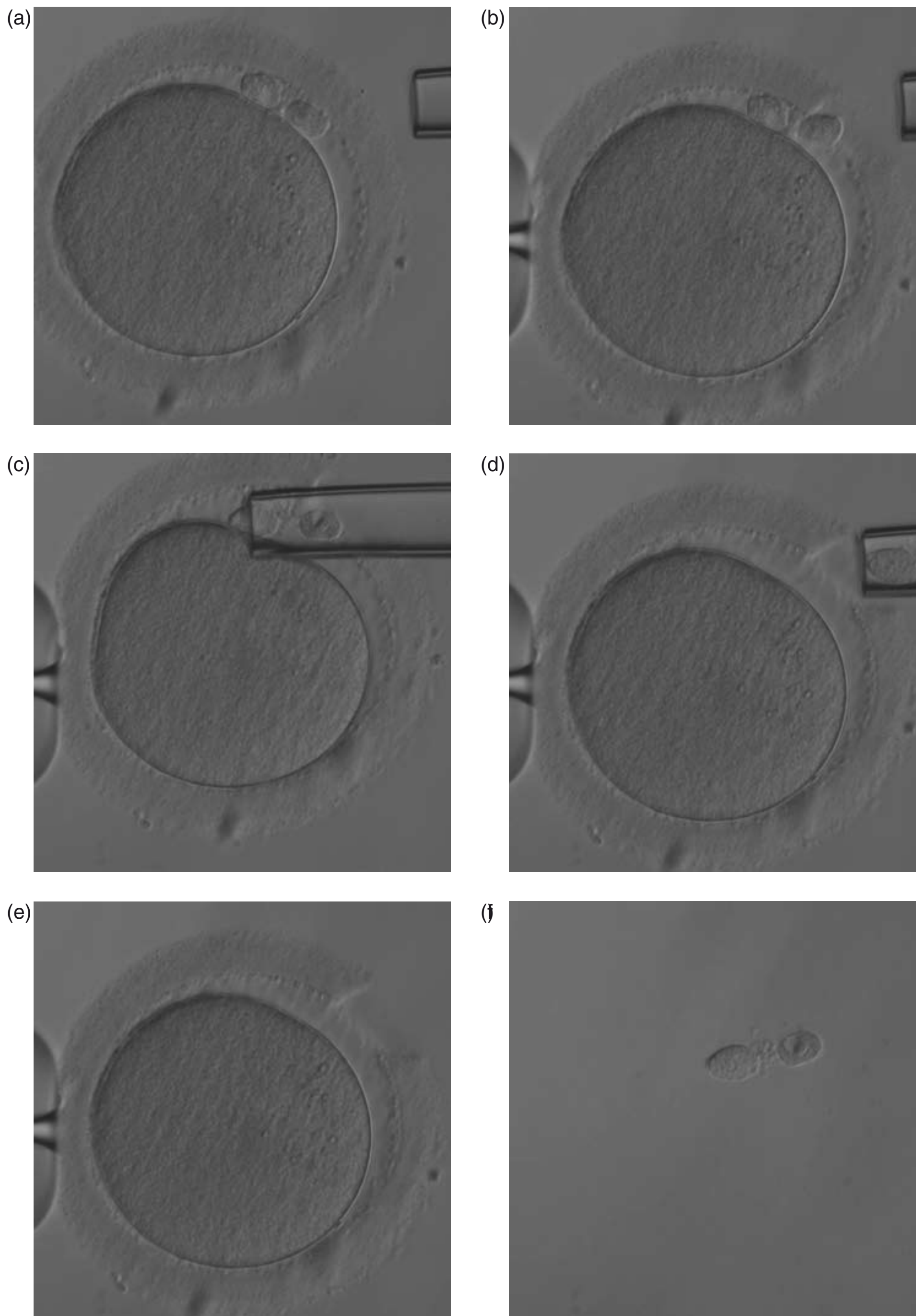

Figure 9.3 Simultaneous biopsy of the first and second polar bodies. For removal of the first and second polar bodies the oocyte is held in a position where the polar bodies are located at 12 o'clock (a). An opening is drilled with a non-contact diode laser (OCTAX, Octax, Landshut, Germany) at 1–2 o'clock which allows retrieval of both polar bodies by sliding the capillary over them (b, c); only then are the polar bodies aspirated with as little suction as necessary (d); the capillary is removed (e); and the polar bodies are expelled in another droplet within the biopsy dish prior to (f) transfer onto the glass slide.

the slide within a small area and that the fluid will dry out very fast, which reduces the risk of a dislocation of the polar bodies on the slide. The drying process should be observed under a stereo microscope and the final location of the polar bodies after air-drying must be marked either by drawing a circle on the underside of the slide or by encircling the polar bodies on top of the slide with a diamond or tungsten carbide marker. Encircling on top of the slide has the advantage that under high magnification using oil immersion allows visualization of the drawing line, which not only denotes the position of the polar body but also indicates the focal plane. With some experience, polar bodies from up to nine oocytes may be placed within a round area of 10 mm, the position of each being marked with a diamond marker. Further processing by fixation and FISH may be performed according to standard protocols and as described previously (Verlinsky *et al.*, 1990; Munné *et al.*, 1995; Montag *et al.*, 2004a).

All manipulation steps during biopsy, and especially during transfer of polar bodies, require a good operating micromanipulation system and extremely fine adjustable micro-injectors. Automatic micromanipulators may be used so that certain positions of the capillaries can be stored in a memory in order to speed up the procedure. This is extremely helpful if several positions in the *z*-direction are needed, for example a position at the bottom of a dish for biopsy, a position outside the dish for changing dishes or placing the slide, and a third position on top of the slide for deposition of the polar bodies.

Diagnostic and strategic considerations

The size and position of openings drilled in the ZP can influence further embryonic development (Montag & van der Ven, 1999) and in particular the mode of hatching at the blastocyst stage (Montag *et al.*, 2000). Although this was described in detail for laser-drilled openings, it may also apply for openings drilled by other means.

Size of drilled openings

When using the laser technique, it is important to generate a sufficiently large opening, which permits consecutive hatching at the blastocyst stage. Smaller openings of less than 15 µm may cause trapping of the embryo followed by degeneration (Montag & van der Ven, 1999). If only a small channel is drilled in order to push the biopsy capillary into the perivitelline space, we should perform an additional zona thinning over this region prior to transfer in order to facilitate successful hatching at a later stage. A small channel could trap the embryo during hatching because the entire thickness of the zona may occasionally be resistant for the embryo.

Number of drilled openings

It is crucial to open the ZP only at one site, as two openings, for example to retrieve both polar bodies through separate openings, may cause problems at the time of hatching. As the hatching process in vitro is a result of blastocyst expansion (Montag *et al.*, 2000), the expanding embryo does not discriminate between one or the other opening and consequently makes use of both openings. The simultaneous hatching through two openings may result in a trapping of the blastocyst within the zona (Montag & van der Ven, 1999). Complete hatching may then not take place, or the embryo may be separated into two parts, which may under certain circumstances both implant, resulting in monozygotic twins.

Polar bodies attached to the oocyte by a cytoplasmic bridge and spindle remnants

As already mentioned, the presence of a cytoplasmic bridge between the first as well as the second polar body and the oocyte can impose during the biopsy procedure. This cytoplasmic bridge cannot be cut by the laser itself due to the nature of the diode laser. Usually the connection will break during the aspiration process. However, this may lead to a severe problem if spindle fibers are present within the cytoplasmic bridge. The presence of spindle fibers between oocytes and the newly formed polar bodies (first and second) is a natural event of the meiotic cell cycle (Montag *et al.*, 2006). In the presence of a strong cytoplasmic bridge, we should refrain from polar body aspiration, as it will often result in the formation of a small ooplasmic droplet which could contain chromosomes or chromatids of the oocyte (Figure 9.4). In order to fully control such a situation, polarization microscopy to identify the location of the spindle remnants may be performed immediately prior to the biopsy procedure.

Polar body fragmentation

A frequent problem in analysis of the first polar body is the high degree of fragmentation observed in human first polar bodies. Obviously, all fragments can contain

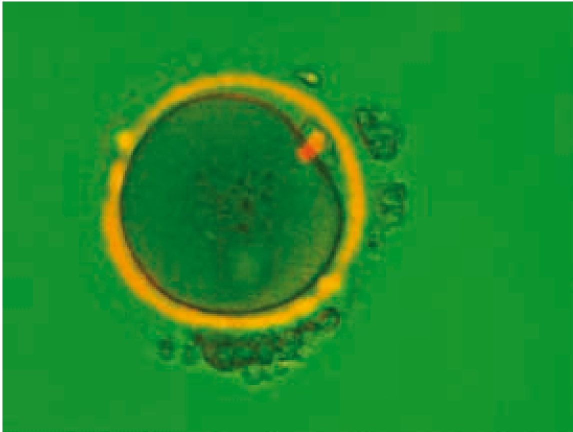

Figure 9.4 Polar bodies and spindle remnants. Using polarization microscopy (OCTAX polarAIDE™, MTG, Landshut, Germany) enables the visualization of spindle remnants (shown in red) between the polar body and the oocyte (shown in green). Biopsy of the polar body shown would result in the formation of an ooplasmic droplet containing the chromosome set of the oocyte, which would be enucleated.

chromatin material and therefore it is essential to remove all fragments. In such a case the process of air-drying on the slide should be observed very carefully; furthermore, a drawing of the location of the fragments within the encircled area is often helpful. If only one fragment is overlooked during FISH analysis we may easily risk a misdiagnosis if one of the chromosomes under investigation is located in the missing fragment.

Oocyte and embryo handling

Openings drilled in the ZP for polar body removal will stay permanently and may reduce the mechanical resistance of the zona. In those cases where oocytes or embryos presenting with an opening in the zona need to be attached to a holding capillary, it is especially important that extreme care is taken not to attach them at the site of the opening as this will result in a leakage of the cytoplasm. Therefore, oocytes and embryos should in general be handled very gently during all subsequent manipulations (e.g. transfer of oocytes or embryos to other media droplets) and especially during embryo transfer.

Safety of laser-assisted biopsy

There is still an ongoing discussion about the safety of the laser technique in combination with embryo or polar body biopsy. In 2003, Joris and coworkers (Joris *et al.*, 2003) compared embryo biopsy using acid Tyrodes solution with laser biopsy. The authors concluded that following laser-assisted embryo biopsy significantly more blastomeres were intact compared with a biopsy using acid Tyrodes solution. Pregnancy and implantation rates did not differ and even showed a trend to higher rates following the use of the laser technique. Another advantage of the laser technique was the ease of the procedure and its time efficiency. More recently, Chatzimeletiou *et al.* (2005) compared the effect of biopsy by acid Tyrodes solution and laser treatment on blastomere viability and embryo development. They clearly demonstrated that a proper laser handling technique does not cause any damage, whereas embryos which were biopsied with the use of acid Tyrodes showed a compromised blastocyst development.

Laser-assisted polar body biopsy, if trained and applied properly, is a very efficient method to reduce loss of polar bodies and trauma of oocytes (Montag *et al.*, 2002). Furthermore, the laser technique does not impair the in vitro fertilization (IVF) success rates (Montag *et al.*, 2004b; Montag *et al.*, 2005). Pregnancy and delivery rates are comparable with those recently reported by the leading group in polar body diagnosis from Chicago (Kuliev *et al.*, 2002), where biopsy is performed using a modified zona drilling technique (Cieslak *et al.*, 1999). Although zona drilling is easily performed by a skilled person, the laser technique may help to reduce the learning curve especially in centers starting with polar body biopsy.

First and/or second polar body biopsy

For polar body biopsy, either the first polar body alone or both the first and second polar bodies may be biopsied to provide genetic information relating to a particular oocyte. Preconception diagnosis concentrated on the former approach. However, biopsy of the first polar body alone has limited applicability for PGD for a number of reasons. The procedure only allows the detection of maternal genetic defects and crossing-over of homologous chromosomes leads to a reduction in the number of embryos available for transfer (Dreesen *et al.*, 1995). Also, there is only the possibility of a single cell for analysis, leading to a lower overall reliability (in contrast to cleavage-stage biopsy, in which two cells may be taken for independent analysis). It is thought that more unacceptable errors would result from polar body analysis when compared with blastomere analysis (Navidi & Arnheim, 1991). To overcome these disadvantages, Verlinsky *et al.* (1990) proposed and undertook to remove both first

and second polar bodies for analysis. After assessing the safety of removing the second polar body in mice (Kaplan *et al.*, 1995) this approach has met with success and has been applied to PGD for the detection of numerous monogenetic diseases (e.g. cystic fibrosis (CF), Strom *et al.*, 1998; beta thalassemia, Kuliev *et al.*, 1999; chromosomal aneuploidies, Verlinsky *et al.*, 1996, 1998; and maternal chromosome translocations, Munné *et al.*, 1998).

Despite the large number of cycles reported with this approach, only a few centers worldwide have used it. This may be attributed to a number of factors. First, the approach may only be applied to maternally inherited diseases. Second, diseases that are detected by assessing changes in gene product (Eldadah *et al.*, 1995) would not be candidates for this approach. Third, polar body biopsy cannot be used for gender determination. Many centers may have established cleavage-stage biopsy as the tool for PGD as a result of the large number of referrals for sexing (see Chapter 14 on FISH and sexing). Finally, biopsy of both the first and second polar bodies is required for optimal diagnostic efficiency and although this can be achieved by either sequential (Strom *et al.*, 1998; Kuliev *et al.*, 1999) or simultaneous (Verlinsky *et al.*, 1998) biopsy with successful results, it is very labor intensive and there have been criticisms.

Conclusions

Owing to its ease, laser-assisted biopsy is now widely used for biopsy of polar bodies (Montag *et al.*, 2004b), blastomeres (Licciardi *et al.*, 1995; Boada *et al.*, 1998), and blastocyst cells (Veiga *et al.*, 1997), and recently its advantage compared to acid Tyrodes solution was reported by Joris *et al.* (2003).

It is important to understand that polar body diagnosis gives direct information about the first and second polar bodies, and therefore only allows an indirect diagnosis of the chromosomal constitution of the corresponding oocyte. In contrast, PGD of the embryo gives a direct diagnosis of the embryo and allows the detection of both maternally and paternally derived genetic or chromosomal contributions. Obviously this is the reason why PGD following removal of one or two blastomeres from an embryo is by far the more commonly applied technique worldwide (Harper *et al.*, 2006). However, in countries with legal restrictions on PGD, such as Austria, Germany, Italy, and Switzerland, only polar body diagnosis within the frame of the existing laws is possible. Furthermore, this technique may be more readily accepted by couples with ethical or moral constraints toward the generation and discarding of supernumerary zygotes and embryos, as in the case of PGD.

Another important aspect to note is that the removal of polar bodies does not influence further developmental potential of the oocyte, as the polar bodies are by-products of the meiotic division cycle and do not contribute to the development of the embryo. This should be kept in mind for the ongoing discussion toward the relevance of polar body biopsy compared with embryo biopsy. Recent findings on cell polarity in the human oocyte and early embryo (Hansis & Edwards, 2002) imply that there could be, at least theoretically, a negative impact of embryo biopsy on embryo and fetal development (Bahce, 2003; Cohen & Munné, 2005). To date there is no proven evidence that removal of one or two blastomeres from an eight-cell embryo is detrimental; however, we cannot be completely sure about this and further studies are required.

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Chapter

10 Cleavage-stage embryo biopsy

Anick De Vos

Key points

- Cleavage-stage embryo biopsy remains the main approach to removing genomic DNA for genetic analysis.
- The biopsy procedure is performed on the morning of day 3, 68–72 hours post-microinjection (or insemination), when the embryos are preferably at the eight-cell stage. The removal of cells requires an opening in the zona pellucida (ZP), which may be created in a mechanical or chemical way, or otherwise by laser energy
- One or two blastomeres may be removed by aspiration, extrusion, or flow displacement
- Incubating the embryos in Ca^{2+} /Mg^{2+}-free decompaction medium may facilitate the biopsy procedure.
- The cellular material removed from the embryo should be suitable for genetic analysis either by fluorescence *in situ* hybridization (FISH) or by polymerase chain reaction (PCR). This means that the cell should be intact and it should contain a single, clearly visible nucleus
- Further development of the embryo may not be impaired as a result of the biopsy procedure.

Introduction

First and second polar body biopsy definitely has certain merits, and has been widely practiced mainly for preconceptional screening but also for genetic testing (Verlinsky *et al*., 2004; Dawson *et al*., 2006) (Chapter 9). However minimally invasive, the major limitation of this approach remains that only the female contribution to the final embryo can be tested. Single blastomere biopsy on day 3 alleviates this limitation and it allows enough time for genetic or chromosomal testing if blastocyst transfer is performed on day 5. Biopsy of trophectoderm cells at the blastocyst stage on day 5 or 6 has started to find clinical application (McArthur *et al*., 2005) (Chapter 11). This approach ensures a larger amount of material for testing; however, the time to reach diagnosis is much shorter if a fresh transfer is still attempted.

The European Society of Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium data collection (Harper *et al*., 2008; Sermon *et al*., 2007) shows that the most widely used biopsy procedure is indeed cleavage-stage biopsy. One or two blastomeres are removed from the embryo on day 3. The embryos are preferably in the third mitotic division at that time, presenting at least five cells. More ideal, of course, is a further advanced division of all blastomeres resulting in synchronous eight-cell embryos at about 68–72 hours after microinjection. The essence of the biopsy procedure is a balance between cell retrieval in order to allow a safe and correct diagnosis of the embryo on the one hand, and safeguarding the implantation potential of the biopsied embryo on the other hand.

Human cleavage-stage embryo biopsy would not have been possible without experimental work on animal (mainly mouse) oocytes and embryos in order to develop the different micromanipulation techniques (reviewed by Tarin & Handyside, 1993), and in order to validate their efficacy, safety, and compatibility with development to term in a preclinical phase (Roudebush *et al*., 1990; Takeuchi *et al*., 1992). In 1990, Hardy and colleagues (Hardy *et al*., 1990) showed that despite reduced cellular mass, the ratio of inner cell mass (ICM) to trophectoderm (TE) cells was maintained in both 7/8 and 6/8 biopsied human embryos. The viability of the biopsied embryos was assessed by measuring glucose and pyruvate uptake, showing a lower uptake, but only in proportion to the reduced cellular mass. From these data, the authors concluded that in vitro preimplantation development of biopsied embryos is not adversely affected by the removal of one or two cells. Clinical application of cleavage-stage biopsy resulted in the first pregnancies (Handyside *et al*., 1990) and birth of a normal girl (Handyside *et al*., 1992). This moment paved

the way for an exponential increment in the number of in vitro fertilization (IVF) cycles with embryo biopsy allowing preimplantation genetic diagnosis (PGD) on the embryos (Verlinsky *et al*., 2004; Harper *et al*., 2006; Sermon *et al*., 2007).

Technical aspects: methodology

Zona drilling

Cleavage-stage biopsy of human preimplantation embryos always involves two steps: opening of the zona pellucida (ZP) and subsequent removal of cellular material. Opening of the ZP may be done in three ways: mechanically (partial zona dissection or zona slitting); chemically (using acid Tyrodes solution); or, more recently introduced, by laser technology. Aspiration is the main technique used to remove blastomeres (Harper *et al*., 2008); however, extrusion or flow displacement is also possible (Tarin & Handyside, 1993). Human ZP opening procedures date from the late 1980s and early 1990s, when applied in order to assist human oocyte fertilization or in order to help the hatching process of the embryo. It was a small step toward removal of cellular material from the embryos in order to allow PGD.

Mechanical

Mechanical zona opening for cleavage-stage biopsy is applied clinically, albeit to a much lesser extent than the two other zona opening methods. Partial zona dissection involves making a slit in the ZP by means of a sharp, closed microneedle (illustrated in Selva, 2000). The size of the slit may depend on the distance between the first and second points at which the zona was pierced by the microneedle. Slits of 20–40 μm long (being only 2 μm wide) have been described. They are most suited for polar body biopsy using a smaller aspiration pipette than cleavage-stage biopsy. When a second slit perpendicular to the first one is created, a larger cross-shaped or V-shaped opening can be obtained (Cieslak *et al*., 1999), suitable for blastomere aspiration pipette entering. The mechanical method seems safe and simple, and may have the advantage that the embryo remains protected until expansion (because the zona flap created as such closes after aspiration pipette removal). This is in contradiction to the chemical zona opening procedure, where an actual hole is created.

Acid Tyrodes

Chemical zona drilling using acid Tyrode's solution (pH 2.3) still remains the most widely used approach in the number of cycles with embryo biopsy (Harper *et al*., 2008), probably because it represents a much cheaper option compared with the more sophisticated laser equipment. Chemical zona drilling creates a larger, rounder hole in the ZP whose size is not always easy to control. Whereas the outer layer of the zona is easily dissolved, the inner layer may vary greatly in its susceptibility to dissolution by acid Tyrodes solution. When the inner layer is refractory to drilling, blastomeres may undergo cell lysis. The target site for drilling therefore is chosen between two blastomeres or in front of anucleate fragments in order to minimize the deleterious effect of the acidic medium on the cells. The rate of dissolution may vary and the time taken to complete this process varies accordingly (between 30 seconds and 2 minutes). Local acidification or even deposition of acid Tyrodes solution in the perivitelline space should be considered when evaluating the safety of this approach. Immediate cell lysis (often lost for diagnosis, see further) or subtle damage to cells may occur as a result of acid exposure, which may interfere with further development or implantation. An intact blastomere rate after biopsy of up to 99 percent may be obtained in experienced, skilled hands (Joris *et al*., 2003; Harper *et al*., 2008).

Laser

The opening of the ZP by laser technology has more recently been introduced in clinical embryo biopsy practice. The use of a 1.48-micron diode noncontact laser in order to open the zona has been shown to be compatible with pregnancy (Boada *et al*., 1998). Several studies compared the laser technology with acid Tyrodes solution for zona opening (Phophong *et al*., 2001, mouse embryos; Joris *et al*., 2003, human embryos; Chang *et al*., 2004, laser biopsy versus control human embryos without biopsy; Chatzimeletiou *et al*., 2005, human embryos; Jones *et al*., 2006, sibling human embryos), showing that laser represents a suitable alternative, being quick, easy and safe, resulting in more intact blastomeres, similar post-biopsy development and similar pregnancy and implantation rates. The laser beam is tangentially guided to the ZP of the embryo. The hole size may be chosen precisely by varying the irradiation time. Typically, a trench-like hole with a diameter of 5–10 μm is produced in 10–15 ms (Germond *et al*., 1996). Larger hole diameters are obtained by increasing the irradiation time. It is important to perforate the zona completely without harming the embryonic cells with the laser shot. Using

infrared non-contact laser at a safe working distance does not cause adverse immediate or longer-term effects on the development of human biopsied embryos (Chatzimeletiou *et al.*, 2005). The holes obtained with laser are more precise than the ones obtained with acid Tyrodes solution.

Cleavage-stage biopsy has been described with all three zona opening approaches; however, the majority of clinical biopsies at the cleavage stage use chemical zona drilling (Harper *et al.*, 2008). It is likely that laser technology will supersede chemical zona drilling. Certainly less detrimental, extremely accurate, and simple in use, a laser system represents quite expensive equipment. However, when the set-up has been established and experience gained, it should take only a few seconds to perform drills in a given population of embryos.

Cell removal

Once the ZP has been opened, we have free access to the blastomeres inside. Several methods have been described in order to remove cells. Aspiration is most widely used, whereas clinical application of extrusion and flow displacement is more limited. For completeness, three other methods are mentioned below; however, these techniques seem less applicable for clinical PGD.

Aspiration

The removal of human blastomeres for clinical PGD, irrespective of the zona opening procedure, is done mainly by aspiration, using an aspiration pipette with an inner diameter of 35–40 μm (illustrated in Gianaroli, 2000). The biopsy pipette is introduced into the perivitelline space through the hole in the zona in order to reach a blastomere. Close location of the blastomeres with respect to the opening allows limited penetration of the microtool. One or two blastomeres are removed by gentle aspiration. Cells may be aspirated completely and then removed, or, alternatively, cells are only partially aspirated and pulled out (given full decompaction and thus no adherence to other blastomeres, see further). See Figure 10.1 and Figure 10.2.

Extrusion

After ZP drilling, the blastomere(s) is extruded through the aperture by pushing against the zona at another site (usually at 90 degrees to the aperture) using a blunt pipette. Acid drilling and extrusion has been described for gender determination of biopsied blastomeres

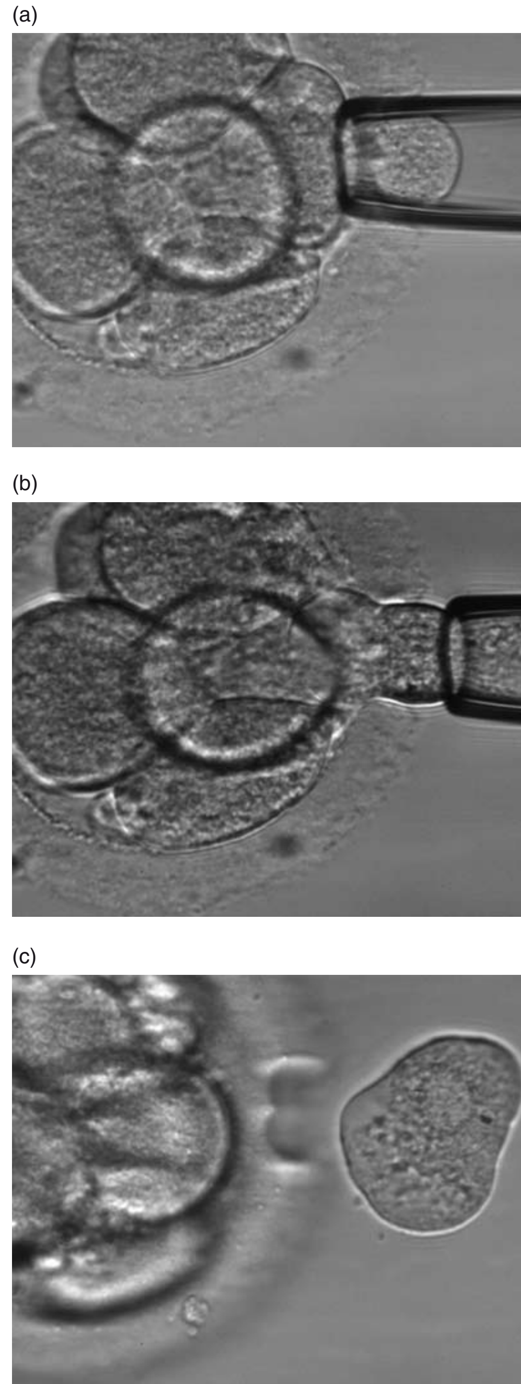

Figure 10.1 One-cell biopsy: (a) laser technology was used to open the zona pellucida (ZP) in front of the single blastomere aimed to be biopsied, limited penetration by the aspiration pipette allows aspiration of the blastomere; (b) the blastomere is partially aspirated and then pulled out; (c) a single nucleus is clearly visible in the biopsied blastomere.

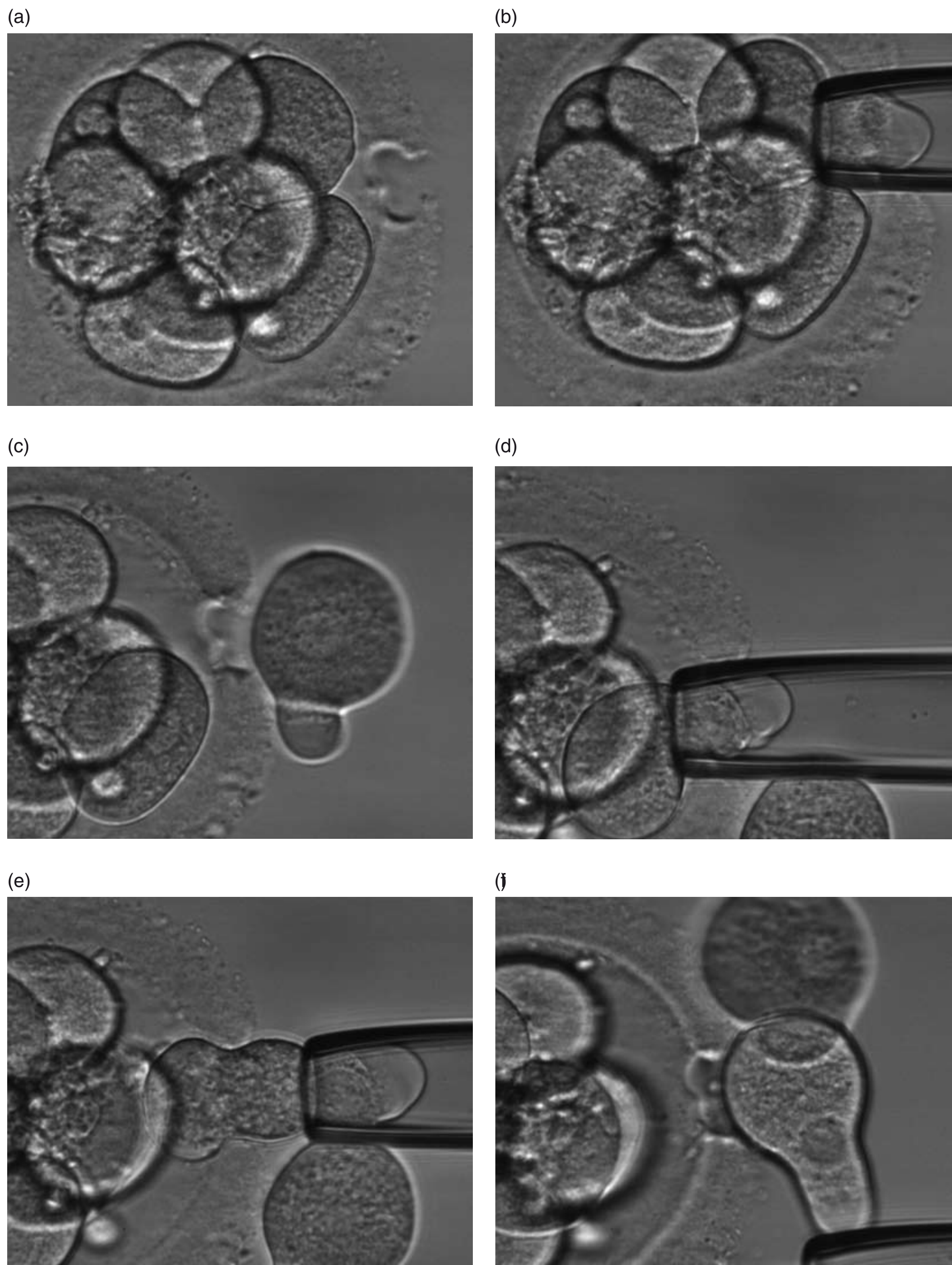

Figure 10.2 Two-cell biopsy: (a) in the case of two-cell biopsy, laser zona opening is done in between two blastomeres; (b) a first blastomere is aspirated into the aspiration pipette and pulled out; (c) a single nucleus is visible in the first blastomere; (d) a second blastomere is aspirated and (e) pulled out; (f) the second blastomere also contains a single nucleus.

in clinical PGD (Levinson *et al*., 1992). Blastomere removal by extrusion is applied clinically albeit to a much lesser extent than blastomere aspiration.

Flow displacement

Roudebush *et al*. (1990) biopsied four- to eight-cell mouse embryos by introducing a slit in the zona with a sharpened needle and, through a second puncture site, injecting medium to dislodge the blastomere through the first puncture site. It was noted that, using this method, it was easier to biopsy eight-cell embryos than four-cell embryos, probably due to blastomere size. This method requires the formation of two separate holes and considerable skill to displace the blastomere of choice. This technique has been used in clinical PGD for the detection of chromosome abnormalities (Pierce *et al*., 1997). However, the routine clinical application of this technique is limited.

Single-needle biopsy

Single-needle biopsy, using the same pipette for acid drilling and blastomere aspiration, has been described to be more efficient (shorter biopsy time per embryo) and equally efficacious as compared with the conventional method using separate pipettes for the two functions, intact blastomere rates being 97 percent and 93 percent, respectively (Chen *et al*., 1998). However, initial clinical application showed a blastomere lysis rate as high as 11 percent (Inzunza *et al*., 1998). Therefore, in order to completely avoid acidic exposure of cells, using separate pipettes for drilling and aspiration (double holder set-up) seems more appropriate.

Stitch and pull

Blastomeres may be removed from cleavage-stage embryos by using stitching movements with a finely sharpened glass needle. The zona is thinned or drilled prior to blastomere removal. As the sharpened needle penetrates the zona, cells are impaled and drawn through the aperture (Muggleton-Harris *et al*., 1995). This method may increase the risk of cell lysis (see later), resulting in lower accuracy and reliability.

Puncture and aspiration

Using a mouse model, Wilton *et al*. (1989) stabilized the embryo with a finely polished blunt pipette with gentle suction and used a beveled micropipette to puncture the zona and aspirate a single cell from four-cell embryos. Ninety-eight percent of controls and 94 percent of the biopsied embryos reached the blastocyst stage and further survived cryopreservation. However, owing to the thickness of the ZP in humans, this technique may not be applicable .

Compaction

Compaction in the mammalian preimplantation embryo is an essential event that leads to the formation of the trophectoderm, the inner cell mass and the blastocele. Whereas full compaction does not occur before the 16–32-cell stage, followed by immediate cavitation and blastocele expansion, relatively early assembly of tight junctions does occur between human blastomeres, as shown by ultrastructural studies (Tesarik, 1989; Dale *et al*., 1991). The presence of a variety of cell adhesion molecules on the preimplantation human embryo has been described (Campbell *et al*., 1995). These membrane adhesion molecules may render the biopsy procedure at the seven- to eight-cell stage rather difficult to perform because the blastomeres show a strong tendency to adhere to each other. Ca^{2+} /Mg^{2+}-free medium has been used to loosen the membrane adhesions between blastomeres (Santaló *et al*., 1996; Dumoulin *et al*., 1998), which allows an easier removal of cells, and results in less blastomere lysis and a shorter procedure time. Embryo biopsy may either be performed completely in this medium or otherwise – in order to limit the exposure time – embryos can be just preincubated for 5–10 minutes (normally sufficient for full decompaction) prior to biopsy in Ca^{2+} /Mg^{2+}-free medium. In clinical PGD practice, some groups prefer to perform the biopsy procedure in Ca^{2+} -containing medium, while others recommend the use of Ca^{2+} /Mg^{2+}-free medium. Because randomized controlled comparisons are not available, it remains unknown whether or not the implantation rate and post-implantation development are affected by the use of Ca^{2+} /Mg^{2+}-free medium for biopsy and the long-term safety of its clinical application should therefore be further substantiated.

Diagnostic and strategic considerations

Embryos for PGD by means of PCR are ideally obtained by micro-injection of a single sperm cell in order to avoid contamination with naked sperm DNA. In cases where diagnosis is carried out using FISH, routine IVF seems acceptable (Thornhill *et al*., 2005). At the moment of oocyte denudation, good care is taken to remove all the remaining cumulus and corona cells, which can also represent a source of maternal DNA contamination (which is important in FISH and PCR diagnosis). Normally developing, good-quality embryos

reach the four- and eight-cell stage, respectively, on day 2 and in the morning of day 3 post-insemination or post-micro-injection. Unfortunately, however, not all human embryos reach the seven- or eight-cell stage on the morning of day 3. Both slow (<7 cells) and fast (>9 cells) cleavage has a significant negative association with normal blastocyst formation (Alikani *et al.*, 2000; Boostanfar *et al.*, 2001; Langley *et al.*, 2001). Despite this knowledge, these slow embryos (six-cell embryos, occasionally four- and five-cell embryos for one-cell removal) as well as fast embryos are indeed included for embryo biopsy in order to optimize the chance for transfer, which is anyway compromised by the fact that genetically non-transferable embryos will be encountered, irrespective of their developmental potential.

The worldwide application of human embryo biopsy is based on the publication of Hardy *et al.* (1990), showing that the development of human embryos to the blastocyst stage in vitro is unaffected by removal of one or two cells at the eight-cell stage, which means that up to one-quarter of the embryo may be removed without impairment of its further in vitro development. An acceptable ongoing pregnancy rate and implantation rate have been reported after two-cell removal from ≥7-cell embryos (Van de Velde *et al.*, 2000).

Number of cells to biopsy

A long-lasting and endless debate, however, exists whether one or two blastomeres should be removed from the embryo. The option to be taken is a delicate balance between a safe, i.e. accurate and efficient diagnosis, on the one hand, and a non-impaired future development of the embryo on the other. Affected pregnancies should be avoided at any price. Multiplex PCR and multiple chromosome FISH analysis represent valuable safety measures (Lewis *et al.*, 2001; Wells, 2004) to ensure an accurate diagnosis on one cell. However, it should be kept in mind that the accuracy and interpretation of certain types of analysis (e.g. if no multiplex PCR is available or translocations) will benefit from, or will even continuously necessitate, having two cells available for diagnosis (Simopoulou *et al.*, 2003; Emiliani *et al.*, 2004). Mosaicism in two- to eight-cell embryos remains a serious obstacle for chromosomal aneuploidy testing, which goes far beyond the discussion of whether to remove only one or two blastomeres. Removal of one cell does not interrogate mosaicism, thus more normal results are encountered, albeit with a lower predictive value. Two-cell analysis results in fewer embryos for transfer but with a higher predictive value. The risk of aggravation of mosaicism is greater in the case of a two-cell than a one-cell biopsy (Los *et al.*, 2004). No matter the strategy followed, the notion holds true that the eight-cell stage does not seem to be the most suitable level for preimplantation genetic screening (PGS) because the highest rates of abnormal and mosaic embryos are present at this stage. The poor representation of a one- or two-cell biopsy for the seven- or six-cell post-biopsy embryo in case of mosaicism leads to a paradoxical effect of an inverse relationship between the developmental prospects of these embryos and their chances for transfer (Los *et al.*, 2004).

As for the adverse effects of the biopsy procedure on the future development of the biopsied embryos, some authors have postulated that the removal of two cells is more harmful than extracting only one cell (Cohen *et al.*, 2007), based on the assumption that cell loss from biopsy may be compared with cell loss after thaw of frozen cleaved embryos. The implantation potential becomes a function of cell loss (Cohen *et al.*, 2007). However, the intrinsic quality of the original embryo does play a role as well. Indeed, embryological data from a prospective randomized controlled comparison removing one or two cells from a clinically representative cohort of cleavage-stage embryos (Goossens *et al.*, 2008), show a strong correlation between the embryonic developmental stage on day 3 and the post-biopsy in-vitro development assessed on day 5 ($p<0.0001$). The influence of the intervention (one-cell or two-cell biopsy day 3) on day 5 development was also significant, but less strong ($p<0.007$). Removal of two blastomeres significantly decreases the likelihood of blastocyst formation compared with removal of one blastomere although this did not translate into a significant decrease in implantation rate (23.5 percent versus 17.3 percent for one-cell and two-cell biopsy, respectively, $p=0.216$). Taking live births per started cycle as an endpoint of this study (including 592 biopsy cycles), no significant difference was obtained between one-cell (20.2 percent) and two-cell biopsy (17.2 percent, $p=0.358$). Because the data show that for every 33 cycles there will be one less live birth delivery following removal of two blastomeres, the removal of only one blastomere is recommended, provided that diagnostic safety measures are in place to ensure a correct diagnosis. If these are not available, we need two cells for diagnosis.

Successful cleavage-stage biopsy involves two aspects. First, the cellular material should be suitable for genetic analysis either by FISH or by PCR. This

means that the cell should be intact and it should contain a single, clearly visible nucleus. Second, further development must not be impaired as a result of the biopsy procedure.

Cell lysis

Although presenting a good quality control measure for the biopsy procedure, blastomere lysis rates (expressed per aspirated blastomere) are hard to find in the literature (Joris *et al.*, 2003). Cell lysis may occur at either step of the procedure: at the moment of zona opening (related to the method used) or when aspirating the blastomeres (related to excessive mechanical stress or to strong adherence of the blastomeres to each other) (De Vos & Van Steirteghem, 2001). Cell lysis most often means the loss of the nuclear material for diagnosis (only occasionally can the nucleus be recovered for FISH analysis). Lysed cells are not used for PCR analysis, because contamination with maternal DNA material cannot be excluded (Sermon *et al.*, 2001). Inevitably, one extra blastomere needs to be removed from the embryo. In cases where cell lysis occurs, it is advised that the aspiration pipette be changed before continuing biopsy of other embryos as a safety measure in order to avoid cross-contamination (especially when PCR is used; however, this measure is also adopted in cases of FISH analysis).

Multinucleation

Multinucleation is a frequently observed phenomenon in cleavage-stage embryos (Van Royen *et al.*, 2003). It may occur as early as the two-cell stage (Staessen & Van Steirteghem, 1998), but has also been observed on day 2 and day 3 of preimplantation development (Pickering *et al.*, 1995; Yakin *et al.*, 2005). Three different mechanisms are held responsible: karyokinesis without cytokinesis (resulting in binucleated blastomeres) (Hardy *et al.*, 1993); partial fragmentation of nuclei (resulting in multinucleated or micronucleated blastomeres); and defective migration of chromosomes at mitotic anaphase (Pickering *et al.*, 1995). In light of these varied mechanisms of formation, it is unlikely that all multinucleated blastomeres will be simply polyploid (Pickering *et al.*, 1995). The knowledge that the chromosome constitution of multinucleated blastomeres is frequently different from that of their sibling blastomeres, casting serious doubt on their representativeness for the entire embryo, makes them unsuitable for PGD (Munné & Cohen, 1993). Of course, the proportion of multinucleated blastomeres within one embryo is an important parameter. Embryos presenting more than 50 percent of their blastomeres with multinucleation are not considered for embryo biopsy, irrespective of the developmental stage at first detection. The presence of one or more multinucleated blastomeres (≤50 percent) has been associated with impaired cleavage and increased fragmentation (Van Royen *et al.*, 2003) and a poor prognosis for blastocyst formation (Yakin *et al.*, 2005), and compromises the ongoing implantation rate (Van Royen *et al.*, 2003), most probably due to chromosomal abnormalities (Kligman *et al.*, 1996). Excluding these embryos for transfer (if other mononucleated embryos are available) seems prudent practice.

Anucleates

The incidence of anucleate blastomeres is also high in normally fertilized embryos, however, especially in those of poor morphology (Hardy *et al.*, 1993; Johansson *et al.*, 2003). Efficient PGD requires a careful assessment of the nuclear status by light microscopy, in order to exclude both multinucleated and anucleate cells from diagnosis (Liu *et al.*, 1993; Cui & Matthews, 1996). Sometimes extreme granularity of the blastomeres may complicate this assessment. A mistaken interpretation of the nuclear status of a blastomere may then be anticipated if a FISH procedure follows (because it would be noticed at fixation): one extra blastomere can be removed. A PCR procedure lacks this intermediate stage because cells are immediately transferred to the PCR tubes and the amplification result is only available later.

Post-biopsy development

Further embryo development should not be impaired as a result of the biopsy procedure. Embryo post-biopsy development can be evaluated on day 4, where a doubling of cells and/or signs of compaction represents a good evolution. Or otherwise, extending the culture toward day 5 allows us to evaluate the quality of inner cell mass and trophectoderm. Data on post-biopsy blastocyst formation are scarce. In 1999, Veiga *et al.* reported a blastocyst rate of 39.3 percent when culturing embryos not used for replacement in sequential media (three PGD cases). Similar results were obtained in 15 couples at risk for beta-thalassemia major: 40/109 (37 percent) embryos biopsied on day 3 developed to blastocysts by day 5 (Palmer *et al.*, 2002). Embryo development characteristics may, however, be related to the genetic condition being tested for (Findikli *et al.*,

2003; Rubio *et al.*, 2003). Two more papers confirm that multiple micromanipulations do not affect embryo development to the morula stage (Magli *et al.*, 2004) or the blastocyst stage (Cieslak-Janzen *et al.*, 2006). In our own paper we report 51.6 percent and 47.0 percent overall blastocyst formation after one- or two-cell biopsy (Goossens *et al.*, 2008).

In addition to the in vitro development, further in vivo development post-biopsy may be reflected by the implantation rates obtained post-PGD. However, since the diagnostic tests are done to help couples to conceive a healthy child, live birth rates per cycle should be the main endpoint relevant to patients, rather than implantation rates per embryo transferred. Large data collections are very much valued to serve as a standard reference of good laboratory and clinical practice: live birth rates per cycle of 15.9 percent (Verlinsky *et al.*, 2004), 17.2 percent (Harper *et al.*, 2006), and 12.5 percent (Sermon *et al.*, 2007) have been reported.

Cryopreservation

After PGD, few embryos usually remain for cryopreservation. The limited numbers of biopsied embryos that have been frozen, show a disappointingly low survival rate (Joris *et al.*, 1999; Magli *et al.*, 1999; Lee & Munné, 2000), resulting in few clinical pregnancies. The increased sensitivity of biopsied embryos may be circumvented by modification of the standard cryopreservation protocol (Jericho *et al.*, 2003; Stachecki *et al.*, 2005). Jericho *et al.* (2003) noted that 75 percent of biopsied embryos survived cryopreservation with ≥50 percent of their blastomeres intact. Six fetal heart-beats have been detected to date following replacement (out of 41 thaw cycles resulting in 36 transfer procedures in which a total of 50 embryos have been transferred). More recent data indicate that vitrification increases the survival rate of human biopsied embryos above standard and modified cryopreservation methods (Zheng *et al.*, 2005). These encouraging results were obtained in non-transferable biopsied embryos; however, reports on clinical application are still lacking. As an alternative to cryopreservation of biopsied embryos at the cleavage stage, embryos diagnosed as normal after PGD can be grown to the blastocyst stage and then cryopreserved (Magli *et al.*, 2006). In 34 thawing cycles, transfer was possible for 18 patients, resulting in six healthy infants born.

Conclusion

Cleavage-stage embryo biopsy on day 3 represents the main approach to obtain cellular material for genetic testing. The main reasons for this are that the paternal contribution to the embryo can be tested and that enough time for testing is available. It is important to obtain intact, single nucleated cells for diagnosis. One-cell biopsy is recommended because it is less invasive to the embryo, provided that diagnostic safety measures are in place to ensure a correct diagnosis. In the absence of these safety measures (e.g. PGD for translocations, no multiplex PCR available), it is advisable to use two blastomeres for diagnosis. Post-biopsy embryo development may be critically evaluated when culturing to the blastocyst stage. Extended culture may serve as an additional selection for the best embryo if several genetically transferable embryos are available. Live birth rate per started cycle represents the main clinical outcome relevant to patients undergoing genetic testing of their embryos. Owing to the valuable nature of embryos diagnosed as genetically transferable, further efforts are needed to improve the cryopreservation results.

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Chapter 11

Blastocyst biopsy

Monica Parriego, Francesca Vidal, and Anna Veiga

Key points

- Blastocyst biopsy for preimplantation genetic diagnosis (PGD) offers a number of advantages, including a higher reliability of the established diagnosis by the analysis of a higher number of cells
- The use of laser, already described in the 1990s, has been demonstrated to be the method of choice for biopsy.
- It involves the removal and subsequent analysis of trophectoderm cells.
- This is especially interesting in the case of PGD for monogenic diseases as more DNA is made available per embryo by blastocyst biopsy.
- It may be possible that certain chromosomal abnormalities are limited to trophectodermal cells Certain anomalies can be confined to extra-embryonic tissues such as the placenta, chorion, or amnios, later during pregnancy
- Blastocyst biopsy has proved to be a successful tool in PGD provided that technical problems related to the methodology are solved
- A high blastocyst rate through an optimized culture system is essential
- The double selection performed by the genetic diagnosis and the culture to the blastocyst stage result in high pregnancy and implantation rates

Introduction

At present, most centers that offer preimplantation genetic diagnosis (PGD) take the biopsies from oocytes or zygotes (polar body biopsy, see Chapter 9), or from early embryos on the third day of development (blastomere biopsy, see Chapter 10). Indeed, the latest data published by the European Society of Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium (Harper *et al.*, 2008) reveal that embryo biopsy was carried out in these developmental stages in 99.8 percent of the cycles performed by the different groups (6.4 percent polar body biopsy and 93.4 percent blastomere biopsy on day 3). There is, however, a third way of obtaining material in PGD cycles: trophectoderm (TE) cells from blastocysts that offer a later-stage option for embryo biopsy (day 5 or day 6). Despite this, blastocyst biopsy was only performed in 0.2 percent of the cycles included in the ESHRE PGD Consortium data collection VII (Harper *et al.*, 2008).

The use of blastocyst TE cells as a source of biopsy material was first suggested over a decade ago (Dokras *et al.*, 1990; Carson *et al.*, 1993), the main aim being to obtain a greater number of cells for diagnosis and thus overcome the difficulties associated with the analysis of single cells and increase reliability while minimizing the risk of diagnostic error. This aspect is particularly important in those cases where diagnosis is based on application of the polymerase chain reaction (PCR – see Chapter 13), which is especially prone to problems derived from having little material to analyze. The amplification failures and allele drop-out (ADO) observed after analysis of a single cell (whether blastomeres or polar bodies) mean that a considerable percentage of embryos remain undiagnosed in PCR–PGD cycles. Furthermore, the biopsy of more cells should enable us to identify various pathologies for diagnosis, or various diagnoses to be combined.

Despite its advantages, blastocyst biopsy has only recently been incorporated into clinical practice (de Boer *et al.*, 2004; Kokkali *et al.*, 2005) and has been closely linked to the use of laser technology (Veiga *et al.*, 1997). The main benefits of using laser technology in the process are speed, accuracy, and the non-acidification of the medium, thus facilitating the biopsy procedure (Boada *et al.*, 1998). Furthermore, its use during blastocyst biopsy is essential in order to

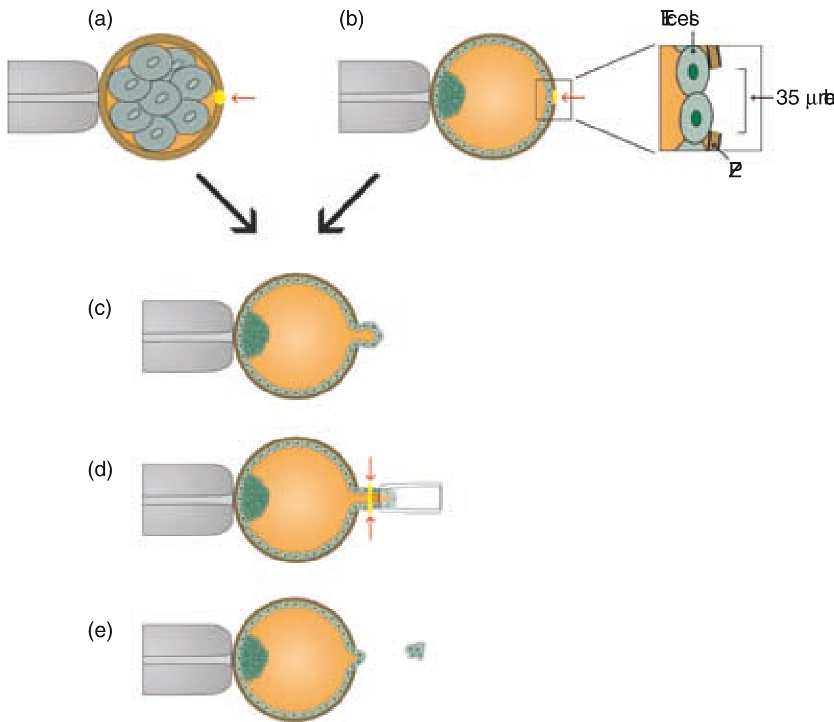

Figure 11.1 Diagram showing the two approaches for blastocyst biopsy: (a) zona pellucida (ZP) opening on a day 3 embryo; (b) ZP opening on a blastocyst; (c) herniation of trophectoderm (TE) cells; (d) biopsy of the herniated cells; (e) biopsied cells. Red arrows indicate the laser shots.

achieve a rapid, easy, and efficient detachment of the cells to be biopsied.

A sufficient number of available blastocysts are needed to increase diagnostic and transfer possibilities when carrying out a PGD cycle with trophectoderm biopsy. Improved culture media (sequential culture), which have led to an increased blastocyst rate after embryo culture, is another parameter that has enabled the clinical application of this type of diagnosis.

The present chapter will discuss current methodology and the advantages and limitations of blastocyst biopsy.

Technical aspects: methodology

The first phase of blastocyst biopsy involves making a hole in the zona pellucida (ZP). The dissection of the ZP may be performed mechanically, by application of acid Tyrodes solution (chemical method), or through the use of laser technology (Tarin & Handyside, 1993). However, all the groups currently performing blastocyst biopsy use the laser method. Thus, this is the procedure that will be described in detail below. The ZP is opened using a near-infrared (NIR) 1.48-μm diode non-contact laser. The exposure time and pulse intensity used must be adjusted to ensure that the hole made in the ZP is of adequate size (10–30 μm), and this may vary in accordance with the thickness of the ZP (Veiga *et al.*, 1997; McArthur *et al.*, 2005).

With respect to the timing, two approaches have successfully been used in clinical practice (Figure 11.1). In the first, the hole is made in early embryos on the third day of development (or occasionally on the fourth day, at the morula stage) (McArthur *et al.*, 2005) (Figure 11.1(a)). The aim of making the hole at this stage is to minimize the risk of damaging the cells adjacent to it, as at the blastocyst stage the perivitelline space is almost nonexistent. In this methodological approach, the ZP of all embryos available on day 3 is drilled. The embryos are then kept in culture until they reach the blastocyst stage. At this point the cells closest to the hole tend to extrude and the herniated cells are then biopsied (it is hoped these are TE cells). Only those embryos which reach the blastocyst stage are biopsied.

The other possibility consists of making the hole at the blastocyst stage, that is, on days 5 or 6 of embryo development (Figure 11.1(b)). The area chosen for ZP drilling must be directly opposite the inner cell mass (ICM) (Figure 11.2). After a few (4–10) hours of culture and the subsequent herniation of a few TE cells, the biopsy may be performed.

In both cases, the biopsies are obtained by securing the blastocyst with a holding pipette (internal diameter, 20–30 μm), with the herniated cells located at the 3 o'clock position (see Figure 11.1 (c)). The TE cells (two to nine cells) are gently aspirated with a biopsy

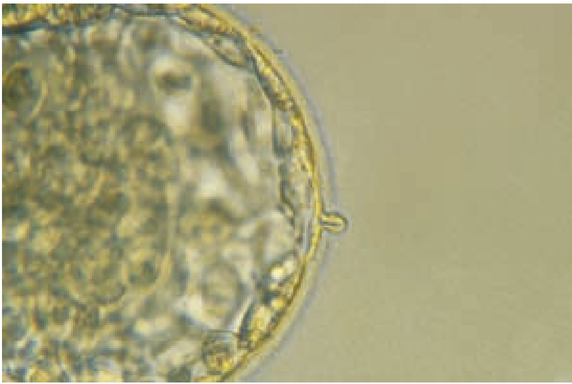

Figure 11.2 Detail of zona pellucida (ZP) drilling on an expanded blastocyst.

pipette (internal diameter 30 μm) and three to five laser pulses are then applied in order to detach them from the blastocyst (see Figure 11.1 (d)). The cells obtained are finally ejected from the biopsy pipette and are ready to be processed (see Figure 11.1 (e)).

Although only a small number of reports have been published to date, the groups applying this technique describe very high efficiency rates: all the blastocysts processed were successfully biopsied and provided material for diagnosis; the viability of the blastocysts after biospy was also maintained and the blastocelic cavity was restored the day after biopsy (McArthur *et al*., 2005; Kokkali *et al*., 2007).

Diagnostic and strategic considerations

Extended culture

As already mentioned, one of the reasons why blastocyst biopsy has not become a routine procedure in PGD is that with current embryo culture techniques only a limited number of embryos reach the blastocyst stage. Recent advances have been made in understanding the metabolic requirements of embryos at different stages of development (Leese *et al*., 1991), and these have led to improvements in culture media (sequential culture) (Gardner & Lane, 1998; Gardner *et al*., 1998a; Gardner *et al*., 1998b; Ménézo *et al*., 1999; Gardner *et al*., 2000). However, the blastocyst rates obtained in vitro remain relatively low, with the number of available embryos that reach this stage being only around half the total number of zygotes.

It therefore seems logical to assume that when biopsy is performed at the blastocyst stage, there will be a higher percentage of PGD cycles in which there will be no embryos suitable for biopsy. Although there are limited data in this regard, McArthur *et al.* (2005) report that in approximately 20 percent of cycles there are no embryos reaching the blastocyst stage and, therefore, PGD cannot be performed. This percentage is much higher than the one reported for early embryos (Sermon *et al*., 2007). However, it has been shown that a considerable percentage of embryos that fail to reach this stage are chromosomally abnormal (Sandalinas *et al*., 2001; Clouston *et al*., 2002), and these abnormalities are likely to be largely responsible for the failure to progress to the blastocyst stage.

We would expect the percentage of transferable embryos to be higher when biopsy is performed after extended culture (selection through blastocyst culture), although studies comparing results after biopsy on day 3 and day 5 provide no data in this regard. This should be particularly evident in cases where PGD is used to screen for aneuploidies. However, the total number of embryos suitable for transfer after blastocyst biopsy continues to be lower owing to the lower number of embryos which may be biopsied. Nevertheless, this difference appears to be compensated for by the greater potential of these embryos once transferred (McArthur *et al*., 2005; Kokkali *et al*., 2007), thus enabling a reduction in the number of embryos to be transferred without reducing the pregnancy rate (McArthur *et al*., 2005), which in turn diminishes the rate of multiple pregnancies.

Type of cells for analysis

In addition to making available a higher number of cells for diagnosis, another advantage attributed to blastocyst biopsy relates to the type of cells used for the analysis (Figure 11.3).

The TE cells used as a source of biopsy material give rise to extra-embryonic tissues while the fetus is originated by the ICM. Therefore, and as it occurs with polar body PGD, the biopsy does not lead to a reduction in total embryo mass, the ICM remaining intact. The diminished implantation potential which results from the reduction of embryo mass constitutes a weak point of early embryo biopsies. Although this reduction may be compensated for by the benefits derived from the transfer of embryos regarded as normal or healthy after PGD, it nevertheless remains an undesirable collateral effect. Although published data are controversial, in a recent report Cohen and coworkers (2007) estimate that, given a theoretical implantation potential of 20 percent for a nonbiopsed embryo, the implantation potential after a single- or two-cell cell biopsy would be reduced to 17.0 percent and 14.0 percent, respectively.

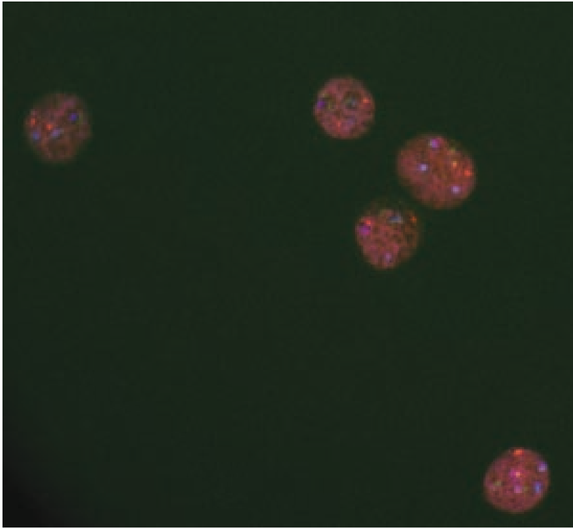

Figure 11.3 Fluorescence *in situ* hybridization (FISH) on trophectoderm (TE) cells.

The biopsy of TE cells enables the detection of anomalies of maternal and paternal origin, as well as fertilization and post-zygotic abnormalities as in the biopsy of early embryos.

The mosaicism observed in embryos obtained through in vitro fertilization (IVF) is the most common chromosomal alteration in early embryos from women aged ≤40 (Márquez *et al.*, 2000). This phenomenon, which is attributed to mitotic errors, is one of the main limitations of PGD as it may mean that an analyzed cell may not be representative of the embryo and thus lead to a diagnostic error. Various published studies that have analyzed the chromosomal content of blastocysts have illustrated that a very high proportion of these (~90 percent) show the coexistence of different cell lines (Veiga *et al.*, 1999; Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a, 2002b; Derhaag *et al.*, 2003; Bielanska *et al.*, 2005), although at this stage the percentage of abnormal cells observed (<30 percent) is lower than in early stages (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a). Although aberrant cell lines are commonly found in blastocysts, data from chorion biopsies, miscarriages, and newborns reveal that their presence is drastically reduced in subsequent stages (Hassold *et al.*, 1980; Hassold & Jacobs, 1984; Association of Clinical Cytogeneticists, 1994). The preferential location of these aneuploid cells in the TE has been suggested as a possible selection mechanism against these cell lines. If this is the case, employing TE cells for PGD would not be useful as they might not be representative of the chromosomal content of the embryo itself. However, these differences in the percentages of normal and abnormal cells between the TE and ICM have not been confirmed (Evsikov & Verlinsky, 1998; Magli *et al.*, 2000; Derhaag *et al.*, 2003).

Unlike other types of mosaicism, the presence of polyploid (mainly tetraploid) cells has been more commonly observed at the blastocyst stage (Munné *et al.*, 1994; Delhanty *et al.*, 1997; Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a, 2002b) and seems to be a normal phenomenon at this stage of development, probably related to implantation. The reported percentages of tetraploid cells vary between 23 percent and 74 percent, being less common in better-quality blastocysts (Bielanska *et al.*, 2002a). Unlike in the case of aneuploid cells, it has been observed that polyploid cells tend to be located in the TE, and this fact must be taken into account during PGD.

Confined placental mosaicism (CPM) arises in 1–2 percent of viable pregnancies studied by chorionic villus sampling (CVS) at 9–11 weeks of gestation (Ledbetter *et al.*, 1992; Kalousek, 1994; Jenkins & Wapner, 1999). These cases reveal chromosomal anomalies (generally trisomies) confined exclusively to the placenta. This phenomenon would originate either in an error in one of the embryo's mitotic divisions (normal diploid), or be caused by the rescue of an originally trisomic embryo. The effect of CPM will depend on the percentage of abnormal cells, as well as on the type of anomaly and tissues affected.

Time available for genetic testing

The time available for analysis in cases of PGD based on blastocyst biopsy is reduced to a maximum of 24 hours. The fact that the results of genetic analysis must be obtained in such a limited time means that high levels of coordination are required between all parties involved in the process. Although most diagnostic protocols can be carried out in this timeframe, it nevertheless restricts the possibility of re-analysis or re-hybridization, when necessary, and limits the advantages associated with PGD in blastocysts.

The fact that not all the embryos from the same cohort reach the blastocyst stage synchronously also means that embryo biopsies must be scheduled at different times, thus affecting the processing and genetic analysis of samples. Even if the final number of embryos to be analyzed is low, this aspect makes organization more difficult and greatly increases the workload of the PGD laboratory. As an alternative, Kokkali and coworkers (2007) opt for freezing – without PGD – embryos

Table 11.1 Results of preimplantation genetic diagnosis (PGD) blastocyst biopsy: clinical experience

	McArthur *et al*. **(2005)**	**Kokkali** *et al*. **(2007)**
No. of cycles started	231	10
No. of blastocysts biopsied	1050	53
No. of cycles with no suitable blastocysts for biopsy (%)	48 (21)	0 (0)
No. of embryos with results (%)	974 (93)	50 (94.3)
No. of cycles with embryo transfer (%)	119 (52)	10 (100)
No. of embryos transferred (mean per embryo transfer)	127 (1.1)	21 (2.1)
Implantation rate (%)	41	47.6
Clinical pregnancies per embryo transfer (%)	43	60
No. of frozen embryos	146	5+7

which reach the blastocyst stage later on (day 6), thus retaining them for analysis and use in subsequent cycles. However, this option will only be effective if blastocysts biopsied at day 5, and characterized as normal or healthy and suitable for transfer, are available.

Cryopreservation

The cryopreservation of biopsied embryos continues to be one of the weak points of PGD. The results published by most groups show that embryos with an opening in the ZP are more sensitive to the freezing process, which is reflected in reduced survival and developmental rates among frozen–thawed biopsied embryos (Joris *et al*., 1999; Magli *et al*., 1999). This negative impact of the freezing process on embryos without an intact ZP is observed in both early embryos and blastocysts.

Vitrification as an alternative to classical freezing techniques is being adopted as part of numerous IVF programs due to its simplicity and, above all, the excellent results obtained not only in oocytes but also in early embryos and blastocysts (Vanderzwalmen *et al*., 2002; Zheng *et al*., 2005). The use of this method should thus be regarded as an option for biopsied embryos (Parriego *et al*., 2007).

In order to improve the cumulative pregnancy rates of PGD cycles, and to be able to reduce the number of embryos to be transferred, further optimization of cryopreservation protocols is required to achieve survival and implantation rates that are comparable with those reported for embryos which have not been subjected to PGD.

Clinical results

The clinical experience of PGD with blastocysts is limited, and only series from two different groups have been published. The most experienced is the Australian group (McArthur *et al*., 2005), which has successfully applied this approach in cases of PGD based on PCR and fluorescence *in situ* hybridization (FISH). Kokkali and coworkers (2007) published a pilot study that included just 10 cycles, all with the aim of detecting beta-thalassemia, and using PCR as the diagnostic technique.

The available data show that the mean number of blastocysts biopsied per patient is five (4.5 in the case of McArthur *et al*. (2005) and 5.3 for Kokkali *et al*. (2007)). In all the cases described by Kokkali *et al*. (2007) there were embryos available for biopsy, whereas in 21 percent of the cycles reported by McArthur *et al*. (2005) no embryo reached the blastocyst stage, meaning that PGD could not be performed in these patients. These differences, which are also reflected in the number of cycles without embryos suitable for transfer, could be due to the low number of cycles included in the study by Kokkali *et al*. (2007), and to differences in the type of indication for PGD between the two series. However, the diagnostic efficiencies described in the two studies are similar (93 percent and 94.3 percent) and are clearly higher than those reported in the PCR–PGD literature for blastomere biopsy on day 3 (Harper *et al*., 2008). Furthermore, both groups report high implantation and pregnancy rates following transfer of these embryos. Table 11.1 summarizes the results published by these groups.

Conclusions

The incorporation of blastocyst biopsy into clinical practice may be considered a valid alternative when performing PGD. The fact that it makes more material available for analysis is of particular value in those cases where the aim is to diagnose monogenic diseases. Couples suffering from such diseases constitute a pool of patients who are generally not infertile and thus higher blastocyst rates can be expected. Furthermore, greater diagnostic efficiency is obtained in PCR–PGD cycles after blastocyst biopsy. Embryos transferred at the blastocyst stage are subjected to a dual selection

process (genetic and through culture) and this is reflected in their greater implantation potential, thus enabling a lower number of embryos to be transferred, which in turn reduces the risk of multiple pregnancy. The availability of a greater number of cells opens the possibility of performing multiple diagnoses in parallel on the same embryo; these could be used to detect multigenic diseases or for the combined diagnosis of different disorders through diagnostic approaches based on both FISH and PCR. In the case of PGD cycles for aneuploidy screening (PGS) that rely exclusively on FISH-based diagnosis (repeated IVF failure, severe male factor infertility, and so on), where the diagnostic efficiency in single cells is very high, the possible negative effect of extended embryo culture in these patients would imply less evident benefits of this methodology. Nevertheless, blastocyst biopsy may be considered a “rescue” protocol in the event of a necessary repeated analysis because of diagnostic failure or for confirmation purposes. Although the clinical application of blastocyst biopsy for PGD remains a limited and recent development, the good results in terms of implantation and pregnancy rates obtained so far, as well as the diagnostic possibilities it opens up, suggest that this technique may become more widely used in the early future.

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Chapter 12

Preimplantation genetic diagnosis for chromosome rearrangements

Caroline Mackie Ogilvie and Paul N. Scriven

Key points

- Chromosome rearrangements include Robertsonian and reciprocal translocations (the most common form of chromosome abnormality in humans, present in approximately one in 500 individuals), peri- and para-centric inversions, inter- and intra-chromosomal insertions, deletions, duplications, and complex chromosome rearrangements (CCRs).
- In all cases presenting for preimplantation genetic diagnosis (PGD), the risk of viable abnormality should have been assessed and the couple counseled appropriately, as for many couples carrying chromosome rearrangements, the best chance of a successful pregnancy outcome may be by natural conception.
- A PGD test using commercially available fluorescence *in situ* hybridization (FISH) probes can generally be designed for all Robertsonian translocations, most reciprocal translocations and inversions, and some CCRs. Specific "home-grown" probes may be required for some insertions.
- By far the largest group presenting for PGD are couples where one partner carriers a reciprocal translocation, which in nearly all cases is unique to that family.
- Most groups use combinations of probes for the different chromosome segments involved in the rearrangement in order to assess dosage of these regions in interphase nuclei or polar body preparations.

Introduction

Couples where one partner carries a balanced chromosome rearrangement or other structural chromosome abnormality are at risk of genetically unbalanced conceptions, which may result in failure to implant, early or late miscarriage, or liveborn children with physical and mental disability. The likely outcome of the conception will depend on the extent and severity of the genetic imbalance, which will in turn depend on the specific rearrangement. Testing embryos for genetic imbalance associated with the familial rearrangement will be appropriate where there is a history of liveborn children with genetic imbalance, an unacceptably large number of miscarriages, or where the couple requires in vitro fertilization (IVF) in order to conceive. For couples where there are no fertility or reproductive problems, and who have a "low-risk" rearrangement (see below), spontaneous conception is likely to be the best option.

Chromosome rearrangements include Robertsonian and reciprocal translocations (the most common form of chromosome abnormality in humans, present in approximately one in 500 individuals), peri- and para-centric inversions, inter- and intra-chromosomal insertions, deletions, duplications, and complex chromosome rearrangements (CCRs). A preimplantation genetic diagnosis (PGD) test using commercially available fluorescence *in situ* hybridization (FISH) probes can generally be designed for all Robertsonian translocations, most reciprocal translocations and inversions, and some of the other more rare rearrangements. Specific "home-grown" probes may be required for some insertions. By far the largest group presenting for PGD are couples where one partner carries a reciprocal translocation, which in nearly all cases is unique to that family.

The material used for testing is usually either polar bodies or biopsied cells from cleavage-stage embryos, although it is also possible to use material from the trophoblast of blastocyst-stage embryos. Most groups use combinations of locus-specific probes (sometimes with whole chromosome paints) for the different chromosome segments involved in the rearrangement,

in order to assess copy number of these regions in interphase nuclei or polar body preparations.

Reciprocal translocations

Balanced reciprocal translocations, which are typically an exchange of two terminal segments from different chromosomes, occur in approximately one in 500 live births (Hook & Hamerton, 1977), and are usually associated with a normal phenotype. (In rare cases, *de novo* reciprocal translocations may be associated with submicroscopic imbalance or gene disruption at the breakpoints, leading to congenital abnormalities.) Reciprocal translocations may be associated with infertility in some carriers, while all carriers have reproductive risks owing to abnormal segregation of the translocation chromosomes at meiosis, resulting in sperm or eggs with chromosome imbalance. The reproductive risks at conception and at term for a reciprocal translocation heterozygote are determined by the chromosomes involved, the different modes of segregation at meiosis, which occur with variable frequency, the number and position of chiasmata, and the size and genetic content of the chromosome segments of the products with chromosome imbalance (Ford & Clegg, 1969; Gardner & Sutherland, 2004).

Translocation segment imbalance

Reciprocal translocations may be described in terms of four chromosome segments (see Figure 12.1): two centric segments that contain the centromeres, and two translocated segments comprising the exchanged material; segregation at meiosis may result in gametes with many different permutations of between zero and three copies of each of the four chromosome segments; deviation from two copies of any segment in the zygote results in chromosome imbalance (Scriven *et al.*, 1998). A zygote with translocation chromosome imbalance has monosomy and/or trisomy or tetrasomy for at least two of the translocation segments. For some translocations, very few or none of the conceptuses with chromosome imbalance are compatible with an ongoing pregnancy; for other translocations, a pregnancy may be established that results in miscarriage, stillbirth, or the live birth of a child with significant mental and physical disability, which may result in early death.

Reproductive risk assessment

Couples presenting with reciprocal translocations may require assisted reproduction because they have male or female factor infertility. However, many couples

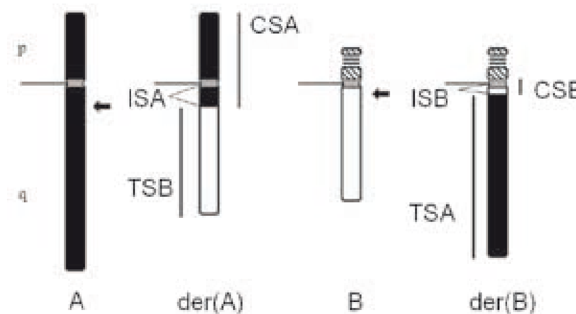

Figure 12.1 Reciprocal translocation segments. CSA(B), centric segment of chromosome A(B); TSA(B), translocated segment of chromosome A(B); ISA(B), interstitial segment of chromosome A(B). An odd number of crossovers in the interstitial segments at meiosis results in recombinant products. All the different segregation products can be expressed in terms of the copy number of the centric and translocated segments (see Table 12.1).

have a reproductive history of recurrent miscarriage, termination of affected pregnancies, or an affected child with profound mental and physical disability. A history of infertility or recurrent pregnancy loss is not necessarily associated with the translocation, and it is often the case that products of conception have not been karyotyped; other potential contributing factors (e.g., antiphospholipid syndrome) should therefore be investigated thoroughly before considering subjecting a fertile woman to assisted reproduction. Similarly, although it is acknowledged that infertility in some male carriers may be attributed to their translocation, it is prudent to investigate other possibilities as appropriate (Stephenson & Sierra, 2006). For a couple presenting with two or three miscarriages, and where a link has not been established with the translocation, and the risk of liveborn offspring with an unbalanced form of the translocation is low, the best chance of a successful outcome may be spontaneous pregnancy with prenatal diagnosis (PND) for reassurance. In a case–control study Franssen *et al.* (2006) showed that in a two-year follow-up period 84 percent of couples are likely to have a successful outcome, although 54 percent had one or more spontaneous abortions. In favor of testing, a prospective study compared with an historic obstetric history of recurrent miscarriage and unsuccessful pregnancies (Otani *et al.*, 2006) argued that testing significantly reduced the risk of miscarriage and increased the chance of a viable pregnancy. Accurate reproductive risk assessment is vitally important in helping couples to decide if PGD is the right way forward. A decision to proceed with PGD should only be taken after all the available reproductive options have been considered.

Gardner and Sutherland (2004) describe an approach to risk assessment which includes empirical single-segment imbalance liveborn data (Stene & Stengel-Rutkowski, 1988; Stengel-Rutkowski *et al.*, 1988; Midro *et al.*, 1992), the predicted mode of segregation most likely to result in liveborn offspring with chromosome imbalance (Jalbert *et al.*, 1980; Jalbert, 1988), family genetic history, and a review of the literature (e.g., *PubMed*) (Borgaonkar, 1997). In addition to estimating the risk of liveborn offspring with chromosome imbalance, a 20–30 percent risk for fetal loss in relation to the general background risk of 15 percent is typically given for most reciprocal translocations (Midro *et al.*, 1992; Gardner & Sutherland, 2004).

Testing for segment imbalance

In PGD clinical practice, one or both polar bodies may be sampled from oocytes, one or two blastomeres may be biopsied from cleavage-stage embryos, or several cells may be sampled from the trophectoderm at the blastocyst stage. Polar body biopsy is only informative for female translocation heterozygotes; however, the other methods sample the zygote and can therefore be used for male or female translocation carriers. The primary technique used for preimplantation genetic testing is FISH. The copy number of the different chromosome segments involved in reciprocal translocations (Table 12.1) is determined by use of target-specific DNA probes labeled with different fluorochromes or haptens. Clinical preimplantation genetic testing has employed chromosome paints and locus-specific probes. Chromosome paints applied to metaphase chromosomes from polar bodies (Munné *et al.*, 1998) can, after blastomere nucleus conversion (Willadsen *et al.*, 1999; Verlinsky *et al.*, 2002a), detect copy number as well as discriminate between normal and heterozygous chromosome complements; the translocation status of the oocyte may thus be inferred. Locus-specific probes applied to interphase nuclei from cleavage-stage blastomeres (Pierce *et al.*, 1998; Munné *et al.*, 2000; Fridstrom *et al.*, 2001; Ogilvie *et al.*, 2001; Simopoulou *et al.*, 2003; Kyu Lim *et al.*, 2004) or cells sampled from the trophectoderm (de Boer *et al.*, 2004) are used primarily to detect copy number of the chromosome segments tested, and will not discriminate between normal and heterozygote chromosome complements unless probes are designed to span the breakpoints (Weier *et al.*, 1999). The development of patient-specific breakpoint-spanning probes is time-consuming and expensive, and is therefore not generally undertaken for routine PGD testing. Commercially available probes specific for subtelomere and centromere regions and directly labeled with different fluorochromes offer the most straightforward approach to PGD for reciprocal translocations (Scriven *et al.*, 1998).

FISH probe selection

Initial selection of potentially informative FISH probes should be made as part of the preliminary assessment as to availability of PGD. Theoretically, in order to maximize sensitivity, every FISH assay would incorporate four probes, one for each centric and translocated segment (see Figure 12.2), and the probe assay would have a hybridization efficiency of 100 percent. However, breakpoints are sometimes distal to available subtelomere probes, unique centromere probes are not available for all the acrocentric chromosomes, and suitable probes are not always available either indirectly labeled with an appropriate hapten, or directly labeled with the necessary fluorochromes. Probe efficiencies are rarely 100 percent, even for a single probe. Incorporating inefficient and possibly unnecessary probes into an assay may therefore significantly reduce the specificity of the PGD test. One probe in each of three out of the four centric and translocated segments is the minimum number of probes required to detect all the theoretical unbalanced forms of a reciprocal translocation (Scriven *et al.*, 1998), and is therefore recommended for most reciprocal translocations. Given the practical limitations discussed above, probe selection should be based primarily on the predicted mode of segregation most likely to result in liveborn offspring with chromosome imbalance, and the segregation mode considered likely to be most frequent.

Adjacent-1 segregation or alternate segregation following an odd number of crossovers in the interstitial segment (see Figure 12.1) results in zygotes with monosomy and trisomy for the translocated segments. Empirically, this is the most frequent reciprocal translocation imbalance found in zygotes (Mackie Ogilvie & Scriven, 2002). For translocations where products of adjacent-1 segregation are likely to lead to embryos with viable segment imbalance, three probes, one for each of the translocated segments and one for one of the centric segments, will give a FISH assay ensuring that only in the case of two signal scoring errors will an embryo with segment imbalance following adjacent-1 segregation be diagnosed as balanced (this requirement for two scoring errors may be described

Table 12.1 Reciprocal translocation segment combinations

Segregation mode	Zygote 2n complement	Segment count			
		TSA	TSB	CSA	CSB
4:0	A,B	1	1	1	1
3:1 Tertiary monosomy	A,der(A),B	1	2	2	1
3:1 Interchange monosomy	A,B,B	1	2	1	2
2:2 Adjacent-2 cross-over in A	A,der(A),der(A),B	1	3	3	1
2:2 Adjacent-1	A,der(A),B,B	1	3	2	2
2:2 Adjacent-2 crossover in B	A,B,B,B	1	3	1	3
3:1 Crossover in A	A,der(A),der(A),B,B	1	4	3	2
3:1 Crossover in B	A,der(A),B,B,B	1	4	2	3
3:1 Interchange monosomy	A,A,B	2	1	2	1
3:1 Tertiary monosomy	A,B,der(B)	2	1	1	2
2:2 Adjacent-2	A,A,der(A),B	2	2	3	1
2:2 Alternate (normal)	A,A,B,B	2	2	2	2
2:2 Alternate (balanced)	A,der(A),B,der(B)	2	2	2	2
2:2 Adjacent-2	A,B,B,der(B)	2	2	1	3
Anaphase II nondisjunction	A,A,der(A),der(A),B	2	3	4	1
3:1 Tertiary trisomy	A,A,der(A),B,B	2	3	3	2
3:1 Crossover in A	A,der(A),der(A),B,der(B)	2	3	3	2
3:1 Interchange trisomy	A,der(A),B,B,der(B)	2	3	2	3
3:1 Crossover in B	A,A,B,B,B	2	3	2	3
Anaphase II nondisjunction	A,B,B,B,der(B)	2	3	1	4
2:2 Adjacent-2 crossover in A	A,A,A,B	3	1	3	1
2:2 Adjacent-1	A,A,B,der(B)	3	1	2	2
2:2 Adjacent-2 crossover in B	A,B,der(B),der(B)	3	1	1	3
Anaphase II nondisjunction	A,A,A,der(A),B	3	2	4	1
3:1 Interchange trisomy	A,A,der(A),B,der(B)	3	2	3	2
3:1 Crossover in A	A,A,A,B,B	3	2	3	2
3:1 Tertiary trisomy	A,A,B,B,der(B)	3	2	2	3
3:1 Crossover in B	A,der(A),B,der(B),der(B)	3	2	2	3
Anaphase II nondisjunction	A,B,B,der(B),der(B)	3	2	1	4
4:0	A,A,der(A),B,B,der(B)	3	3	3	3
3:1 Crossover in A	A,A,A,B,der(B)	4	1	3	2
3:1 Crossover in B	A,A,B,der(B),der(B)	4	1	2	3

as an “internal check”, see Figure 12.2). This therefore provides a robust test with a very low false normal error rate.

Viable chromosome imbalance consistent with 3:1 segregation is usually associated with monosomy or trisomy for the smallest chromosome (Jalbert, 1988). For translocations with a risk of viable 3:1 segregation products, the smallest chromosome usually therefore dictates the choice of centric segment probe, and a second probe on the smallest chromosome will provide two probes for potentially viable imbalance (see above).

Very few reciprocal translocations result in viable offspring with imbalance due to adjacent-2 segregation (monosomy and trisomy for the centric segments); however, where this is a possibility, probes for both centric segments, with a single probe in one of the translocated segments, should be incorporated into the assay.

Available commercial FISH probes include those produced by Abbott (formerly Vysis) and Cytocell. The FISH assays may combine directly labeled and indirectly labeled probes, and probes from different manufacturers. Probes with known polymorphisms, or those known to cross-hybridize significantly with other chromosomes (Knight & Flint, 2000), should be avoided where possible as first-choice probes. It is not always possible to design assays using three probes

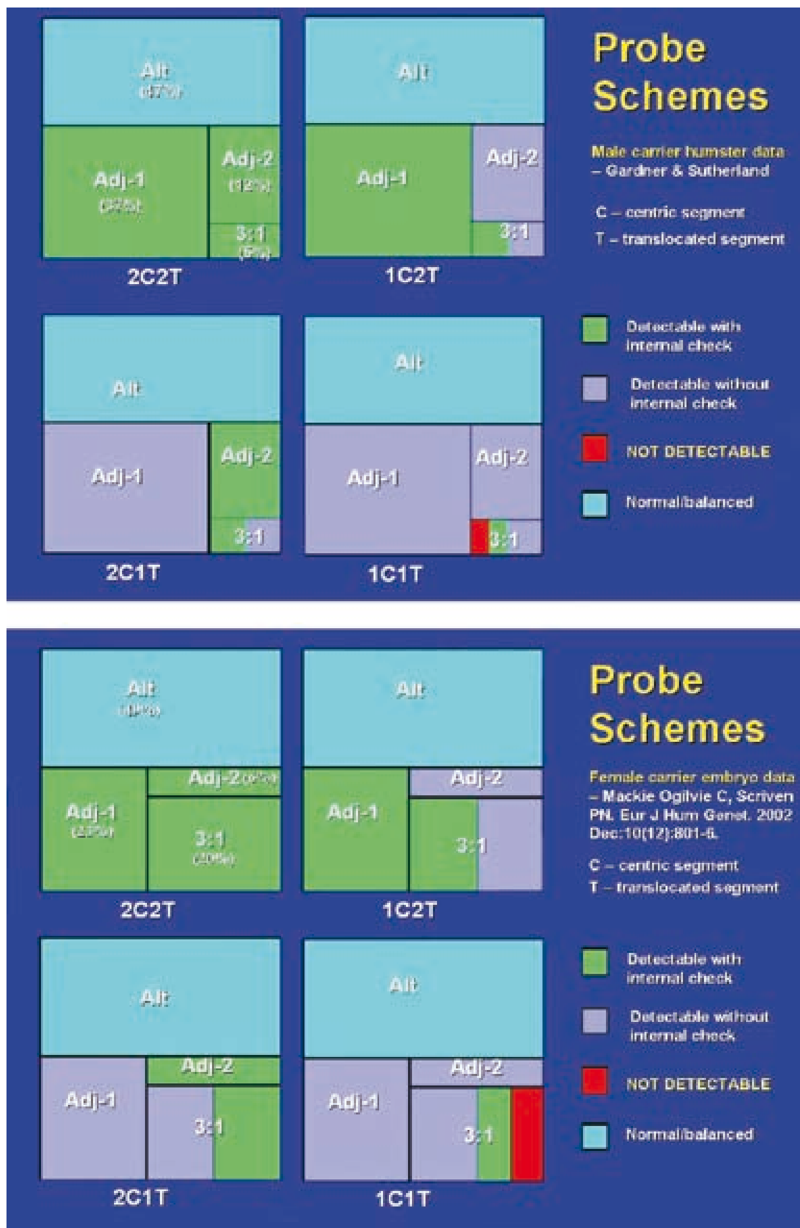

Figure 12.2 Reciprocal translocation probe schemes used in clinical practice. The proportions of segregation products are different for male and female carriers. An ideal probe mix would include a probe for both centric segments and both translocated segments (2C2T); two scoring errors are therefore required to misdiagnose an unbalanced product as normal/balanced; however, suitable probes may not be available. Probes for three out of the four segments are necessary to detect all the unbalanced products. A 1C2T mix, with careful selection of which centric segment, is preferred for single-cell biopsy to ensure two informative probes for the unbalanced products most likely to be frequent and viable; two-cell biopsy is recommended for 2C1T and 1C1T probe schemes. Some 3:1 segregation products are not detectable with a 1C1T probe scheme.

directly labeled with different fluorochromes. Where Abbott CEP probes labeled with SpectrumAqua™ are not available, biotinylated alphasatellite and classical-satellite probes detected with Cy-5 streptavidin (Amersham) and visualized using a FarRed filter provide an alternative strategy. However, other groups choose to mix red and green probes or use a second round of hybridization (Ogur *et al.*, 2002). An elegant solution to the third color problem exploits the observation that Abbott SpectrumOrange probes may be visualized using a TexasRed Filter and a SpectrumGold filter, whilst Cytocell TexasRed-labeled probes may only be seen using the TexasRed filter; probes labeled with these fluorochromes can therefore be used together in an assay (see Figure 12.3).

Lymphocyte FISH work-up

It is necessary to assess each probe combination prior to clinical treatment (Thornhill *et al.*, 2005). Metaphase spreads and interphase nuclei should be analyzed from

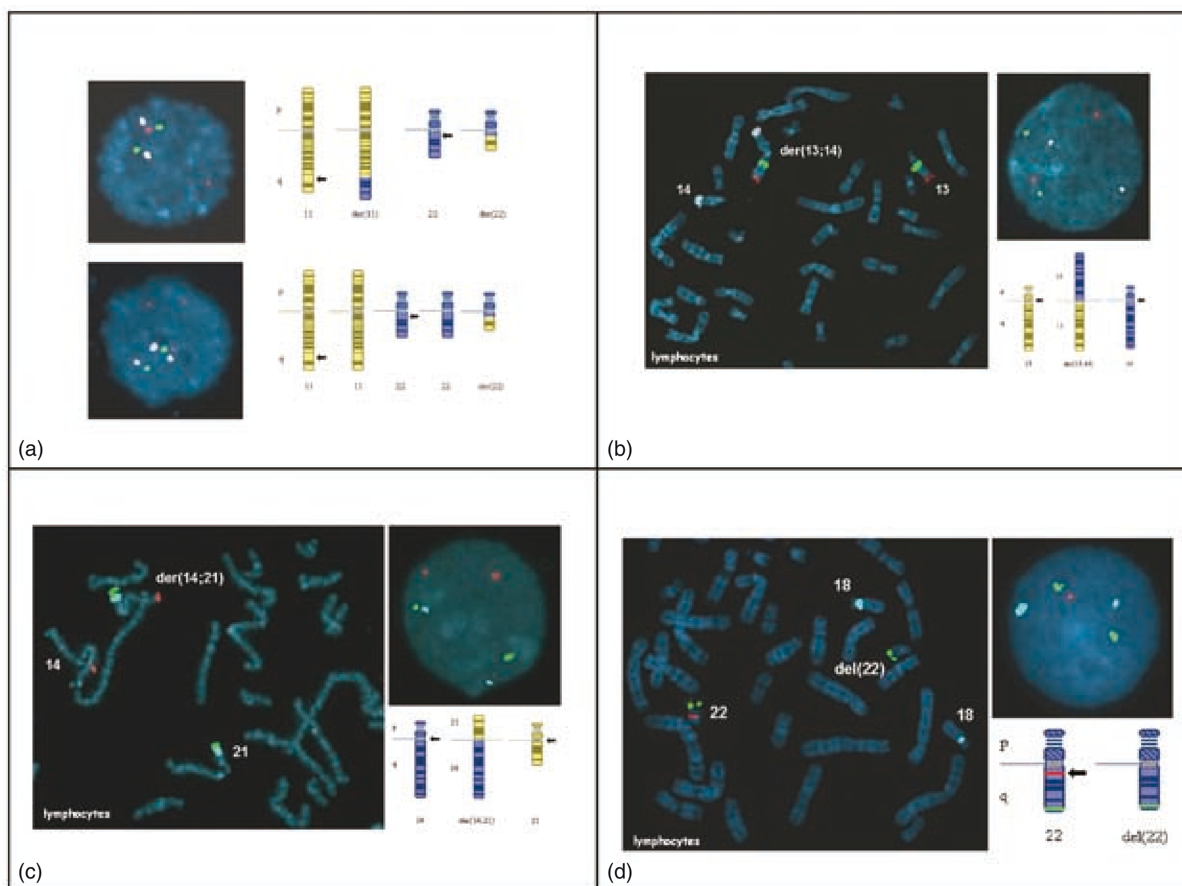

Figure 12.3 Recommended FISH probe combinations for: (a) the "common" 11;22 translocation: 46,Xn,t(11;22)(q23.3;q11.2); 11qsubtel (11q25; TexasRed, Cytocell), TUPLE1 (22q11.2; SpectrumOrange, Abbott), ARSA (22q13.3; SpectrumGreen, Abbott). Top row: normal/balanced blastomere nucleus and ideogram of balanced rearrangement; bottom row: 3:1 tertiary trisomy product (blastomere and ideogram), demonstrating "internal check" for the supernumerary chromosome. (b) Robertsonian translocation between chromosomes 13 and 14: 45,Xn,der(13;14)(q10;q10); RB1 (13q14; SpectrumGreen, Abbott), D13S1825 (13q34; TexasRed, Cytocell), D14S308 (14q32.3; SpectrumOrange, Abbott). Metaphase spread and interphase nucleus from carrier lymphocytes, and ideogram of the balanced rearrangement. (c) Robertsonian translocation between chromosomes 14 and 21: 45,Xn,der(14;21)(q10;q10); D14S1420 (14q32.3; TexasRed, Cytocell), LSI21 (21q22.13-q22.2; SpectrumOrange, Abbott), D21S1575 (21q22.3; FITC, Cytocell). Metaphase spread and interphase nucleus from carrier lymphocytes, and ideogram of the balanced rearrangement. (d) 22q11 deletion (46,Xn,del(22)(q11.2q11.2)): TUPLE1 (22q11.2; SpectrumOrange, Abbott), ARSA (22q13.3; SpectrumGreen, Abbott); and to confirm diploidy D18Z1 (cen 18, SpectrumAqua, Abbott). Metaphase spread and interphase nucleus from carrier lymphocytes, and ideogram illustrating the deletion.

each reproductive partner. Metaphase spreads should be analyzed from the translocation carrier to ensure that the probes selected hybridize as expected and are informative for the translocation segments. Metaphase spreads should be analyzed from the non-carrier reproductive partner to ensure that the probes hybridize normally. Interphase nuclei should be scored to make a quantitative assessment of the assay and a qualitative assessment of FISH signal intensity and discreteness. The quantitative data collected may be used to assess the potential analytical performance of the assay. The false-abnormal and false-normal error rates may be estimated to assess the test specificity and sensitivity. The negative predictive accuracy (the likelihood that a normal test result represents a normal or balanced chromosome complement) and the positive predictive accuracy (the likelihood that an abnormal test result represents segment imbalance) may be used to decide if the probe combination is suitable for a clinical PGD cycle (Scriven, 2003). Test analytical performance is a function of individual probe efficiencies, the prevalence of the 32 different reciprocal translocation segregation products, and the fidelity of embryo sampling.

For the purpose of assessing the analytical performance of a probe assay, it is assumed that random sampling of a cell from a day 3 embryo is representative.

Efficiencies of at least 95 percent are realistically achievable for individual probes. The prevalence in day 3 embryos of the different translocation forms can at best only be estimated. Each translocation, with very few exceptions, is unique to one family, and therefore pedigree information is usually very limited. Although it is possible to undertake sperm FISH studies for male carriers, it may be impractical to offer this on a routine basis. The inaccessibility of the female germline means that, for practical purposes, only estimation is possible. However, pachytene shape analysis appears useful not only in predicting the mode of segregation associated with liveborn imbalance, but also in predicting which translocations are likely to produce a high prevalence of 3:1 or adjacent-2 segregation products (Mackie Ogilvie & Scriven, 2002).

Careful probe selection, with two informative probes for potentially viable chromosome imbalance (Thornhill *et al*., 2005), and an estimated negative predictive value of at least 95 percent and a positive predictive value of at least 85 percent, are generally considered acceptable in order to offer a clinical PGD cycle using single cell biopsy (Scriven, 2003). Figure 12.3 (a) shows the recommended FISH probe combination for the "common" 11/22 reciprocal translocation.

Number of blastomeres to test

Mosaicism (the presence in an embryo of two or more genetically different cell lines) is well described (Munné *et al*., 1994; Kuo *et al*., 1998; Daphnis *et al*., 2005). Some groups therefore choose to biopsy and test two cells from cleavage-stage embryos, only transferring those embryos where there is a concordant result between the two cells tested. However, this may in many cases be overcautious and result in unnecessary exclusion of embryos from the cohort suitable for transfer. Inherent errors in the test may result in a false-abnormal result for one of the two cells. In addition, for most translocations, mosaicism for the chromosomes involved is unlikely, and cells carrying such imbalance are likely to be selected against in favor of the normal cell line. However, where the FISH assay has no internal check for a potentially viable product, or where a small derivative chromosome may be lost, giving rise to a viable minor cell line, two-cell biopsy and testing is prudent.

Robertsonian translocations

Introduction

Robertsonian translocations (centric fusion of two acrocentric chromosomes) occur with a prevalence of one in 1000 in the general population (Gardner & Sutherland, 2004). By far the most common are the nonhomologous forms, that is those involving two different acrocentric chromosomes – either two different D group chromosomes (chromosomes 13, 14, and 15), two different G group chromosomes (21 and 22), or a D group and a G group chromosome. At meiosis, these rearrangements form trivalents, segregation of which may result in gametes, nullisomic or disomic, for one of the chromosomes involved in the rearrangement and consequently to a zygote with trisomy or monosomy for one of the chromosomes involved. Zygotes with monosomy are not compatible with life and most translocation trisomy conceptuses are expected to result in first-trimester loss or earlier; however, some survive beyond the second trimester and to term.

Reproductive risks

The most common Robertsonian translocation is between chromosomes 13 and 14. This D/D translocation makes up ~75 percent of all Robertsonians (Gardner & Sutherland, 2004). The potential liveborn chromosomally unbalanced outcome of this is translocation trisomy 13 (Patau syndrome); there is an empirical risk of occurrence at second-trimester, prenatal diagnosis of <0.4 percent (Boue & Gallano, 1984; Gardner & Sutherland, 2004). There is also potential for uniparental disomy (UPD) for chromosome 14 after trisomy rescue with an estimated risk of ~0.1–0.5 percent (Gardner & Sutherland, 2004). Translocation trisomy 14 is expected to result in first-trimester loss. For der(13;14) carriers the overall risk of miscarriage is not expected to be significantly different from the background risk of 15 percent (up to two miscarriages); however, some individuals with a der(13;14) present with infertility or recurrent spontaneous abortions. The empirical reproductive risks for male carriers of 13;14 Robertsonian translocation carriers are low. Sperm FISH using probes for the translocation chromosomes may be used to establish the level of aneuploidy and, in the case of a normal sperm count and low aneuploid levels, PGD may not be indicated (Scriven *et al*., 2001). For some males who present with oligozoospermia and a Robertsonian translocation, intracytomasmic sperm injection (ICSI) may be necessary to overcome the infertility, in which case PGD would be indicated, to exclude the possibility of transferring abnormal embryos, and therefore to increase the chances of establishing a normal pregnancy.

Other D/D Robertsonians are much less frequent and specific risks have not been derived; however,

der(13;15) and der(14;15) might be expected to have similar risks to the der(13;14) (Gardner & Sutherland, 2004).

The most common Robertsonian after the der(13;14) is the der(14;21). The potential liveborn unbalanced outcome of this D/G Robertsonian is translocation trisomy 21 resulting in Down syndrome; for female carriers, the empirical risk of occurrence at second-trimester prenatal diagnosis is 15 percent, with a 10 percent risk of liveborn trisomy 21 plus a small risk of UPD 14, as before. For male carriers, the second-trimester risk of translocation trisomy 21 is <0.5 percent (Boue & Gallano, 1984; Gardner & Sutherland, 2004).

Other D/G Robertsonians which involve chromosome 21 may be expected to have similar reproductive risks to the der(14; 21); those involving chromosome 22 have a lower risk since trisomy 22 has very limited potential to be viable.

FISH probe selection

Any combination of two probes specific for the long arms of the two chromosomes involved in the Robertsonian translocation (for instance, a subtelomere probe for each chromosome) may be used as a FISH test; however, where there is a likely viable outcome, such as for Robertsonian translocations involving chromosomes 13 or 21, the use of two different probes for the viable trisomy, plus a probe for the other chromosome involved in the rearrangement, is considered prudent, as this gives an internal check (see above). (See Figure 12.3 (b) and Figure 12.3 (c) for the recommended FISH probe mixes for the two most common Robertsonian translocations.)

Indications and counseling

For infertile couples undergoing assisted conception, where one partner carries a Robertsonian translocation, the screening out of embryos with an unbalanced translocation prior to transfer is likely to be beneficial, regardless of the chromosomes involved. However, where a couple is referred for recurrent miscarriages, careful counseling is required prior to PGD; the translocation may not be causative of the miscarriages, and subjecting a fertile couple to invasive procedures in these cases should be avoided. This is particularly the case for the der(13;14) translocations, where empiric data suggest that the translocation is unlikely to be associated with recurrent miscarriage. Karyotyping products of conception may be a worthwhile pathway to follow for the couple and their counselor. Further discussion of this issue, and a description of cases tested for Robertsonian translocation, may be found in Scriven *et al.* (2001).

Other chromosome rearrangements

Carriers of balanced pericentric inversions are at risk of pregnancies with imbalance for both the non-inverted segments. This risk will depend on the length of the inverted segment, which will be related to the likelihood of an odd number of crossovers within the inversion loop. Imbalance for the noninverted segments may be ascertained using subtelomeric probes for the relevant segments (Escudero *et al.*, 2001; Fridstrom *et al.*, 2001). Melotte *et al.* (2004) describe the design and execution of a PGD cycle for an insertional translocation, demonstrating the care that is needed in considering all the possible outcomes of such rearrangements, and the need to design a PGD strategy that adequately detects chromosome imbalance in these cases. For complex rearrangements, such as insertional translocations, custom probes may need to be worked up so that informative probes are available for segments at risk of imbalance. Three- or four-way translocations may be approached either by selection of probes labeled with appropriate fluorochromes, or, if this is not possible, rehybridization of biopsied nuclei with a second probe set. Carriers of deletions or duplications have a 50 percent risk of transmitting the abnormal chromosome to their embryos. Where the effect of the imbalance may be variable (for instance the 22q11 microdeletion syndrome), PGD may be sought to avoid a child more severely affected than the parent. In these cases, there may be only a single probe available for the deleted or duplicated segment, in which case two-cell biopsy would be recommended, with transfer only of embryos with a concordant normal result. Figure 12.3 (d) shows the FISH work-up for a clinical diagnostic cycle for a carrier of a 22q11 microdeletion.

Conclusion

There is considerable heterogeneity in the approach to this application of PGD: some centers use polar body analysis (Munné *et al.*, 1998; Verlinsky *et al.*, 2002b), whilst others use labeled probes to ascertain copy number of rearrangement segments in blastomere nuclei. For reciprocal translocations, some groups favor a two-centromere and one-telomere combination (Simopoulou *et al.*, 2003), or even one centromere and one telomere (Kyu Lim *et al.*, 2004) rather

than the two-telomere, one-centromere approach described above. Additionally, the routine biopsy and testing of two cells for every chromosome rearrangement is carried out at some centers (Van de Velde *et al*., 2000; Simopoulou *et al*., 2003). European Society of Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium data collection (Harper *et al*., 2006) indicated that from within the Consortium, 33 centers routinely carried out PGD for chromosome abnormalities, with an average clinical pregnancy success rate of 24 percent per embryo transfer. Overall, individual success rates for different centers ranged from 0 percent to 100 percent (median 12.5 percent); the variation is likely to be due to small numbers, the differences in patient groups and practice described above, and it may reflect the quality of the assisted reproduction technology (ART) performance in the center in which PGD is based. Our own experience, based on a large number of cycles, is 33 percent per embryo transfer (Grace *et al*., 2006); outside the Consortium other large centers have reported a similar success rate of 35 percent per embryo transfer (de Boer *et al*., 2004; Verlinsky *et al*., 2004).

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Chapter 13

Preimplantation genetic diagnosis for infertility (preimplantation genetic screening)

Santiago Munné

Key points

- Many studies have shown that the incidence of chromosome abnormalities in early human embryos is high (50–70 percent). Data from oocyte donation shows that women of advanced maternal age (AMA) have decreased delivery rates owing to poor oocyte quality.
- Therefore preimplantation genetic diagnosis (PGD) for infertility (or aneuploidy screening – PGS) has been developed to help women of AMA, recurrent implantation failure (RIF), repeated miscarriage (RM), and other indications.
- Selecting chromosomally normal embryos for replacement should: (i) increase the implantation rates; (ii) reduce spontaneous miscarriage rates; (iii) reduce aneuploid conceptions; and (iv) improve the delivery rates in assisted reproduction technologies (ART) cycles.
- Deficiencies in methods may preclude an improvement in ART outcome. Some key factors are the biopsy of single cells; the analysis of at least chromosomes X, Y, 13, 15, 16, 18, 21, and 22; an error rate based on re-analysis results of less than 10 percent; reduced embryo damage even when one cell is biopsied; and a minimum number of embryos produced to allow for the selection potential to fully compensate for the embryo damage caused by the biopsy.
- Methods are being developed toward a full chromosome count, such as metaphase and array comparative genomic hybridization (CGH).

Introduction

The incidence of chromosomal abnormalities in cleavage-stage embryos produced in vitro, 50–70 percent depending on maternal age, is considerably higher than that of spontaneous abortions, indicating that a sizeable percentage of chromosomally abnormal embryos are eliminated before any prenatal diagnosis. In addition, data from oocyte donation show that in women of advanced maternal age (AMA) the decline in pregnancy is largely caused by failing oocyte quality (Navot *et al.*, 1991). Thus, by selecting chromosomally normal embryos for replacement, preimplantation genetic diagnosis (PGD) for infertility (or preimplantation genetic screening, PGS) should, using appropriate methods: (i) increase implantation rates; (ii) reduce spontaneous abortion rates; (iii) reduce aneuploid conceptions; and (iv) improve delivery rates in ART cycles (Munné *et al.*, 1993). Despite large studies indicating the advantages of this approach, the notion that PGS is beneficial is not yet uniformly shared, and results vary between PGS centers.

In this chapter, the different steps that compose PGS will be evaluated to determine which are appropriate for ART improvement.

There is new evidence that biopsy of two cells per embryo, while not reducing error rates, may reduce the implantation potential of an embryo. How the cells are biopsied may also negatively affect embryo development. Biopsied cells must be fixed for fluorescence *in-situ* hybridization (FISH) analysis and some fixation methods produce fewer false-positive errors than others. Once fixed the FISH procedure needs to analyze a minimum number of chromosomes, apparently at least eight chromosome pairs, to result in an improvement of implantation rates. But not all analyzers do the same good job. There are reports indicating FISH error rates between 5 percent and 50 percent, this sometimes being confused with true mosaicism (Baart *et al.*, 2004; Colls *et al.*, 2007). Obviously, high error rates will preclude any benefit of a screening technique. Finally, once chromosomally normal embryos have been selected for replacement, special care should be taken when doing the transfer. Reports following appropriate methods have shown that PGS does indeed increase implantation rates and reduce spontaneous abortion and trisomic conception rates (Gianaroli *et al.*, 1999; Munné *et al.*, 1999; Munné *et al.*, 2003; Munné *et al.*, 2006; Colls

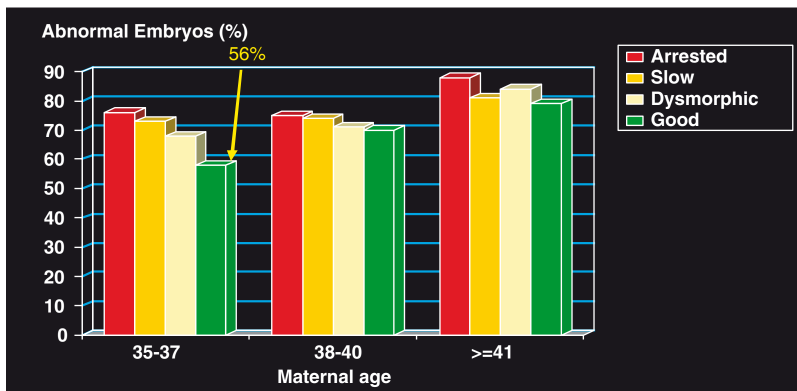

Figure 13.1 Chromosome abnormalities by age and morphology. Data from Munné *et al.* (2007a). With permission. Copyright © 2007 American Society for Reproductive Medicine.

et al., 2007), but large studies are still needed to show a significant improvement in delivery rates. However, in a single- or double-embryo transfer (SET/DET) system, this should be achieved in centers with no limit on the number of embryos that can be replaced.

In addition to infertile couples, PGS has proven to significantly reduce the risk of miscarriage in women with idiopathic repeated miscarriage (RM) or for patients with RM caused by chromosome translocations (Munné *et al.*, 2005).

Chromosome abnormalities and embryo selection

In most in vitro fertilization (IVF) laboratories one of the most powerful tools to improve results is embryo selection, based on morphological and developmental characteristics (Gardner *et al.*, 1998; Alikani *et al.*, 2000; Racowsky *et al.*, 2003). However, the implantation potential of human embryos produced in vitro remains low even when most laboratories use morphological selection, generally about 29 percent for patients aged <35 years, 21.8 percent for those aged 35–37, 14 percent for those aged 38–40, and 7.7 percent for those aged 41–42 years (SART, 2005).

One reason for the limited potential of morphological selection is that the majority of human embryos produced in vitro are chromosomally abnormal (Munné *et al.*, 1995a; Delhanty *et al.*, 1997; Márquez *et al.*, 2000; Kuliev *et al.*, 2002; Verlinsky *et al.*, 2001; Bielanska *et al.*, 2002; Magli *et al.*, 2001a; Magli *et al.*, 2007; Munné *et al.*, 2007a). In a recent study involving over 6000 embryos (Munne *et al.*, 2007a), it was shown that chromosome abnormalities were widespread regardless of maternal age and morphology. For example, only 44 percent of the best embryos, according to morphology, in young patients (aged <35 years) were chromosomally normal, and the proportion decreased subsequently with age and decreasing embryo developmental characteristics (Figure 13.1) (Munné *et al.*, 2007a). Thus, in that study, although morphology is correlated with euploidy and implantation potential, its use as a selection tool for replaced embryos may only enrich the implantation potential by a few percentage points (i.e. for patients of 35–37 years of age, from an average of 37 percent normal embryos to 44 percent in those with the best morphology).

Extending culture to day five may improve implantation rates for patients with multiple day three embryos and good morphology. However, embryos of lower quality can implant and develop successfully if replaced on day three, while delay to day five might be deleterious in these embryos (Alikani *et al.*, 2000).

Studies on chromosome abnormalities in blastocysts derived from PGS cases indicate that some chromosome abnormalities do not reach blastocyst, such as pure monosomies and haploidies, but these studies have not been fully confirmed (Sandalinas *et al.*, 2001; Magli *et al.*, 2000; Li *et al.*, 2005).

There are emerging technologies attempting to assess embryo quality non-invasively (see Elder & Cohen, 2007). As with extended culture, it seems rationally unlikely that all chromosome abnormalities could be detected. To start with, there are two types of chromosome abnormalities of very different origins: one is aneuploidy, generated during gametogenesis and accounting for 20–50 percent of chromosome abnormalities depending on maternal age (Munné *et al.*, 2007a). The other is postzygotic abnormality, accounting for 30 percent of chromosome abnormalities and not linked to maternal age but to dysmorphism (Munné *et al.*, 2007a), and is probably caused by spindle abnormality that also causes dysmorphism (Alikani *et al.*, 2005). The latter are likely to be more correlated with abnormal development, and screened against by noninvasive methods,

Table 13.1 Effect of cell loss on implantation*

Cells survived after thaw (%)	Procedures with unique transfers (*n*)	Implantation rate (%)		Pregnancy rate with fetal heart beat (%)
		Fetal sac	Fetal heart beat	
≤50	9	7.7	7.7	11.11
51–75	32	15.3	13.7	18.75
76–99	32	27.6	27.6	37.50
100	103	33.6	30.0	49.51

*Only thaws and transfers with uniform embryo cohorts are included.
From: Cohen *et al.* (2007) with permission. Copyright © 2007 American Society for Reproductive Medicine.

morphological selection, and extended culture, but not most trisomies, which can and do implant.

Thus PGS, alone or in combination with other selection techniques, has the potential to select the most euploid embryos and improve ART outcome. A growing body of evidence shows that PGS increases the implantation rate while reducing trisomic conceptions and miscarriage. However, despite large studies indicating the advantages of aneuploidy screening, the notion that PGS is beneficial is not yet uniformly shared, which is largely caused by the differing results of studies that used different procedures and methods, some more effective than others.

Differences in methods between optimal and poor PGS results

PGS results do not depend solely on the PGS laboratory; instead, hormone stimulation, biopsy techniques, fixation methods, and embryo replacement skills may substantially affect the outcome of a cycle. In this chapter several key aspects needed to ensure ART improvement through PGS will be discussed.

Which cells to biopsy?

First, or first and second, polar body (Verlinsky *et al.*, 1995; Munné *et al.*, 1995b; Verlinsky *et al.*, 1996), and day three single-cell embryo biopsy are the most common methods for assessment of euploidy in human embryos (Munné *et al.*, 1993; Harper *et al.*, 2008a). More recently, some programs have applied polar body biopsy combined with day three single-cell analysis either with acid Tyrodes (Magli *et al.*, 2004) or mechanical (Cieslak-Janzen *et al.*, 2006), two-cell day three biopsy (Staessen *et al.*, 2004), or blastocyst biopsy (De Boer *et al.*, 2004).

Each method has advantages and disadvantages with regard to the damage caused, the quality of information obtained, and the quantity of cells analyzed. While first and second polar body analyses are the less invasive techniques, especially if the biopsy is performed mechanically, they do not provide information on postzygotic abnormalities, which affect 30 percent of embryos (Munné *et al.*, 1995b). Blastocyst biopsy is theoretically more benign than cleavage-stage biopsy, since the inner cell mass is not biopsied, but not all embryos that are euploid reach the blastocyst stage (while if transferred before they could implant – Alikani *et al.*, 2000), and the techniques for trophectoderm biopsy are unknown to the vast majority of embryologists. Furthermore, if PGS was to be performed by a reference laboratory (e.g. transporting the fixed cells for analysis) the technique would require day six replacement.

There are no reports on the effect of embryo biopsy done without PGD or PGS selection on implantation. Some studies followed embryos only to blastocyst formation (Hardy *et al.*, 1990). The best indirect assessment of the damage caused by embryo biopsy is the study by Cohen *et al.* (2007) comparing the effect of cell loss after freezing and thawing. In that study, cycles in which “pure” replacements (all embryos match the criteria) of embryos with all cells intact, embryos with one cell lost, with two cells lost, and with three cells lost were compared. As seen in Table 13.1, cycles that had embryos replaced with no cells lost had a 30 percent implantation (plus fetal heartbeat), compared with 28 percent if all embryos replaced had lost one cell, but only 14 percent if all embryos replaced had lost two cells. In some studies, single-cell embryo biopsy appears to minimize reduction in implantation potential that is more than compensated for by the selection

of euploid embryos (Gianaroli *et al.*, 1999; Munné *et al.*, 1999; Munné *et al.*, 2003). However, two-cell biopsy seems to reduce the implantation potential by more than half (Cohen *et al.*, 2007), and embryo biopsy by inexperienced hands can be very detrimental, even when withdrawing a single cell (Mastenbroek *et al.*, 2007; Munné *et al.*, 2007d; Cohen & Grifo, 2007; Kuliev & Verlinsky, 2007).

Blastocyst biopsy is starting to be used clinically (De Boer *et al.*, 2004), producing several cells for analysis; while this certainly will reduce the risk of misdiagnosis, it is unlikely to show good implantation but no controlled studies are available yet about its effect on outcome.

How to open the zona

Mechanical biopsy (Wilton *et al.*, 1989) seems intuitively to be the less detrimental means of embryo biopsy, but is the hardest to learn. Chemical zona opening using Tyrode's solution (Gordon & Talansky, 1986) is widely used, although both methods are being progressively substituted by laser biopsy (Germond *et al.*, 1995), which is the simplest to use. Mouse models are not appropriate for evaluation of human embryo biopsy because of the difference in ZP characteristics and the inability of mouse blastomeres to recover following acidosis (Dale *et al.*, 1998). There are very few publications comparing the three methods in relation to ART outcome (Joris *et al.*, 2003; Chatzimeletiou *et al.*, 2005).

One study suggests that a safe working distance for the laser is crucial in order to prevent immediate or long-term adverse effects on the development of lased and biopsied human embryos (Chatzimeletiou *et al.*, 2005). Another study compared acidified Tyrodes medium and laser biopsy and found that making the hole between two cells obtained identical pregnancy rates, but with a slight increase in broken cells (5 percent) after acid than after laser (2 percent) ($p<0.05$) (Joris *et al.*, 2003).

These observations certainly suggest that specialized training is necessary for the correct performance of zona opening, regardless of the method used, and lack of such training is surely one of the primary reasons for unsatisfactory implantation obtained in some centers after PGS.

How to remove the cell

Once the ZP has been breached, blastomeres may be biopsied either by suction, liquid displacement (Roudebush *et al.*, 1990) or by exerting pressure against the ZP with the micropipette (Gordon & Gang, 1990). The first method seems the less detrimental since it hardly disturbs the other cells, unless the embryo is compacted; while the last is certainly the most disruptive. No studies have compared the three methods.

The majority of normally developing human embryos have been found to undergo compaction on day four of development (Nikas *et al.*, 1996) but the first immature tight junctions appear at the six-cell stage (Gualtieri *et al.*, 1992). Compaction complicates cell biopsy and results in high rates of cell lysis. This has been circumvented by using Ca^{2+} /Mg^{2+}-free medium, which breaks the tight junctions. However, extended time in such medium was found to impair embryo development (Reeve, 1981). Thus the embryos must be less than five minutes in Ca^{2+} /Mg^{2+}-free medium before the biopsy and after the biopsy the embryos should immediately be washed to remove this medium (Santaló *et al.*, 1996; Dumoulin *et al.*, 1998; Kahraman *et al.*, 2000).

The composition of the Ca^{2+} /Mg^{2+}-free medium is also important. Hill and Li (2004) indicated that a Ca^{2+}- or Mg^{2+}-free biopsy medium with sodium lactate, sodium pyruvate, and alanyl-glutamine, produced significantly higher pregnancy rates than simpler media.

Polar body biopsy is usually done by suction or liquid displacement (Verlinksy & Kuliev, 2005). A specific problem of first polar body biopsy is to coordinate it with ICSI. Inexperienced programs are reluctant to perform polar body biopsy before ICSI since the position of the first polar body is indicative of the spindle position and results may be jeopardized if the sperm injection disturbs the spindle (Blake *et al.*, 2000). In certain cases, results must be obtained before ICSI (Munné *et al.*, 2000a), but a bridge of microtubules between the egg chromosome spindle and the polar body may exist, and if the polar body is pulled the egg may be damaged. If ICSI is performed too late, in vitro oocyte aging may occur, producing nondisjunction. Experienced programs usually perform first and second polar body biopsy together at the zygote stage (Verlinksy & Kuliev, 2005). Again it is clear that inexperienced hands doing biopsy may easily cause very low implantation.

Cell fixation

Biopsied polar bodies are extremely small and are therefore usually fixed by letting the cell dry on the slide then applying Carnoy fixative (3:1 methanol:acetic acid). Since the chromatin structure of the first polar body degenerates within 24 hours after egg retrieval (and sometimes is already degenerated) blastomere fixation methods are not effective. However, if the first

polar bodies are fixed a few hours after retrieval and have not degenerated, metaphase chromosomes may be obtained using improved techniques (Pujol *et al.*, 2003) and FISH signals can be better observed. This method is also suitable for chromosome painting for PGS of maternal translocations when the translocation breakpoints are not terminal.

For blastomere fixation the original method using Carnoy fixative (Tarkowski, 1966) (method 1), although improved with several modifications (Munné *et al.*, 1998a), is still considered difficult to master for people who use it sporadically, and two alternative methods have been developed (Coonen *et al.*, 1994; Harper *et al.*, 1994; Dozortsev & McGinnis, 2001). Method 2 uses Tween-20, a detergent, and HCl and method 3 uses a combination of Tween-20/HCl and Carnoy fixative.

The mixture in method 1 of fixative with the drop of hypotonic containing the blastomere produces turbulences in which the cell may be lost. This risk is about 3 percent in expert hands (Velilla *et al.*, 2002), but it may be higher for technicians using this method sporadically (Xu *et al.*, 1998; Dozortsev & McGinnis, 2001). In contrast, methods 2 and 3 overcome the turbulence step and are easily learned. However, the purpose is to provide a fixed nucleus with the highest chance of producing reliable FISH results. Comparing the three techniques, Velilla *et al.* (2002) found that the average diameters of the fixed nuclei were 59, 31, and 46 microns, respectively, resulting in 14 percent, 58 percent, and 39 percent overlaps between chromosomes, and 10 percent, 30 percent, and 17 percent errors, respectively. So it appears that method 1 is more effective in reducing errors and minimizing no-results but has a higher learning curve. It is also possible that changes in FISH protocols may allow better results using the other two methods.

Number of chromosomes analyzed

FISH is currently the best method to analyze polar bodies and blastomeres since the former have poor-quality metaphases and in the latter metaphases are produced in low rates even after culture (Santaló *et al.*, 1995). FISH methods have been properly standardized but remain limited because the human eye can only detect five fluorescence dyes in the visible spectrum, which limits the number of chromosome DNA probes used simultaneously; thus requiring one or more extra rounds of hybridization to analyze more chromosomes. However, more hybridization rounds on the same cell means more errors, and therefore PGS laboratories usually analyze a maximum of 12 chromosomes. The number and types of chromosomes analyzed are, of course, critical for ART improvement. The chromosomes most involved in aneuploidy at the cleavage stage are in order 22, 16, 21, and 15 (Munné *et al.*, 2004a). When combined with those whose abnormalities may reach term (X, Y, 13, 18, 21) they make eight as the minimum number of probes that should be used for PGS of aneuploidy. Although a reduction in spontaneous abortions has been reported by use of only five probes (X, Y, 13, 18, 21; see Munné *et al.*, 1999), any studies obtaining improved implantation have used probes for at least the eight critical chromosomes (X, Y, 13, 15, 16, 18, 21, 22) (Gianaroli *et al.*, 1999; Munné *et al.*, 2003; Munné *et al.*, 2005; Munné *et al.*, 2006). This is because the five-chromosome test detects only 28–31 percent of chromosome abnormalities detected in fetuses, 70–72 percent with nine probes (the eight plus 17), and 79–80 percent with the 12-probe test (the eight plus 17, 14, 8, and 20) (Jobanputra *et al.*, 2002; Lathi *et al.*, 2007). However, spontaneous abortion data do not coincide entirely with that obtained by metaphase comparative genomic hybridization (m-CGH) in oocytes, where the nine-probe test only detects 57 percent of abnormal eggs and the 12-probe test only 67 percent (Gutiérrez-Mateo *et al.*, 2004a; Gutiérrez-Mateo *et al.*, 2004b; Fragouli *et al.*, 2006a) (Table 13.2). This is because some trisomies and monosomies survive better than others to the first trimester (Sandalinas *et al.*, 2001; Munné *et al.*, 2004b).

PGS analysis of all chromosomes has been attempted using m-CGH. Because of the time taken, initially embryo biopsy was followed by freezing (Voullaire *et al.*, 2000; Wilton *et al.*, 2001; Wilton *et al.*, 2003), but thawing attrition was found to eliminate the advantages of the PGS selection. Next, attempts were made to analyze first polar bodies and replace on day five (Wells *et al.*, 2002), but the technique was too labor-intense to be practical and was also abandoned. With the advent of better freezing methods, m-CGH is being revisited (Sher *et al.*, 2007; Voullaire *et al.*, 2007).

Once more there is no standard number of chromosomes analyzed between IVF programs, which will mean yet another variation in PGS results between centers, and m-CGH is not yet a practical alternative.

Figure 13.2 (a), (b), (c), and (d) show blastomeres analyzed with the first panel of probes for chromosomes 13, 16, 18, 21, and 22.

Table 13.2 Abnormalities detected by FISH and m-CGH

Reference	Eggs + polar body analyzed		Egg/polar body that will be classified abnormal by FISH using:				
			Four chromosomes	Six chromosomes	Eight chromosomes	Eleven chromosomes	Abnormals missed
	Total	Abnormal	XY, 13, 15, 18	Five chromosomes + 16, 22	Six chromsomes + 15, 17	Eight chromsoms + 14, 20, 8	
Gutiérrez-Mateo *et al.* (2004a)	25	11	4	4	6	6	5
Gutiérrez-Mateo *et al.* (2004b)	21	21	3	9	11	12	9
Fragouli *et al.* (2006a)	100	22	13	13	14	18	4
Total	146	54	20 (37%)	26 (48%)	31 (57%)	36 (67%)	18

No results and result rescue

The rate of undiagnosed cells mostly depends on proper biopsy and fixation techniques. The rate of no diagnosis ranges from a very low 1 percent of embryos undiagnosed (Gianaroli *et al.*, 2004) to a huge 20 percent of embryos undiagnosed (Mastenbroek *et al.*, 2007).

FISH signals may overlap or split. Size analysis and distance between signals have been used in the past as criteria for scoring dubious results (Munné & Weier, 1996; Munné *et al.*, 1998a). A better alternative is to use "no-result rescue" (NRR), which consists of rehybridizing a nucleus for which there is a dubious signal for a specific chromosome with a probe for the same chromosome which binds to a different locus (Magli *et al.*, 2001b; Colls *et al.*, 2004; Colls *et al.*, 2007). A recent study by Colls *et al.* (2007) involving 34 225 embryos indicates that with this method, nuclei with inconclusive results are reduced from 7.5 percent to 3.1 percent, and FISH errors from 13.6 percent to 4.7 percent.

As yet, this is by no means a standard procedure, and shows yet another area of potential error in the application of PGS.

Number of analyzers

Counting FISH dots is not as simple as it seems. There is a blind spot where the optical nerve is collected and passed through the human retina; in certain situations a FISH signal becomes invisible. Also, signals on the periphery of the nucleus, usually in micronuclei, may be missed by an analyzer. Thus it is paramount that two analyzers score all samples independently. In one study, using NRR using two analyzers who scored the signals independently, the individual error rate was 10.3 percent compared with the combined analysis of 4.7 percent (Zheng *et al.*, personal communication). This is quite a surprising outcome, and is not generally recognized; obviously, this is an important factor.

Error rate criteria and differences between centers

Fixation and FISH techniques may be evaluated by the error rate of the PGS laboratory. Reasonable and continuous evaluation of the error rate should include:

- re-analysis of all embryos not replaced after PGS
- re-analysis on day three or day four
- use of standard FISH and fixation methods
- clinical confirmation of abnormality
- previously published criteria of defining a mosaic embryo as abnormal (i.e., Munné *et al.*, 1994), although this point may be debatable and instead the previously used <3/8 abnormal cells to define normality could be raised to 50 percent since

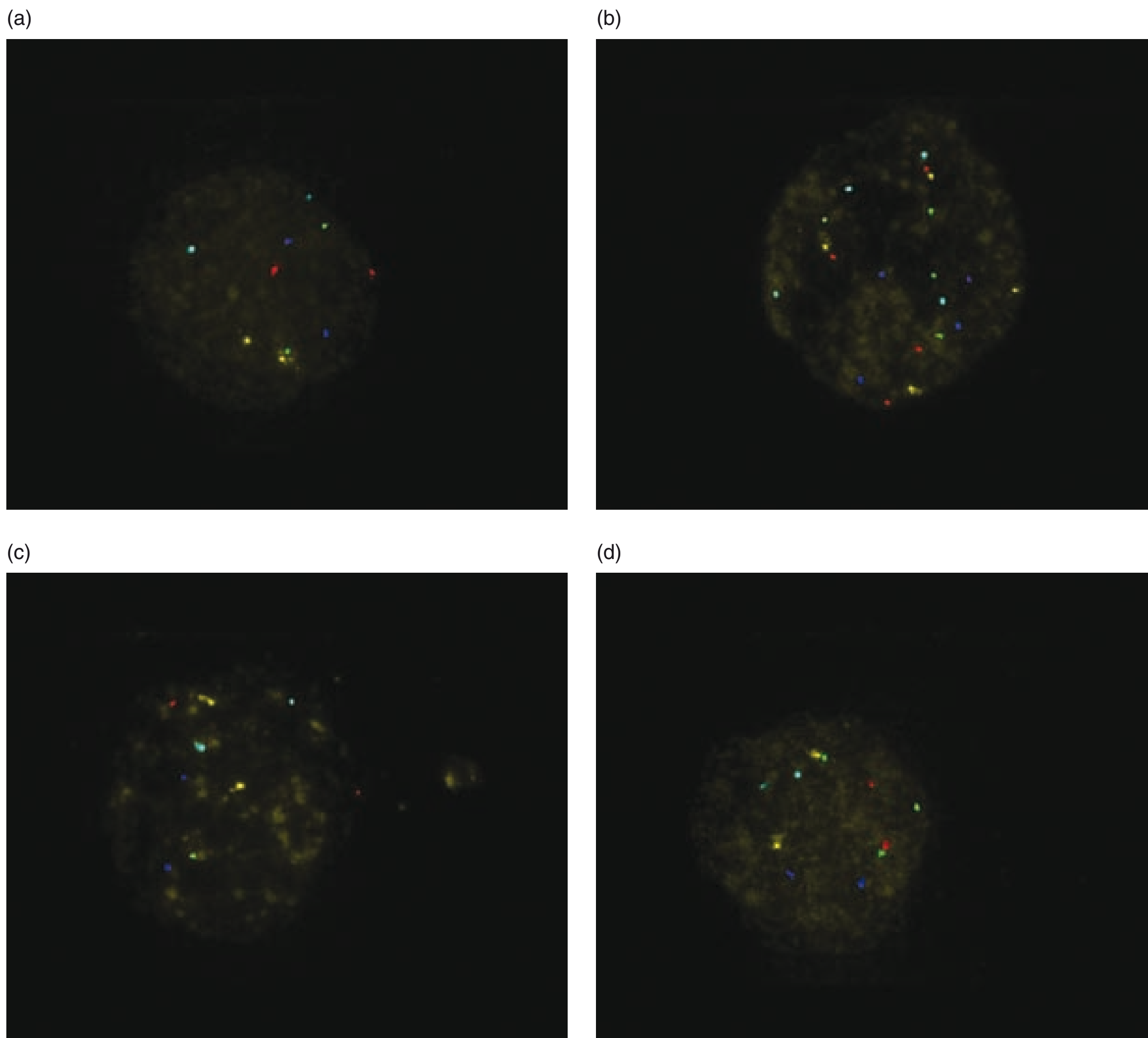

Figure 13.2 Single blastomere analyzed by fluorescence *in situ* hybridization (FISH) with probes for chromosomes 13 (red), 16 (light blue), 18 (aqua), 21 (green), 22 (yellow): (a) normal; (b) polyploidy; (c) monosomy 21; and (d) trisomy 21.

hardly anyone would accept replacement of such an embryo.

Once similar criteria are used, errors will then be caused, either by the same technique or by mosaicism.

Each PGS laboratory has a different error rate, and its value ranges from 4 percent to 7 percent (Munné *et al.*, 2002; Colls *et al.*, 2007; Magli *et al.*, 2007) to 50 percent (Baart *et al.*, 2004; Li *et al.*, 2005; Coulam *et al.*, 2007) (Table 13.3). An error rate close to 50 percent is in fact equivalent to no diagnosis. Thus it is paramount for a patient to be aware of the error rate of the PGS center that will perform the procedure. Some centers quote error rates from the literature instead of their own error rate, which may be misleading.

Differences in error rate among PGS centers are not always caused by the differences mentioned above, but by how the re-analysis is performed. Overall, these differences may be:

- unsuitable fixation techniques more prone to errors
- use of unsuitable probe combinations, including mixtures of colors, or polymorphic probes which are more prone to errors
- analyzing only spare embryos and failing to re-analyze all embryos not replaced after PGS,

Table 13.3 Error rates reported by different centers, and differences between studies

Reference	No. of probes	Embryos re-analyzed	Type of embryos	Day re-analyzed	Fixation type	Embryos without results (%)	Discordance** (%)
Baart *et al.* (2004)	10	29	Discarded	5	Tween-20/HCl	24	50.0
Li *et al.* (2005)	5	55	PGS*	5	Tween-20/HCl	NA	40.0
Coulam *et al.* (2007)	8	228	Discarded	3	Tween-20/HCl	28	24.5
Munné *et al.* (2002)	6–9	885	Discarded, PGS	4	Carnoy	NA	7.2
Magli *et al.* (2007	8	853	PGS	4	Carnoy	NA	8.6
Colls *et al.* (2007)	9+NRR	212	PGS	4	Carnoy	3	5.2

*PGS: embryos classified by PGS as chromosomally abnormal.
**Discordance: normal for abnormal or abnormal for normal, NRR: no-result rescue (Colls *et al.*, 2007).

thus overestimating chromosome abnormalities by analyzing embryos with a higher likelihood of being abnormal

- re-analyzing on day five instead of day three or four; some abnormal embryos will not survive to day five (Sandalinas *et al.*, 2001), a misdiagnosed embryo as abnormal (but being normal) will be more likely to reach blastocyst stage, but the ones arresting will not be counted in the denominator, thus overestimating the frequency of errors
- use of "cytogenetics confirmation" criteria (an abnormal to be confirmed should have the same identical abnormality detected by PGS) instead of a "clinical confirmation" criteria (e.g., monosomy 18 by PGS and chaotic 100 percent abnormal by re-analysis, abnormality is confirmed).

So there are plenty of reasons why patients should investigate the details of actual procedures and results at the clinic they attend.

Mosaicism and error rates

Mosaicism rates vary widely in the literature. Some of the differences between centers are attributed to the population; others to hormonal stimulation and the general quality of embryos produced in those centers. For example, it is well known that changing intrafollicular and laboratory conditions may alter the rate of mosaicism (Munné *et al.*, 1997). Also, as mentioned previously, mosaicism frequencies often change according to the developmental stage of the embryo.

Most large studies on cleavage-stage embryos indicate mosaicism rates around 25–30 percent (Delhanty *et al.*, 1993; Harper *et al.*, 1995; Munné *et al.*, 1995a; Delhanty *et al.*, 1997; Márquez *et al.*, 2000; Munné *et al.*, 2002; Magli *et al.*, 2007; Munné *et al.*, 2007a).

Mosaicism may produce false-positive or false-negative results. It has been estimated that the false-positive error rate was 4.3 percent and the false-negative error rate was 1.3 percent (Munné *et al.*, 2002) (Table 13.4).

The low error rate caused by mosaicism compared with its 30 percent frequency may be explained by the fact that the majority of mosaic embryos have only abnormal cells (Table 13.5) (Colls *et al.*, 2007). Even considering 50 percent abnormal cells as the cut-off for defining normalcy, the total error rate may be as low as 7 percent (Colls *et al.*, 2007) (Table 13.5), of which 5 percent is due to mosaicism and the rest to other technical problems. Even lower error rates have recently been reported by Magli *et al.* (2007).

Thus, the effect of mosaicism on PGS errors has been overestimated, and, provided appropriate methods are used it is included in the overall <10 percent error rate.

Table 13.4 Estimated risk of PGS misdiagnosis due to mosaicism

	Risk of classifying an abnormal embryo as normal		
	Overall frequency (A)	**Percentage of normal cells (B)**	**Risk of misdiagnosis (AxB) (%)**
2N/POL (detrimental)	3.7% (70/1903)	34.8	1.3
Chaotic (detrimental)	12.7% (242/1903)	9.8	1.2
Split (detrimental)	0.3% (5/1903)	29.8	0.1
Mitotic aneupl (all)	6.6% (126/1903)	24.2	1.6
Meiotic & mitotic aneupl (all)*	0.6% (12/1903)	12.2	0.1
TOTAL	23.9% (455/1903)	18.0	4.3
	Risk of classifying a mostly normal embryo as abnormal		
	Overall frequency (A)	**Percentage of abnormal cells (B)**	**Risk of misdiagnosis (AxB) (%)**
2N/POL (benign)	3.9% (74/1903)	23.1	0.9
Chaotic (benign)	1.3% (24/1903)	24.9	0.3
Split (benign)	0.2% (3/1903)	26.7	0.1
TOTAL	5.3%(101/1903)	23.6	1.3
Total misdiagnosis rate due to mosaicism		5.6 percent	

These were embryos fully trisomic or monosomic for a specific chromosome and, in addition, mosaic for the same chromosome.
Data from: Munné *et al.* (2002). The risk of misdiagnosis was calculated by multiplying column A by column B.

Table 13.5 Error rate and percentage of abnormal cells in mosaics

592 abnormal embryos from PGS were re-analyzed and found to be:	
Normal	13
Mosaic <38% abnormal	15
Mosaic 38–49% abnormal	12
Mosaic 50–99% abnormal	124
Mosaic 100% abnormal	297
Homogeneously abnormal	131
False-positive error with 38% threshold as abnormal	4.7%
False-positive error with 50% threshold as abnormal	6.8%

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Results of PGS for aneuploidy: trisomic offspring, spontaneous abortions, and implantation

Reduction in trisomic offspring

The first proposed use of PGS for chromosome abnormalities was as an alternative to prenatal diagnosis. In order to determine if PGS significantly reduces the risk of aneuploid conceptions, a suitable control group is needed. However, there are scant data on chromosomally abnormal conceptions, stratified by age, from IVF databases such as that of the Society for Assisted Reproductive Technology (SART). Since IVF pregnancies are usually closely monitored, a second-best control group would be pregnancies diagnosed through early prenatal diagnosis. By use of such a control population, Munné *et al.* (2006) determined that the expected risk of aneuploid conceptions for chromosomes X, Y, 13, 18, and 21 in a group of 2279 PGS cycles was 4.7 percent, based on the data of Eiben *et al.* (1994), but after PGS the observed rate of trisomic conceptions was significantly lower, 1.2 percent ($p<0.001$). A continuation study on 2300 fetuses resulting from PGS cycles, including the previously published data of Munné *et al.* (2006) indicated a reduction in aneuploid conceptions from an expected rate of 2.6 percent to an observed rate of 0.5 percent ($p<0.001$) (Table 13.6).

Data from other centers include a 1.2 percent error rate in 170 pregnancies from women with an average age of 36 years (Gianaroli *et al.*, 2004) while data from polar body analysis indicated no errors from 376 babies born (Verlinsky *et al.*, 2005).

Figure 13.2 (d) shows a cell from a trisomy 21 embryo. Although the prevention of trisomic conceptions is usually lumped together with the concept of improving ART outcome, it is an indication in itself.

Table 13.6 Expected and observed aneuploid conceptions after preimplantation genetic selection

Age	Conceptions after PGS	Expected aneuplodies*	Observed aneuploidies**
<35	891 x 1.2% = 10.7		
35–39	906 x 1.3% = 11.8		
>39	503 x 7.3% =36.7		
Total		59.2 / 2300 (2.6%)	11/2300 (0.5%)***

* Eiben *et al.* (1994);
** Munné *et al.* (2006) and unpublished data. $p<0.001$
*** Observed aneuplodies: four XO; one XXY; one T13; one T18; four T21.

For example, a recent study by Twisk *et al.* (2007) reports that in a survey conducted in Holland, where PGS is covered by health insurance, 87 percent of subfertile patients would undergo PGS if pregnancy rates were unchanged and 100 percent of Down syndrome were detected, and 36 percent if pregnancy was reduced from 20 percent to 14 percent. With 80 percent of Down syndrome screened for, and no change in pregnancy rate, 75 percent of subfertile women would do PGS. That is akin to the 81 percent screening potential reported by Munné *et al.* (2006).

Decrease in spontaneous abortions

Most studies about pregnancy loss agree that maternal age, followed by previous miscarriage, are the major risk factors (Regan *et al.*, 1989; Fretts *et al.*, 1995; Nybo Andersen *et al.*, 2000; La Rochebrochard & Thonneau, 2000; Kupka *et al.*, 2004).

SART (2005) data indicate that 13.3 percent of ART pregnancies in patients younger than 35 years of age result in miscarriage, 17.7 percent in patients aged 35–37, 26.2 percent in those aged 38–40, 39.4 percent in 41–42-year-olds, and 53.3 percent in patients 43–44 years old. The German registry (Kupka *et al.*, 2004) reports even higher rates of miscarriage after IVF, with 23.9 percent in patients aged 35–36, 26.3 percent in those aged 37–38, 36.6 percent in 39–40-year-olds, 43.1 percent in those aged 41–42, and 56.1 percent in women over 42 years of age.

In sporadic miscarriages among the general population, chromosome abnormalities range from 39 percent to 76 percent, depending on the study (Warburton *et al.*, 1986; Jacobs & Hassold, 1987; Stern *et al.*, 1996; Ogasawara *et al.*, 2000; Carp *et al.*, 2001; Stephenson *et al.*, 2002). Some of these studies, particularly the earlier ones, appear to underestimate chromosome abnormalities. This is because conventional karyotyping requires tissue culture, which is prone to maternal contamination and fails up to 25 percent of the time, failing more often if the embryo is chromosomally abnormal (Qumsiyeh *et al.*, 2000; Carp *et al.*, 2001).

Chromosome studies in spontaneous abortions of ART patients also indicate a high rate of chromosome abnormalities (Spandorfer *et al.*, 2004; Lathi *et al.*, 2007), with 65–71 percent of spontaneous abortions being chromosomally abnormal, and increasing with maternal age, from 65 percent in women aged 39 and younger to 82 percent in women aged 40 and older (Spandorfer *et al.*, 2004).

Because of the high rate of chromosome abnormalities in spontaneous abortions, PGS should substantially reduce the rate of miscarriage in infertile patients undergoing ART. Indeed, FISH with probes for chromosomes 13, 15, 16, 18, 21, 22, X, and Y can detect 72–83 percent of the chromosomally abnormal fetuses routinely detected by karyotyping in women of advanced maternal age (Jobanputra *et al.*, 2002; Lathi *et al.*, 2007). Since this combination of probes is our current standard, PGS should also eliminate close to 80 percent of all chromosomally abnormal embryos at risk of causing a miscarriage.

In a multicenter study control subjects were compared with a test group undergoing embryo biopsy and PGS for aneuploidy of chromosomes X, Y, 13, 18, and 21 (Munné *et al.*, 1999), and the results revealed a significant reduction in spontaneous abortions, from 23 percent in the control subjects to 9 percent in the PGS group ($p<0.05$) and a significant increase in ongoing pregnancies (10.5 percent versus 16.1 percent, $p<0.05$). In another study, an abortion rate of only 9 percent was reported after PGS of aneuploidy for 343 cases in women aged >36 years (Gianaroli *et al.*, 2001a; Gianaroli *et al.*, 2001b) instead of an expected 16 percent.

In two studies, pregnancy outcome in 191 couples after PGS was compared with previous pregnancy history. In one study Gianaroli *et al.* (2004) reported an 11.4 percent pregnancy loss compared with 88.5 percent before PGS ($p<0.001$). Verlinsky *et al.* (2005) reported a pregnancy loss of 28 percent after PGS of

Table 13.7 Comparison of non-PGS and PGS cycles from five IVF centers

IVF clinic	Age group	Non-PGD cycles	Loss rate (%)	Live birth (%)	PGD cycles	Loss rate (%)	Live birth (%)
1	38–42	505	27	35	70	22	40
2	38–42	210	36	14	72	27	15
3	38–42	1204	34	12	120	15	23
4	38–42	509	29	15	236	26	22
5	38–42	191	25	17	208	16	25
Total	38–42	2619	30	18	706	21	24

PGD, preimplantation genetic diagnosis.
Total PGS results were significantly better for pregnancy rate ($p<0.01$), miscarriage rate ($p<0.01$), and pregnancy to term ($p<0.001$) (Munne *et al.* 2007b).

aneuploidy in a population with an average maternal age of 37, and compared that with the previous reproductive history of that same group (68 percent, $p<0.001$). These two studies have been criticized on grounds of comparing a self-selected population with prior negative reproductive history with their next cycle, overestimating the true baseline of spontaneous abortions in that group.

A more recent study (Munné *et al.*, 2006a) comparing SART data (2005) on spontaneous abortions with PGS data on 522 pregnancies from 100 IVF centers, revealed a significant decrease in spontaneous abortions after PGS, from 19 percent to 14.1 percent ($n=382$) ($p<0.05$) in women aged 35–40, and from 40.6 percent to 22.2 percent ($n=140$) ($p<0.001$) in women >40 years old. This study had the limitation that not all centers in the SART database contributed to the PGS group. To solve that problem a similar study (Munné *et al.*, 2007b) compared SART data from 2003–2005 from five IVF centers with extensive experience in biopsy (at least 50 cases in that period and at least 10 percent of their IVF cycles being PGS), with the PGS data from those same centers and same years, for patients aged 38–42 years. There was a significant reduction in spontaneous abortions from 30 percent in non-PGS cycles to 21 percent in PGS cycles ($p<0.01$) (Table 13.7).

In each of the studies showing an improvement after PGS, the protocols used were of the highest standards, and the personnel well-practiced in the PGS procedures.

Increase in implantation, pregnancy, and take-home baby rates

Reviewing the literature there have been two starkly different conclusions: a first group of investigators represents and supports the hypothesis that PGS for aneuploidy improves implantation and reduces miscarriage rates (Munné *et al.*, 1999; Gianaroli *et al.*, 1999; Munné *et al.*, 2003; Gianaroli *et al.*, 2004; Munné *et al.*, 2005; Verlinsky *et al.*, 2005; Munné *et al.*, 2006a, Colls *et al.*, 2007; Munné *et al.*, 2007b), and a second group who were not able to demonstrate any significant differences between control and PGS patients (Staessen *et al.*, 2004; Platteau *et al.*, 2005) or showed a negative effect (Mastenbroek *et al.*, 2007). There are critical differences between these studies, which will be reviewed in this section.

There have been basically three types of comparative trial investigations of PGS: trials that used retrospective analyses or compared patients only to their prior reproductive history (Gianaroli *et al.*, 2004; Munné *et al.*, 2005; Taranissi *et al.*, 2005; Verlinsky *et al.*, 2005; Munné *et al.*, 2006; Munné *et al.*, 2007b); those that used prospective datasets comparing patients who accepted PGS with patients who declined it (Munné *et al.*, 1999; Gianaroli *et al.*, 1999; Munné *et al.*, 2003; Colls *et al.*, 2007); and prospective data in a randomized controlled trial (Staessen *et al.*, 2004; Platteau *et al.*, 2005; Mastenbroek *et al.*, 2007).

Some retrospective studies are problematic. For instance, studies comparing previous pregnancy history before and after PGS have the obvious bias that most patients choosing PGS had prior poor results without PGS (Gianaroli *et al.*, 2004; Verlinsky *et al.*, 2005).

Other retrospective studies may contain inclusion biases. For instance, Munné *et al.* (2006) compared cycles from 100 IVF clinics performing PGS to the SART 2000 database (>300 clinics), detecting a significant decrease in spontaneous abortions after PGS. It may be argued that IVF centers performing PGS were not representative of the whole IVF population represented by SART. Possibly that problem was solved in a later study, which compared PGS cycles from five IVF centers, to cycles from those same centers that

Table 13.8 Summary of prospective studies comparing preimplantation genetic screening and control assisted reproductive technology outcome

Study Characteristics	1	2	3	4	5	6	7
Cells biopsied	One	One	One	One	Two	One	One
Chromosomes analyzed	4–8**	8	8	8	6	8	8
Analysis of 15, 22 chromosomes	No**	Yes	Yes	Yes	No	Yes	No
Fixation type appropriate?	Yes	Yes	Yes	Yes/ No†	No	Yes	No
No result rate	NA	3.1%	4.4%	N/A	4.7%	4.7%	20.1%
Average no. of embryos, PGS group	N/A	6.7	8.9	N/A	5.9	7.2	5.4
Type of study	Comp	Comp	Comp	Randzd	Randzd	Comp	Randzd
Results							
Cycles retrieved, control	117	127	138	28	141	100	402
Cycles retrieved, PGS	117	135	138	29	148	100	Unclear***
Average embryos replaced, control	3.6	3.0[a]	3.7[a]	N/A	2.8[a]	2.4[a]	1.9
Average embryos replaced, PGS	3.1	1.8[a]	2.0[a]	N/A	2.0[a]	1.5[a]	1.8
Implantation rate, control (%)	13.7	12.4[a]	10.6[c]	N/A	11.5	20[d]	14.7[e]
Implantation rate, PGS (%)	17.6	24.2[a]	17.6[c]	N/A	17.1	31[d]	16.8–6[e¥]
Pregnancy rate, control (%)	29.9	25.1	N/A	20.7	27.7	32	84
Pregnancy rate, PGS (%)	35.9	29.1	N/A	43.0	19.6	35	Unclear***
Pregnancy loss rate, control (%)	33.8[c]	20.6	N/A	N/A	25.6	28[d]	21.4
Pregnancy loss rate, PGS (%)	15.0[c]	5.4	N/A	N/A	25.0	6[d]	Unclear***
Ongoing implantation rate,* control (%)	10.6[c]	10.2[a]	N/A	N/A	10.4[b]	14.1[d]	N/A
Ongoing implantation rate,* PGS (%)	15.9[c]	22.5[a]	N/A	N/A	16.5[b]	28.9[d]	N/A
Ongoing pregnancy rate, control (%)	22.2	20.0	N/A	N/A	20.6	26	16.4

Table 13.8 (Cont.)

Study Characteristics	1	2	3	4	5	6	7
Ongoing pregnancy rate, PGS (%)	32.5	27.6	N/A	N/A	14.9	31	Unclear***

1. Munné *et al.* (1999); 2. Gianaroli *et al.* (1999); 3. Munné *et al.* (2003); 4. Werlin *et al.* (2003); 5. Staessen *et al.* (2004); 6. Colls *et al.* (2007); 7. Mastenbroek *et al.* (2007).
Comp, prospective non-randomized comparative study; Randzd, prospective randomized study. *Fetus ongoing ≥12 weeks/ embryos replaced.
[a] $p<0.001$; [b] $p=0.06$; [c] $p<0.05$; [d] $p<0.025$; [e] =59 percent implantation reduction caused by the biopsy alone, when no PGS analysis was performed.
** Only 31/117 cycles with eight probes.
[†]Different centers in this multicenter study used different fixation types.
[¥] There were three subgroups of cycles in the PGS group, the first with two normal embryos replaced (16.8 percent implantation rate), the second with two undetermined embryos replaced (6 percent implantation rate), and the rest.
*** Because PGS cycles were reported together with a subgroup of cycles with no PGS analysis (see ¥), PGS pregnancy, miscarriage, and ongoing pregnancy rates cannot be assessed properly.

did not have PGS, again showing a decrease in spontaneous abortions and an increase in take-home baby rates (Munné *et al.*, 2007b). Although that paper may be criticized on the grounds that PGS cycles usually should have larger cohorts of embryos than those without PGS, the literature indicates higher rates of chromosome abnormalities in large cohorts of embryos than in small ones (Reis Soares *et al.*, 2003; Munné *et al.*, 2006c), which should affect negatively more PGS cycles. This is, of course, one of the prerequisites for optimal PGS results, a larger cohort of embryos per procedure.

Regarding prospective non-randomized and prospective randomized studies, there are many differences between them, some extremely important: the number of cells being biopsied; the number and type of chromosome probes applied; the form of randomization; the type of cell fixation used; the error rate and undiagnosed rate of embryos; and the number of embryos available for biopsy (Table 13.8).

As shown in Table 13.8 studies in group 2 used inappropriate methodology with the result of a lack of positive results. These methodology problems included for example biopsying two cells per embryo (Staessen *et al.*, 2004), which according to Cohen *et al.* (2007) most probably eliminates any potential beneficial effect of the PGS selection. Indeed, the same group of Staessen *et al.* (2004) recently published a study on a clinically randomized controlled trial comparing PGS results applying one-cell and two-cell biopsy (Goossens *et al.*, 2008). In this new study Goossens *et al.* (2008) found a significant negative influence on embryo development from day three to day five after two-cell biopsy compared with one-cell biopsy ($p<0.007$), particularly in embryos with poorer morphology ($p<0.0001$). The implantation rate between one-cell biopsy (23.5 percent) and two-cell biopsy (17.3 percent) was not significant, and pregnancy rate was just below significance ($p=0.068$). Interestingly, error rates for FISH were similar between one-cell and two-cell biopsies, thus making it unnecessary to biopsy two cells (Goossens *et al.*, 2008).

Several type-2 studies have a low average number of embryos produced, specifically Mastenbroek *et al.* (2007), resulting in an extremely limited potential selection. Prior studies have indicated that in order to improve pregnancy outcomes, a minimum number of embryos ($n=8$) is necessary (Munné *et al.*, 2003) (Table 13.9). Similarly, Tur-Kaspa (2007) indicated that best results are obtained with a minimum of 13 oocytes. This is because when the total number of embryos is low (four or fewer), most control-group embryos are replaced and the total number of normal embryos replaced is similar in control and PGS groups. PGS is a selection tool to improve ART outcome; if there are not enough embryos to make a selection pregnancy rates cannot improve.

Other methodology problems in type-2 studies were that chromosomes 15 and 22 were not analyzed (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007). These chromosomes account for 24 percent of chromosome abnormalities detected in IVF spontaneous abortions (Lathi *et al.*, 2007) and at least 10 percent of day three abnormal embryos (Munné *et al.*, 2004a) (Table 13.10). Also, fixation methods were not the ones producing the lowest error rates in the studies by Staessen *et al.* (2004) and Mastenbroek *et al.* (2007).

Table 13.9 Pregnancy and implantation rates in PGS patients and their respective controls, depending on the number of 2PN zygotes

	Pregnancy[a]		Implantation[b]	
Groups	Control	PGS	Control	PGS
Total***	29% (30/103)	31% (32/103)	10% (38/367)**	19% (39/201)**
<8 zygotes	25% (14/55)	14% (6/43)	9% (17/194)	19% (10/54)
≥8 zygotes	33% (16/48)	43% (26/60)	12% (21/176)	20% (29/147)

[a] Cycles pregnant with fetal heartbeat; [b] Fetal heartbeats per embryos replaced.
** $p<0.005$.
*** Patients aged 35 and older with fewer than two previously failed in vitro fertilization (IVF) cycles. Average maternal age in both groups was 40 years. From Munné *et al.* (2003).

Finally, probably the most damaging methodological shortcoming of the study by Mastenbroek *et al.* (2007) is its fourfold higher rate of undiagnosed embryos (20 percent) compared with other studies in Table 13.8 (3–5 percent) and the 1.8–2.5 percent of Goossens *et al.* (2008). In addition, by replacing these undiagnosed embryos, Goossens *et al.* (2008) created a third arm in their study, that is, embryos biopsied but undiagnosed. No other study to date had this arm, and it would be unethical to design a priori such a study knowing that biopsy may be detrimental, but not offering PGS to balance or compensate in excess that damage. Interestingly, this third arm (biopsy, no PGS) showed in their hands that the biopsy method used by Mastenbroek *et al.* (2007) produced a 59 percent reduction in implantation potential (from 14.7 percent in control subjects to 6 percent in this group), something unheard of in other studies. After PGS, and even not analyzing chromosomes 15 and 22, and offering to patients with very few embryos, PGS selection was able to compensate the biopsy damage (assuming that was similar in the "biopsy and no PGS" and "biopsy and PGS" groups), producing 16.8 percent implantation rates, but not enough to be better than controls.

None of the studies mentioned above is perfect, but comparison studies using appropriate methodology do indicate that PGS may be beneficial to some patients. Randomized studies with appropriate technology and skills are needed (Harper *et al.*, 2008b).

These are some of the characteristics for a randomized study with appropriate methodology.

- A patient's maternal age should be 35 and older, although a perfect study might limit the age to 38–42, where chromosomes abnormalities are higher.
- A minimum of eight zygotes or six embryos with six or more cells each on day three should be available.
- Chromosomes analyzed should at least include X, Y, 13, 15, 16, 18, 21, 22, using a maximum of two hybridization steps; and also using "no-result rescue" to reduce error rates in a third hybridization if needed (Colls *et al.*, 2007).
- The PGS laboratory used should have a low error rate, confirmed by re-analyzing abnormal embryos previously analyzed by PGS. The error rate should be <10 percent, and obviously would indicate that the fixation method used is appropriate.
- The PGS center should be skilled and have a proven low rate of undiagnosed embryos (<5 percent).
- A biopsy method that minimizes embryo damage: IVF centers should have previous experience with PGD, either showing positive results with comparison studies or showing similar pregnancy rates between PGD of single gene defects and non-biopsied cycles.

Other indications for PGS

As well as advanced maternal age and prevention of trisomic conceptions, there are two other indications for PGS, being recurrent miscarriage (RM) and repeated implantation failure (RIF). Another indication, male factor infertility in males of reproductive age undergoing IVF, has had such a limited number of cases studied that the ART outcome cannot be assessed as yet. Studies on RIF (Gianaroli *et al.*, 1999; Kahraman *et al.*, 2000; Pehlivan *et al.*, 2002; Munné *et al.*, 2003; Werlin *et al.*, 2003; Vouillaire *et al.*, 2007), male factor (Silber *et al.*, 2003; Platteau *et al.*, 2004; Donoso *et al.*, 2006), previous trisomic conception (Munné *et al.*, 2004b), and even egg donation (Munné *et al.*, 2006c) have been mostly limited to analyzing chromosome abnormalities in these embryos, so little evidence has been obtained regarding its effect

Table 13.10 Chromosome-specific aneuploidy rates in human cleavage-stage embryos

Chromosome	No. of analyzed embryos	Aneuploid (%)[∞]
XY	1308	0.8
14	279	1.1
6	194	1.5
18	1607	1.5
1	550	2.0
4	236	2.5
7	235	2.6
13	1350	2.8
17	218	2.8
15	638	3.6
21	1548	3.7
16	1209	4.4
22	818	6.2
Total	1607	

[∞] Double aneuploidies counted twice, once for each chromosome.
From Munné *et al.* (2004a).

on ART outcome, which in any case may have other causes (Cohen *et al.*, 1998). Thus, in this section only RM will be discussed.

Recurrent miscarriage

RM in patients with normal karyotype is defined as three or more consecutive spontaneous abortions of less than 20–28 weeks' gestation (Crosignani & Rubin, 1991; Stephenson, 1996). There is little evidence of endometrial rejection or a defective endometrium and 50 percent of cases remain classified as having unknown etiology (Simpson, 1990; Clifford *et al.*, 1994; Quenby *et al.*, 2002). The studies suggested that couples experiencing RM may be more prone to producing chromosomally abnormal conceptions.

Chromosome abnormalities are the major cause of miscarriage, with 99 percent of chromosomally abnormal pregnancies miscarrying (Jacobs & Hassold, 1987) compared with 7 percent of chromosomally normal (McFadyen, 1989). Also, about 85 percent of miscarriages are embryonic losses with <9 weeks of gestation, before fetal heartbeat is detected (Bricker & Farquharson, 2002), and of those, karyotype studies report an 87 percent rate of chromosome abnormalities (Burgoyne *et al.*, 1991). M-CGH studies, which preclude maternal contamination, report even higher rates (Daniely *et al.*, 1998; Fritz *et al.*, 2001; Stephenson *et al.*, 2002).

Other studies suggest that not only chromosome abnormalities are involved. The frequency of abnormal embryonic karyotypes has been found to be higher in sporadic abortions (63–76 percent) than in RM (40–60 percent) (Jacobs & Hassold, 1987; Stern *et al.*, 1996; Ogasawara *et al.*, 2000; Stephenson *et al.*, 2002). In addition, women aborting two to four consecutive pregnancies had 60 percent chromosomally abnormal abortuses but women with more than four miscarriages had only 29 percent abnormal (Ogasawara *et al.*, 2000). It is important to remark that the study by Ogasawara *et al.* (2000) involved all kinds of RM patients, and not only idiopathic ones.

All the previously mentioned studies were performed on products of conception of clinically recognized pregnancies. Studies on RM cleavage-stage embryos have consistently indicated either more chromosome abnormalities than in control groups (Simon *et al.*, 1998; Vidal *et al.*, 1998; Pellicer *et al.*, 1999; Rubio *et al.*, 2003) or similar rates in fertile RM patients and infertile ones (Munné *et al.*, 2005; Munné *et al.*, 2006).

Reduction in spontaneous abortions in patients with idiopathic RM

The main goal of most patients undergoing PGS for RM is to prevent another miscarriage and a few studies have evaluated spontaneous abortion rates after PGS (Munné *et al.*, 2005; Garrisi *et al.*, 2007). Some other studies were uncontrolled (Platteau *et al.*, 2005) and/or did not assess miscarriages (Rubio *et al.*, 2003; Werlin *et al.*, 2003).

Munné *et al.* (2005) offered PGS to idiopathic RM patients and compared pregnancy loss with that expected according to their previous number of miscarriages

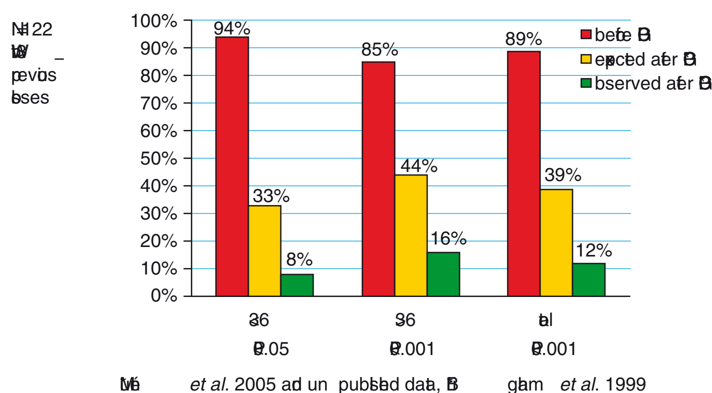

Figure 13.3 Reduction in spontaneous abortions after preimplantation genetic screening (PGS).

Table 13.11 PGS results according to previous number of miscarriages

No. of previous SAB	Cycles	Percentage lost expected	Percentage lost after PGS	
2	95	32	26	NS
3–5	169	41	23	$p<0.025$
>5	15	44	47	NS

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Table 13.12 Preimplantation genetic screening results according to fertility

Method of conception	Cycles	Percentage lost expected	Percentage lost after PGS	*P*	Percentage deliveries
IVF	56	40	14	NS	34
Natural	113	41	17	$p<0.005$	38

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and maternal age, and according to prediction from the study by Brigham *et al.* (1999). These RM patients had experienced an average of 3.9 previous pregnancies before the PGS cycle, of which 87 percent were lost. Based on the formula of Brigham *et al.* (1999) the RM group expected losses of 36.5 percent; after PGS the observed loss rate was only 16.7 percent (p=0.028). In the subgroup aged ≥35, the expected loss in the next pregnancy was 44.5 percent compared with an observed 12 percent (2/17) (p=0.007) after PGS. When the data of Munné *et al.* (2005) were combined with newer unpublished in-house data, the results were even better, showing that PGS for idiopathic RM may reduce miscarriage rates in all age groups (Figure 13.3).

Recently, Garrisi *et al.* (2007) have studied the effect of PGS reduction of spontaneous abortions in relation to previous miscarriages. Their study found that PGS significantly reduces miscarriage rates in patients with three to five previous miscarriages, but not significantly in patients with two or more than five miscarriages (Table 13.11). That is in agreement with Ogasawara *et al.* (2000), who showed that women with five or more miscarriages had more chromosomally normal miscarriages than those with three or four.

In addition, Garrisi *et al.* (2007) analyzed the effect of PGS reduction in miscarriages in relation to the fertility status of the patient. They found that patients with RM who were fertile after treatment had a significant reduction in miscarriages after PGS, from 41 percent to 17 percent (p<0.005) (Table 13.12). Overall, it is reasonable to conclude that RM with idiopathic etiology in women of advanced maternal age is mostly

Table 13.13 Outcome of preimplantation genetic screening for translocations

Type	Average age	Cycles	No. transferred	Not pregnant	Pregnant	Miscarried	Ongoing pregnancy	Percent loss	Percent ongoing
ROB	34.0	133	16	67	50	2	48	2	36.1
REC	36.1	338	106	153	79	7	72	2	21.3

ROB, Robertsonian translocation; REC, reciprocal translocation.
Reproduced from Munné (2006d), with permission from Oxford University Press.

a problem of recurrent chromosomally abnormal embryos. This is in agreement with previous observations in translocation carriers in which PGD of translocations significantly reduced spontaneous abortions (Munné *et al.*, 1998b; Munné *et al.*, 2000b).

Based on existing evidence at the time, which supported the concept that PGS may reduce spontaneous abortions whilst no other studies supported the contrary, the Preimplantation Genetic Diagnosis International Society (PGDIS) guidelines indicate that PGS should be offered for idiopathic RM (PGDIS, 2007). Other guidelines that only take into consideration randomized clinical trials do not agree (the practice committee of SART and the American Society for Reproductive Medicine (ASRM), 2007).

Reduction in spontaneous abortions in patients with RM caused by translocations

The unbalanced products of a translocation are usually lethal and present a real risk to the patient with RM (see Chapter 12). Among 1284 couples with recurrent miscarriage, 58 (4.5 percent) were carriers of translocations. In the next pregnancy the reciprocal translocation patients miscarried significantly more often (68 percent) than couples without structural abnormalities (28 percent) (n=1184) (p<0.001) (Sugiura-Ogasawara *et al.*, 2004). It has been shown that PGD for patients with translocations substantially increases their chances of sustaining a pregnancy to full term (Munné *et al.*, 1998b; Munné *et al.*, 2000b). Data from another group showed that in 45 pregnancies, 18 percent spontaneously aborted, much lower than the 88 percent of pregnancy loss in these patients prior to undertaking PGD procedures (p<0.001) (Verlinksy *et al.*, 2002; Verlinsky *et al.*, 2005).

For most translocation patients, the risk of consecutive pregnancy loss is their major incentive in enrolling in a PGD program. Indeed, about 2.7 percent to 4.7 percent of RM patients carry structural chromosome abnormalities (De Braekeleer & Dao, 1990; Carp *et al.*, 2004; Goddijn *et al.*, 2004; Sugiura-Ogasawara *et al.*, 2004; Stephenson & Sierra, 2006).

There is ample evidence that PGD of translocations substantially increases a couple's chances of sustaining a pregnancy to full term, with couples with translocations showing, on average, 85 percent pregnancies lost before PGD, and 0–25 percent after PGD (Munné *et al.*, 1998b; Munné *et al.*, 2000; Munné *et al.*, 2005; Munné *et al.*, 2006d; Verlinsky *et al.*, 2002; Gianaroli *et al.*, 2003; Lim *et al.*, 2004; Verlinsky *et al.*, 2005; Otani *et al.*, 2006). In the last review of 471 PGD translocation patients, we observed only 7 percent (9/129) pregnancy loss after PGD (Munné, 2006) (Table 13.13). A study specific for patients with RM attributed to translocations and with no history of live births (0/117) showed that after PGD only 5.3 percent were lost (Otani *et al.*, 2006). In addition, the cumulative pregnancy rate was 57.6 percent and a cumulative ongoing pregnancy rate was 54.5 percent in the short period of time of 1.24 IVF cycles.

PGD results may be compared to an expected 26–68 percent pregnancy loss (p<0.001) for patients when they are not treated by PGD (Goddijn *et al.*, 2004; Sugiura-Ogasawara *et al.*, 2004; Stephenson & Sierra, 2006), with the advantage that time to conception is much faster with IVF–PGD than without it (Table 13.14). Although patients may have a successful outcome without PGD, the psychological pain inflicted by repeated spontaneous abortions is considerable (Aoki *et al.*, 1998); so the shorter this period, the better.

Toward a full chromosome count

Current FISH tests can effectively analyze up to 12 probes, which means that only about 67 percent (Lathi *et al.*, 2007) of known chromosome abnormalities in spontaneous abortions are detected. Tests that can score all 24 chromosome types in a timeframe compatible with days three to five transfer are highly desirable.

Several strategies have been suggested (Harper & Wells, 1999), including: quantitative fluorescence multiplex PCR (QF-PCR) (Mansfield, 1993; Sherlock *et al.*, 1998); cell conversion (Verlinsky & Evsikov, 1999; Willadsen *et al.*, 1999); metaphase comparative genomic hybridization (m-CGH) (Kallioniemi *et al.*, 1992; Voullaire *et al.*, 1999; Wells *et al.*, 1999); FISH or

Table 13.14 Pregnancy outcome in translocation carriers after PGD or no-PGD treatment

	Patients (cycles)	Patients conceiving a child (per cycle; cumulative)	Risk of miscarriage	Time frame
Reciprocal translocations With PGD				
Lim *et al.* (2004)	43 (64)	14 (22%, 33%)	17% (3/18)**	1.4 cycles
Munné *et al.* (2006d)	239 (338)	71 (21%, 30%)	9% (7/79)	1.4 cycles
Otani *et al.* (2006)	29 (36)	17 (47%, 59%)	0% (0/17)	1.2 cycles
Without PGD				
Sugiura-Ogasawara *et al.* (2004)[a]	47	15 (32%, N/A)	32 (15/47)	11.5 months[d]
Sugiura-Ogasawara *et al.* (2004)[b]	47	32 (N/A, 68%)	65% (62/95)	23.3 months[c]
Goddjin *et al.* (2004)[e]	41	25 (N/A, 74%)	26% (11/43)	6 years
Stephenson and Sierra (2006)[f]	28	18 (N/A, 64%)	38% (13/34)	4.2 years[g]
Robertsonian translocations With PGD				
Munné *et al.* (2006d)	88 (133)	48 (36%, 55%)	4% (2/50)	1.4 cycles
Without PGD				
Sugiura-Ogasawara *et al.* (2004)[b]	11	7 (N/A, 64%)	36% (4/11)	23.3 months[h]
Stephenson and Sierra (2006)[f]	12	8 (N/A, 67%)	31% (4/13)	4.2 years[g]

[a] First pregnancy after ascertainment of carrier status.
[b] Cumulative success rate.
[c] As reported by Ogasawara and Suzumori (2005).
[d] Although Ogasawara and Suzumori (2005) do not mention it, if to achieve 95 pregnancies took 23.3 months, to achieve 47 is estimated to take about 11.5 months.
[e] Data include: 28 reciprocal translocations; five pericentric inversions; four paracentric inversions; three Robertsonian translocations; and one marker chromosome. Inversions and Robertsonian translocations produce fewer chromosomally abnormal pregnancies and therefore are more likely to succeed.
[f] While Sugiura-Ogasawara *et al.* (2004) patients were not treated for RM, Stephenson and Sierra (2006) patients were treated for different conditions other than translocations, thus the lower miscarriage rate compared with Sugiura-Ogasawara *et al.* (2004).
[g] Average age at start 29.8 years and 34 years at end, without differentiating reciprocal and Robertsonian.
[h] As reported by Ogasawara and Suzumori (2005) for reciprocal translocations, and thus it could be different.
** Lim *et al.* (2004) did not use appropriate methods of PGS for translocation, which, for interphase FISH analyses requires the use of two proximal and one distal probe to the breakpoint or one proximal and two distal, in order to detect all possible unbalanced embryos; hence the high rate of spontaneous abortions in this study.

spectral karyotyping (SKY) with 24 probes (Márquez *et al.*, 1998; Fung *et al.*, 2000; Sandalinas *et al.*, 2002; Weier *et al.*, 2001); and DNA micro-arrays (array-CGH) (Pinkel *et al.*, 1988).

Of these techniques, only m-CGH has been applied clinically (Wilton *et al.*, 2001; Wells *et al.*, 2002; Wilton *et al.*, 2003; Sher *et al.*, 2007), although DNA micro-arrays still hold real potential for clinical application. Of the others, QF-PCR has not been able to give results on single-cell, and cell conversion, which involves the fusion of cells to oocytes or polar bodies to convert them to metaphase stage, is not conducive for application in reference laboratories and requires highly skilled personnel; the rate of conversion to obtain karyotypable or SKY-quality metaphases is too low, and the same problem applies to polar bodies fixed in different conditions (Márquez *et al.*, 1998; Evsikov *et al.*, 2000).

Comparative genomic hybridization

m-CGH is promising (Kallioniemi *et al.*,1992), as it is a DNA-based method capable of accurately determining total or partial aneuploidy by detecting losses or gains of all 46 chromosomes. The combination of a specific type of PCR which amplifies the entire genome (whole-genome amplification) and FISH technology, enabled the application of m-CGH for the detailed investigation of oocytes, polar bodies, and embryos (Voullaire *et al.*,, 2000; Wells & Delhanty, 2000; Gutiérrez-Mateo

Table 13.15 Embryos classified on day 3 by PGS that reach blastocyst stage

Study	Average maternal age	Technique (chromosomes analyzed)	Percent euploid reaching blastocyst %	Percent abnormal reaching blastocyst %	Percent blastocyst that are: Euploid	Abnormal
Li *et al.* (2005)	38.8	FISH (5)	63 (54/86)	20 (55/281)	N/A*	N/A*
Magli *et al.* (2000)	34.8	FISH (6)	34 (24/70)	19 (14/73)	63 (24/38)	37 (14/38)
Sandalinas *et al.* (2001)	38.5	FISH (9)	68 (40/59)	16 (30/185)	57 (40/70)	43 (30/70)
Sher *et al.* (2007)	27.5	CGH (24)	93 (25/27)	21 (11/52)	69 (25/36)	31 (11/36)
Total			59 (143/242)	19 (110/591)	62 (89/144)	38 (55/144)

* Not all euploid replaced on day 5, thus percent blastocyst cannot be calculated.

et al., 2004a; Gutiérrez-Mateo *et al.*,2004b; Fragouli *et al.*, 2006a; Fragouli *et al.*, 2006b). The technique has been successfully tested in human blastomeres and babies have been born after PGS using m-CGH (Voullaire *et al.*, 1999; Voullaire *et al.*, 2000; Wells & Delhanty, 2000; Wilton *et al.*, 2001;Wilton *et al.*, 2003). However, m-CGH is labor-intensive and the technique still requires five days to yield results; so after biopsy, the embryos are frozen and then thawed for replacement, bringing increased embryo loss, but not compensating by the improved embryo selection potential (Magli *et al.*, 1999; Munné & Wells, 2003; Hill, 2003; Verlinsky & Kuliev, 2003). It was therefore mostly abandoned. To avoid cryopreservation, m-CGH analysis of first polar bodies was proposed and applied (Wells *et al.*, 2002), but M-II errors, which some studies indicate account for half of the chromosome abnormalities (Verlinsky & Kuliev, 2003), were not detected and the time still required made its widespread application unrealistic.

Recently, Sher and coworkers (2007) used an improved method of oocyte and embryo freezing, termed "vitrification" (Yoon *et al.*, 2000; Yoon *et al.*, 2007), which recently produced >85 percent survival rates, and combined it with m-CGH for the analysis of first polar bodies. Even though the pregnancy rates reported in this study were acceptable, the methodology was not validated, as it was in other investigations involving m-CGH examination of oocytes and polar bodies (Gutiérrez-Mateo *et al.*, 2004a; Gutiérrez-Mateo *et al.*, 2004b; Fragouli *et al.*, 2006; Fragouli *et al.*, 2007). Additionally, examination of first polar bodies only, meant that, as with the Wells *et al.* (2002) investigation, only errors affecting chromosomes during meiosis-I were detected. However, an ongoing clinical trial involving the use of m-CGH for the analysis of both first and second polar bodies, in combination with vitrification of zygotes generated from poor prognosis IVF patients, has demonstrated that meiosis-II errors are actually more frequent than meiosis-I (55.3 percent versus 39.2 percent) (Fragouli & Wells, unpublished). This suggests that in order for m-CGH to significantly improve clinical outcome examination of both polar bodies is necessary. It therefore remains to be seen if m-CGH of polar bodies is capable of improving ART outcome more than the FISH technology currently used.

Interestingly, it seems that zygotes diagnosed as normal by m-CGH of both polar bodies reach blastocyst stage in much higher numbers than those selected for FISH (Table 13.15), thus indicating a better selection, since all chromosomes are analyzed with m-CGH. However, the studies' differences in maternal age compared in Table 13.15 may also play a part.

DNA micro-arrays

The use of DNA micro-arrays in PGS to determine chromosomal copy number has been proposed (Harper & Wells, 1999; Weier *et al.*, 2001). However, this technology has only recently been successfully applied to single cells (Kearns *et al.*, 2007; Munné *et al.*, 2007c; Treff *et al.*, 2007), although not yet clinically. Two types of DNA micro-array platforms have been utilized for this purpose: array comparative genomic hybridization (a-CGH) and single nucleotide polymorphism (SNP) arrays. Both approaches require a whole-genome amplification (WGA) step prior to use with single cells. Copy number aberrations are detected in much the same way using array CGH as in conventional m-CGH. Namely, test and reference DNA are differentially labeled, co-precipitated, and co-hybridized onto

a target representative of the genome. However, the targets used in a-CGH are bacterial artificial chromosome (BAC) clones spotted onto the arrays, permitting a much higher degree of resolution than is possible by cytogenetic means. Using optical methods, the relative abundance of each probe is measured, intensity ratios are calculated, and chromosomal status is determined. SNP arrays are spotted with oligonucleotide probes targeting regions of copy number variation. Prior to hybridization onto the array, the test sample's DNA is digested with a restriction enzyme and the resulting adaptor-ligated fragments are amplified. Thereafter, the amplified DNA is fragmented and labeled. Copy-number assessment of scanned images is performed using genotyping software. There are advantages and disadvantages to the use of each platform. For example, SNP chips are particularly susceptible to noise and bias owing to their short target size. This drawback would be particularly pronounced in amplified single-cell preparations. BAC CGH arrays are very robust, producing a high signal to noise ratio, better reproducibility, and lower standard deviations. The use of FISH mapped BACs further improves the reliability and specificity of CGH arrays, which are preferable to the use of genomic databases and representations as is the case with SNP arrays. However, CGH arrays cannot detect copy-neutral events (such as the loss of heterozygosity resulting from duplications). However, this limitation is of little relevance in reproductive medicine and more of a concern for cancer geneticists.

PGS versus non-invasive embryo selection techniques

A wide variety of techniques is used to select human embryos for the highest implantation potential. The oldest methods are based on morphology assessment (reviewed by Alikani, 2001) and have been studied extensively. But chromosome abnormalities bear little relation to morphology (Munné *et al.*, 1995a; Márquez *et al.*, 2000; Magli *et al.*, 2007); thus, the best morphology embryos in women aged 35–37 and older are 56 percent chromosomally abnormal compared with 75 percent for those that arrest (Munné *et al.*, 2007b). In these studies poor morphology is often linked to mosaicism and other post-meiotic abnormalities, but not to aneuploidy. Another selection technique that can screen for some chromosome abnormalities is blastocyst morphology, but, as Table 13.15 above shows, 38 percent of all blastocysts are still aneuploid in older patients. A recent book edited by Elder and Cohen (2007) reviews other selection techniques such as metabolomics, but as with blastocyst selection, embryos implanting can still miscarry in high numbers or carry chromosome abnormalities (see Table 13.15).

Conclusion

A sizeable fraction of embryos produced during ART procedures are chromosomally abnormal. Current techniques based on morphology and developmental assessment do not screen for the majority of these chromosome abnormalities; thus PGS selects chromosomally normal embryos for replacement, expecting an improvement in implantation, a reduction in spontaneous abortions and trisomic offspring, and an improvement in so-called take-home babies.

However, differences in techniques have produced conflicting results. Some factors seem to be better than others and may be summarized as: appropriate maternal age; minimum number of embryos to be biopsied; a single cell biopsied; extensive experience in biopsy and good control results; the analysis of at least chromosomes X, Y, 13, 15, 16, 18, 21, and 22; cell fixation, fluorescence scoring, and NRR techniques to produce FISH error rates below 10 percent. Under these conditions PGS seems to produce positive ART outcomes. Still, no fully randomized study has been performed to date under these conditions.

For a fraction of patients choosing PGS, high take-home baby rate is not the major goal; we see that 83 percent of patients wish to prevent trisomic conceptions as long as pregnancy rates do not decrease; while 36 percent would choose it even if there were a reduction in pregnancy rates (Twisk *et al.*, 2007). Also, patients with idiopathic RM or previous miscarriages are reluctant to experience the trauma again and choose PGS to avoid pregnancy loss if at all possible, irrespective of potentially lower pregnancy rates.

Techniques analyzing all chromosomes have the promise of furthering the selection potential of the technique while minimizing error rates.

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Chapter 14

Preimplantation genetic diagnosis for sex-linked diseases and sex selection for non-medical reasons

Caroline Mackie Ogilvie and Paul N. Scriven

Key points

- Sex-linked diseases are caused by mutations in genes carried on the X chromosome. Although the penetrance of the trait is typically high in males and low in females, caution should be exercised in classifying sex-linked diseases as recessive or dominant.
- Fluorescence *in situ* hybridization (FISH) is the technique of choice to sex embryos. FISH provides a very robust test with a low risk of misdiagnosis; however, testing that only determines the sex of the embryos cannot discriminate between normal and affected male embryos or between normal and carrier female embryos, which is wasteful of male embryos and accepts a level of risk that carrier females may have clinical features of the trait.
- Polymerase chain reaction (PCR) methodology has been used to develop direct tests for known mutations associated with a range of X-linked diseases. Protocols generally utilize a nested PCR approach, to include one, two, or three microsatellite markers closely linked to the disease gene, in order to monitor contamination and allele drop-out, potential sources of misdiagnosis when using PCR-based testing.
- Preimplantation genetic haplotyping (PGH) is a rapid way of developing indirect tests of X-linked disease. The same marker multiplex may be used for all families with the same disease, without the need for the development of family-specific mutation testing. PGH relies on the identification of the chromosome carrying the familial mutation by haplotype analysis of family members. The presence or absence of this chromosome in embryos can then be established by genotyping the biopsied cell. This approach has the added advantage of effectively "fingerprinting" each embryo, which obviates the need for washdrop blanks normally required to monitor contamination.
- Testing for X-linked disease provides special ethical, counseling, and decision-making challenges beyond those posed by autosomal monogenic defects and chromosome rearrangements.
- Many professionals involved in the preimplantation genetic diagnosis (PGD) field take the view that sex selection for a non-medical reason is unacceptable. For others there are varying degrees of ethical risk, and the balance between regulation and reproductive freedom is difficult to assess.

Introduction

Sex-linked diseases are caused by mutations in genes carried on the X chromosome. Typically, the penetrance of the sex-linked trait is high in males and low in females; however, the penetrance for some traits can be moderate or high in females. Caution should be exercised in classifying sex-linked diseases to have a recessive or dominant pattern of inheritance as the degree of variability in heterozygotes is greater than for autosomal traits due to skewed X inactivation, clonal expansion, and somatic mosaicism (see Chapter 3). Online Mendelian Inheritance in Man (OMIM, 2007) currently lists 1006 entries for genes and/or phenotypes with X-linked inheritance.

X-linked diseases with a recessive pattern of inheritance are the most common. The defective gene on the X chromosome tends to have little effect on heterozygote females because there is a second normal copy of the gene on the other X chromosome. However, males with an X chromosome carrying the defective gene are affected with the disease, as there is no second, normal, X chromosome. The affected male who reproduces will have carrier daughters and normal sons. Relatively

common X-linked diseases with a recessive pattern of inheritance are muscular dystrophy and hemophilia A. The muscular dystrophies are a group of hereditary diseases characterized by the progressive loss of muscle cells. Duchenne muscular dystrophy (DMD; OMIM#310200) is the most common and severe form. Progressive muscle wasting results in loss of mobility and frequently death as a result of respiratory failure by the late teens or early twenties. Hemophilia A (OMIM#306700) is a hereditary blood disorder characterized by a deficiency of the Factor VIII blood clotting protein that results in abnormal bleeding; the clinical severity may be mild, moderate, or severe.

X-linked diseases with a dominant pattern of inheritance are less common and can be lethal to males *in utero*. An example is incontinentia pigmenti (IP; OMIM#308300), which is a disorder that affects the skin, hair, teeth, and nails. IP is lethal in most males although there are a few reports of surviving males with a 47,XXY karyotype, somatic mosaicism, or different mutations in the IKBKG gene associated with IP.

Sex-linked diseases were the first target of PGD, and the first successful clinical case used PCR to amplify a specific repeat on the Y chromosome to sex embryos in the presence of an X-linked genetic condition (adrenoleukodystrophy; OMIM#300100) (Handyside *et al.*, 1990). Failure of the Y chromosome sequence to amplify in some cases led to misdiagnosis (Hardy & Handyside, 1992), and this approach is now therefore no longer recommended. Instead, fluorescence *in situ* hybridization (FISH) -based preimplantation genetic diagnosis (PGD) was adopted as a more robust technique for the identification of female embryos (Griffin *et al.,* 1994; Munné *et al.,* 1994); however, a small number of centers still use polymerase chain reaction (PCR) for sexing (Goossens *et al.*, 2008). Although most PGD centers use sexing only, many specific mutation analyses for X-linked disease genes have been developed and applied successfully (Verlinsky *et al.*, 2002; Sermon *et al.*, 2007) and the recent introduction of preimplantation genetic haplotyping (PGH) (Renwick *et al.,* 2006) has opened the door to rapid and straightforward development and application of further specific tests for X-linked disease.

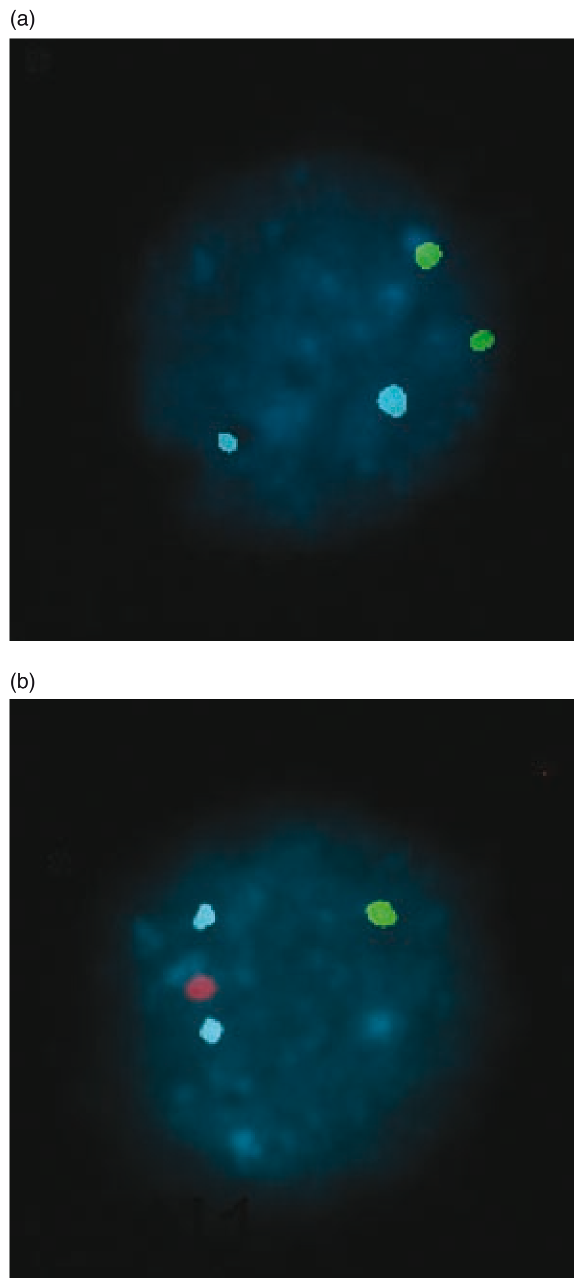

Figure 14.1 Sexing using fluorescence *in situ* hybridization (FISH). Vysis AneuVysion alphasatellite probes for the centromere regions of the X chromosome (SpectrumGreen), Y chromosome (SpectrumOrange) and chromosome 18 (SpectrumAqua). (a) Normal female (XX, 18, 18); (b) Normal male signal pattern (XY, 18, 18).

Approaches to PGD for X-linked diseases

FISH: sexing only

The identification of female embryos using FISH is a standard technique in most PGD centers. Commercial probe sets (e.g., the Abbott Vysis AneuVysion) are generally used for the centromeres of the X chromosome, Y chromosome, and chromosome 18, or another autosome. The different centromeres are discriminated by the different-colored fluorescent tags associated with the specific DNA probes (see Figure 14.1). This test is

extremely robust as in order to misdiagnose a male chromosome complement as female two errors have to occur: first, failure to score the signal for the Y centromere, and then second, to score a single X chromosome signal as two signals. For this reason, embryo misdiagnosis is extremely unlikely using FISH for sex determination. There has been one case of misdiagnosis reported to the European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium (Goossens *et al.*, 2008), which was associated with social sexing; the pregnancy was terminated. The full etiology of this misdiagnosis is unknown, but in addition to the accuracy of scoring FISH signals, other potential sources of error include: polymorphism of the probe binding regions; single cells with more than one nucleus or nuclear fragments; contamination with nuclei from maternal cumulus cells associated with the ovum; and incorrect embryo identity at transfer.

In addition to establishing the sex of the embryo, this test will also identify abnormalities of sex chromosome copy number (for instance, a single X chromosome, which is associated with Turner syndrome, and XXY, which is associated with Klinefelter syndrome), as well as embryos with aneuploidy (monosomy or trisomy) for the autosome. The presence of an autosome probe indicates the ploidy of the embryo, which allows discrimination between a single X chromosome and haploidy (one copy of every chromosome), XXX or XXY and triploidy (three copies of every chromosome), and XXXX or XXYY and tetraploidy (four copies of every chromosome).

FISH: testing for specific deletion

An elegant FISH approach to PGD for Duchenne muscular dystrophy tests for the deletion of exon 45, the causative mutation in approximately two-thirds of Duchenne muscular dystrophy cases, by using an exon 45-specific FISH probe, in combination with probes for the X and Y centromeres (Malmgren *et al.*, 2006), making it possible to distinguish between affected and unaffected male embryos, and between carrier female and normal female embryos.

PCR: sexing only

PCR may be used to amplify a specific repeat on the Y chromosome to sex embryos. However, failure of the Y chromosome sequence to amplify in some cases can lead – and has led – to misdiagnosis (Hardy & Handyside, 1992). This approach is not recommended (Thornhill *et al.*, 2005) and is now used infrequently by centers which contribute data to the ESHRE PGD Consortium (Goossens *et al.*, 2008).

PCR: mutation-specific diagnosis

The disadvantage of the FISH-based sexing approach to X-linked disease is that all male embryos are discarded; on average, half of these discarded embryos will be normal males, and the potential population of embryos available for transfer is diminished. For this reason, PCR methodology has been used to develop direct tests for known mutations associated with a range of X-linked diseases (Verlinsky *et al.*, 2002; Goossens *et al.*, 2008). These protocols generally utilize a nested PCR approach, to include one, two, or three microsatellite markers closely linked to the disease gene, in order to monitor contamination and allele drop-out, potential sources of misdiagnosis when using PCR-based testing. General principles and approaches to direct mutation detection using single-cell PCR are described in Chapter 15.

PCR: preimplantation genetic haplotyping

Work-up of specific mutation tests such as those described above can be lengthy and technically challenging, and for many PGD centers, beyond the scope of their funding and staffing constraints. The introduction of PGH (Renwick *et al.*, 2006) has allowed more rapid development of indirect tests of X-linked disease. PGH relies on the identification of the chromosome carrying the familial mutation by haplotype analysis of family members. The presence or absence of this chromosome in embryos may then be established by genotyping the biopsied cells (see Figure 14.2). This development was made possible by the recent availability of commercial kits allowing extremely effective whole genome amplification, usually by multiple displacement amplification (MDA). MDA is an isothermal technique that uses an φ polymerase, which amplifies the single-cell DNA approximately 10^9 -fold (see Chapter 15). This amplification then provides sufficient product for the application of multiplex marker panels for genotyping. This approach has the added advantage of effectively “fingerprinting” each embryo, thus obviating the need for washdrop blanks normally required to monitor contamination.

Allele drop-out associated with MDA is usually around 20–30 percent, but may in some cases be as high as 40 percent, probably due to a poor-quality starting template, for instance in biopsied cells in which the DNA is degrading; however, the large number of markers that may be tested means that in the vast majority of reported cases, at least one marker

Figure 14.2 Preimplantation genetic haplotyping (PGH) for Duchenne muscular dystrophy. Parental haplotypes were inferred from the genotype of the affected child in the family (black square). Green boxes – markers associated with the paternal haplotype; pink boxes – markers associated with the low-risk maternal haplotype; blue boxes – markers associated with the high-risk maternal haplotype. No affected males were present in the embryo cohort in this cycle.

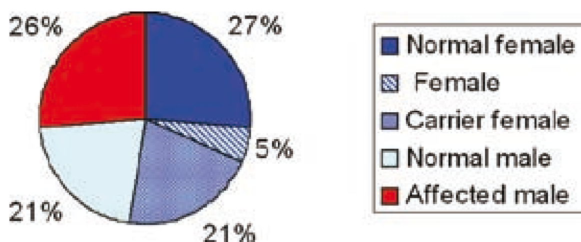

Figure 14.3 Distribution of embryo categories following preimplantation genetic haplotyping (PGH) cycles for X-linked disease. In cycles where fluorescence *in situ* hybridization (FISH) for sex determination is used, the pale blue segment (normal males) would be discarded.

is amplified on each side of the disease gene, thus reducing the risk of misdiagnosis to that of a double recombination event (<0.1 percent). A further advantage of this approach is that the same marker multiplex may be used for all families with the same disease, without the need for the development of family-specific mutation testing. In our experience to date, PGH for DMD results in 56 percent of biopsied embryos with "transferable" results (normal female, carrier female, and normal male) (see Figure 14.3) compared with 33 percent when FISH is used for sex determination.

Indirect testing has also been reported using single-cell protocols without prior whole-genome amplification. For instance, six microsatellite markers were identified in the region of the X chromosome long arm containing the genes for hemophilia A, adrenoleukodystrophy, hydrocephalus, and IP. These genes are in close proximity to each other, and the authors describe using combinations of these markers, found to be informative for the individual families tested, to identify embryos free of disease (Gigarel *et al.*, 2004). Two cells were biopsied from each embryo, and only those with concordant results were transferred, presumably because of the possibility of allele drop-out or contamination, which may be undetected owing to the small number of markers used.

Sexing for non-medical reasons (social sexing, gender balancing)

Many professionals involved in the PGD field take the view that sex selection for a non-medical reason is unacceptable, and few centers reporting to the ESHRE Consortium accept such patients (Goossens *et al.*, 2008). Nevertheless, cycles for social sexing continue to be reported to the ESHRE Consortium. The ethical issues associated with this topic are discussed below.

Current status of PGD for sex-linked disease and sex determination

The latest ESHRE data collection (Goossens *et al.*, 2008) reports on 816 cycles to oocyte retrieval (OR) of sexing only for X-linked diseases, 225 cycles to OR using a specific diagnosis of an X-linked disease, and 412 cycles to OR for social sexing.

The most common diseases tested by centers contributing cycle data to the ESHRE PGD Consortium using a specific diagnosis were Duchenne muscular dystrophy, hemophilia A, and Fragile X. The most common X-linked diseases where sexing only was used were Duchenne muscular dystrophy, hemophilia, and X-linked mental retardation.

Ethical issues

Discarding normal male embryos

PGD for X-linked disease based on identification and transfer of female embryos only is also ethically contentious because of the destruction of potentially normal male embryos. One approach to this dilemma is to sort the sperm prior to the PGD cycle, in order to enrich for X-bearing sperm using flow cytometry (Fugger *et al.*, 1998), and hence to minimize the production of male embryos and increase the number of embryos available for transfer. Flow cytometric separation of X- and Y-bearing spermatozoa is still experimental and the subject of an ongoing clinical trial.

Unusual PGD requests

Ethically difficult requests for PGD include those for male selection in X-linked dominant diseases, such as IP, where the mutation is lethal to male embryos, but carrier females are likely to manifest symptoms. By selecting male embryos, only those without the mutation will reach term, but the transfer of embryos that have a 50 percent chance of abnormality is ethically difficult for PGD staff. Similarly, requests have been made for transfer of male embryos where the male partner carries a mutation of variable penetrance, or an "intermediate" expansion in the Fragile X gene, for instance. This strategy ensures that the mutation is not passed to the next generation (all male embryos will inherit their single X chromosome from their mother, and will therefore be free of the mutation), but involves the destruction of female embryos which may carry only a very small risk of phenotypic abnormality. Conversely, selection of female embryos has been requested for families where X-linked mental retardation is suspected from the family history, but the specific etiology is unknown. In these cases, the condition may not in fact be X-linked, and the association with gender purely coincidental. This possibility means that the decision to accept such families for PGD may be particularly difficult.

Allowing choice of sex after PGD for X-linked disease

Couples embarking on PGD cycles for X-linked disease involving sexing only are clearly aware that any child resulting from a successful cycle will be female. The introduction of direct mutation testing with linked markers, and indirect testing such as PGH, which permits selection and transfer of normal male embryos, has meant that the sex of each embryo is likely to be known at the time of embryo transfer. In the UK, testing to select embryos of a particular sex for social reasons is prohibited by the Human Fertilisation and Embryology Authority (HFEA) Code of Practice. The Human Fertilisation and Embryology Act (1990) empowers an HFEA license committee to vary or revoke a license if they consider there has been a failure to observe the code. The code is designed to prevent social sex selection "by the back door." However, there may be good reasons why, for instance, a male would be preferred to a heterozygote female; while both such embryos would be considered "transferable" by most centers, in some cases there is the possibility that a heterozygote female may show some manifestations of the disease. The legal rights of the couple to have all the information concerning the status of each of their embryos, in order to weigh up the balance of risks for themselves, may outweigh legislation aimed at reducing social sex selection; withholding these rights may be considered unethical as well as illegal. Where a couple has embarked on a PGD cycle because of an X-linked disease in the family, rather than to choose a child of a certain sex, it is usually the case that their

overriding motivation is to have a healthy baby, regardless of gender. Providing potentially relevant information on the status of their embryos after genetic testing may therefore be considered reasonable and responsible. In any case, only in a very small number of cases is it likely that there will be a choice between embryos of equal morphological quality, and the recommendation of embryos for transfer from the available cohort must continue to be based on morphological considerations. Europe-wide quality control of PGD is currently being formulated, and this is an area that is likely to be covered by any best practice and quality guidelines that emerge.

Sexing for non-medical reasons

The Ethics Committee of the American Society for Reproductive Medicine (ASRM) reported on sex selection and PGD (ASRM, 1999) and recognized that "individuals and couples have wide discretion and liberty in making reproductive choices, even if others object." The committee identified that sex selection might provide "perceived individual and social goods such as gender balance or distribution in a family with more than one child, parental companionship with a child of one's own gender, and a preferred gender order among one's children;" the report also highlighted ethical concerns, which include "the potential for inherent gender discrimination, inappropriate control over nonessential characteristics of children, unnecessary medical burdens and costs for parents, and inappropriate and potentially unfair use of limited medical resources."

The ASRM report concluded that PGD to prevent the transmission of a serious sex-linked disease is ethically acceptable, and identified four classes of embryo sex identification by PGD for non-medical reasons with varying degrees of ethical risk. The recommendation was made that PGD "should not be encouraged" where the patient learns the sex of an embryo "as part of, or as a by-product of" PGD done for medical reasons; where the patient requests that sex identification is added to the test that is being done for a medical reason, and where PGD is not a necessary part of the treatment for patients undergoing ART. The recommendation was made that PGD "should be discouraged" where ART and PGD are initiated only for the purpose of sex selection.

For some who consider sex selection for non-medical reasons to be unethical, there is no room for flexibility; however, others may see that in some situations their personal viewpoint may conflict with reasonable freedom of choice for the couples involved. It certainly seems excessively harsh to discourage a couple from having PGD for an X-linked condition, for the simple reason that the test will identify the sex of the embryos available for transfer (see previous section). However, nearly all professionals in the field agree with the final recommendation of the ASRM report, that is, that testing should be discouraged (and is in fact regulated against in many countries) where ART and PGD are not required for any other reason than to select the sex of a child.

Conclusion

The introduction and development of new tests for X-linked disease mean that the range of these conditions for which PGD is available will continue to widen. However, X-linked disease provides special ethical, counseling, and decision-making challenges beyond those posed by autosomal monogenic defects and chromosome rearrangements. PGD to determine the sex of embryos for non-medical reasons continues to be illegal or highly controversial in most countries.

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Chapter 15

Preimplantation genetic diagnosis for monogenic disorders: multiplex PCR and whole-genome amplification for gene analysis at the single cell level

Karen Sermon

Key points

- The first autosomal disease to be diagnosed was cystic fibrosis, followed by reports of a myriad of other monogenic diseases.
- Nowadays, several laboratories offer custom-made preimplantation genetic diagnosis (PGD) for monogenic disease to their patients, and so the exact number of indications for which PGD is offered would be difficult to establish.
- Technically, PGD for monogenic diseases is particularly challenging because the accurate diagnosis on one single blastomere may be hampered by variables that are difficult to control, such as amplification failure, extraneous DNA contamination, and allele drop-out.
- In a constant effort to minimize or detect these problems, single-cell polymerase chain reaction (PCR) has constantly evolved, and currently the "gold standard" is multiplex fluorescent PCR. Here, at least two loci (e.g. the mutation and a linked marker such as a microsatellite, or two linked microsatellite markers) are used to establish a diagnosis, while a nonlinked microsatellite may be used to detect contamination or as a simple form of aneuploidy screening. Because of this, preliminary set-up of diagnosis has become more labor-intensive and complex.
- Recently, a possible solution to this increasing complexity has presented itself. Multiple displacement amplification (MDA) allows that several nanograms (ng) of total pre-amplified DNA are obtained from a single cell. This pre-amplification is followed by analysis of several linked markers, yielding the haplotype (i.e. the paternal or maternal origin of the chromosome may be established) of the analyzed cell around the mutation(s) of interest. This particular form of PGD has been coined preimplantation genetic haplotyping (PGH).

Introduction

The first preimplantation genetic diagnosis (PGD) for monogenic disease was polymerase chain reaction-(PCR-) based. The diseases for which the couples were at risk were X-linked, and the amplification of repetitive Y-sequences was the only technology available at that moment, as fluorescence *in situ* hybridization (FISH) for chromosomal analysis was introduced only some years later (Handyside *et al.*, 1990). It soon became clear that this type of diagnosis was hazardous when a first misdiagnosis occurred. Because the Y-sequence failed to amplify, a male embryo was diagnosed as female (since the diagnosis of female was based on the absence of amplification), was transferred, and led to an affected pregnancy (Hardy & Handyside, 1992). This has prompted the development of schemes and strategies to avoid this type of misdiagnosis: most notably, PCR is now not recommended any more for sexing, also because chromosome abnormalities such as 45, XO may be missed, and diagnosis based on the absence of amplification should be avoided. Other types of misdiagnoses were described later. Examples of these are a partial failure of amplification that caused a misdiagnosis in an embryo that was a compound heterozygote for cystic fibrosis (Grifo *et al.*, 1994), and a contamination that most probably caused a misdiagnosis in myotonic dystrophy type I (DM1, Sermon *et al.*, 1998a). Important lessons were learned from these mistakes: single-cell PCR is a difficult enterprise

at best, and three major phenomena have to be dealt with. First, failure of amplification possibly reduces the number of embryos in which diagnosis can be reached; second, allele drop-out or partial amplification failure can lead to erroneous conclusions about the genotype of the embryo; and third, contamination with extraneous DNA must be avoided at all costs, while it must always be kept in mind that it can occur.

Multiplex PCR has the advantage of presenting answers to all three obstacles, and that is why it has become the "gold standard." Multiple displacement amplification (MDA) may be seen as a special type of multiplex PCR, but has its own limitations.

Single-cell PCR: beware of the traps

What is single-cell PCR?

"What is single-cell PCR?" is a misleadingly simple question. Molecular biologists with no special expertise in single-cell PCR will tell you it is only a matter of doing more PCR cycles. And of course it is true that the principle is the same: the sample to be amplified is mixed with specific primers that will anneal next to the area of interest, dinucleotides to build up the new DNA strand, and a thermostable DNA polymerase. The mix is subjected to several rounds of denaturation of first the native DNA and then the PCR fragments, annealing of the primers, followed by extension and synthesis of the second DNA strand. More information on how PCR works is available online (http://en.wikipedia.org/wiki/Polymerase_chain_reaction). However, so much more may go wrong with single-cell PCR, that in order to achieve the same accuracy as obtained on genomic DNA extracted from peripheral blood, additional safeguards have to be built in. The main reason why "do more cycles" does not work, is that regular thermostable DNA polymerases do not copy DNA faithfully, so more and more mistakes are incorporated as the number of cycles is increased, leading to artifactual bands and smears. The more recently developed DNA polymerases have proofreading activity, and are thus less sensitive to smearing (Sermon *et al.*, 1999). Moreover, nested PCR has long since proven to be an elegant method to increase both the yield of PCR product and the sensitivity at the same time (Sermon *et al.*, 1997; De Rycke *et al.*, 2001; Verlinsky *et al.*, 2002).

Failure of amplification

It is not surprising that, when starting from one single diploid cell containing about 6 pg of DNA, PCR amplification sometimes fails. Several reasons for this can be brought forward: the cell was not introduced in the PCR tube; the lysis of the cell did not occur properly, so the DNA could not be sufficiently reached by the PCR machinery. The DNA of the cell could have been damaged, e.g. if the cell was not viable any more, and so the sequence where the DNA primers were to bind was nonexistent. For purely random reasons, the primers may not find the two DNA strands present in the solution, and so the PCR reaction cannot be initiated. The (part of the) chromosome on which the gene of interest lies may be absent in the blastomere that is biopsied, although it is present in the rest of the embryo. This is particularly important in X-linked diseases, where in male embryos there is only one of each chromosome (X and Y) present. The first misdiagnosis reported was attributed to failure of amplification, while a diagnosis was based on the absence of amplification. For the sexing of embryos, amplification of repetitive sequences on the Y-chromosome was carried out (Handyside *et al.*, 1990; Hardy & Handyside, 1992). If a band was produced after the PCR, the embryo was thought to be male; if no amplification occurred, the embryo was diagnosed as female and transferred. In one blastomere, failure of amplification occurred in a male embryo that was transferred and implanted. Another example of this type of diagnosis was reported by Liu *et al.* (1995). A specific DNA diagnosis was developed for a family at risk for Duchenne muscular dystrophy. The PCR was designed so that it amplified the DNA within the deletion present in the dystrophin gene of the carrier mother. If no band was present, the embryo was diagnosed as affected male. If amplification occurred, it was assumed that the embryo was either a healthy male, or a carrier or homozygous normal female. The situation is the reverse of the first example, because embryos without amplification are not transferred. However, contamination with the gene fragment of interest could lead to misdiagnosis. This is why it is felt that this type of diagnosis offers too little certainty as to the real genotype of the embryo (Thornhill *et al.*, 2005).

Multiplex PCR could offer a solution for amplification failure because if several loci are analyzed the loss of information from one of them would not directly rule out a diagnosis. Even when multiplex PCR is used total failure of amplification is still the most important reason for failure of diagnosis and occurs in about 4 percent of the embryos (Goossens *et al.*, 2008).

Partial failure of amplification: allele drop-out and preferential amplification

Although described earlier on a theoretical basis (Navidi & Arnheim, 1991), it was Ray and Handyside (1996) who first described and characterized allele drop-out. These authors found that blastomeres thought to be homozygous at PGD, were later shown at confirmatory testing to be heterozygous. One of the alleles in a heterozygous blastomere had failed to amplify, leading to the erroneous conclusion that the embryo was homozygous. The phenomenon was dubbed allele drop-out, or ADO. These authors convincingly showed that raising the denaturing temperature in the first PCR cycles significantly decreases the occurrence of ADO, indicating that correct denaturation of the native DNA during the first PCR cycles played a crucial role. Another reason for ADO that was proposed was the chromosomal abnormality of the blastomere.

In cases where couples who both carry the same mutation (e.g. p.F508del in cystic fibrosis) for an autosomal recessive disease are treated, ADO will not lead to misdiagnosis, because the transfer of a heterozygous (phenotypically normal) embryo, even misdiagnosed as homozygous normal, is allowed. However, one misdiagnosis for cystic fibrosis due to ADO was reported in a compound heterozygous embryo which had inherited two different mutations, one paternal and one maternal. A duplex PCR had been devised that independently amplified both mutations. ADO for one of the mutations led to the conclusion that the embryo was not carrying that mutation, although the other mutation was shown to be present, and so this embryo was diagnosed as heterozygous carrier, transferred, and led to an affected pregnancy (Grifo *et al.*, 1994).

All earlier publications on PGD for monogenic disease dealt with single-cell PCR followed by analysis of the fragments on ethidium bromide-stained gels. Because the sensitivity of these gels is rather low, many of the instances of ADO may in fact have been undetected preferential amplification (PA), i.e., one allele was only weakly amplified, so weakly in fact that it was not detected at analysis. With the introduction in the mid-1990s of fluorescent PCR for single-cell genetics, by Findlay and Quirke (1996), it was shown that ADO could significantly be reduced to a more acceptable rate. ADO rates could drop from around 25 percent to 5 percent in PGD of DM1 (Sermon *et al.*, 1998b). Part of the reduction in ADO rates was attributed to the better detection of PA because of the much better sensitivity of fluorescent PCR. PA nowadays only presents a problem when microsatellites are used for diagnosis and when the two alleles are only one repeat apart. The combination of stutter bands and PA may make it impossible to differentiate a homozygous genotype with stutters from a heterozygous genotype.

Contamination with extraneous DNA

Contamination with extraneous DNA was recognized very early on after the development of PCR as a cause of erroneous diagnosis (Kwok & Higuchi, 1989). When PCR is carried out on large quantities of genomic DNA, carryover contamination, where products from a previous PCR reaction enter a next PCR reaction, is the main problem. Extensive precautions against contamination have been given in the literature (Kwok & Higuchi, 1989) such as strict separation of pre- and post-PCR area, use of laminar airflows and positive pressure rooms, and regular decontamination with appropriate products or ultraviolet lamps. Carryover contamination may also occur in single-cell PCR, and can be avoided by the same strict precautions. In single-cell PCR, contamination with native DNA also has to be taken into account. Examples of this type are sperm or cumulus cells stuck to the zona pellucida (ZP) after in vitro fertilization (IVF), which may inadvertently be taken up in the PCR tube. This is why intracytoplasmic sperm injection (ICSI) is recommended as the method of choice for fertilization in cycles for PGD for monogenic diseases (Thornhill *et al.*, 2005) and measures should be taken to detect possible maternal contamination from cumulus cells.

Even when using very strict precautions, contamination can never totally be ruled out, and measures to detect contamination should also be implemented. A generally used way to detect contamination has been to use blanks, which contain all components of a PCR except the test sample. The drawback of the use of blanks is that although it gives an overall idea of the level of contamination, it does not give any information on the contamination of a particular test sample. The best way to detect contamination within a sample is again multiplex PCR, either with linked or unlinked microsatellite markers. Contaminating DNA from the couple undergoing PGD will show alleles that may be expected in an embryo, but there will be one too many. Contamination from other sources will show up as alleles unrelated to the couple (Harper *et al.*, 2002).

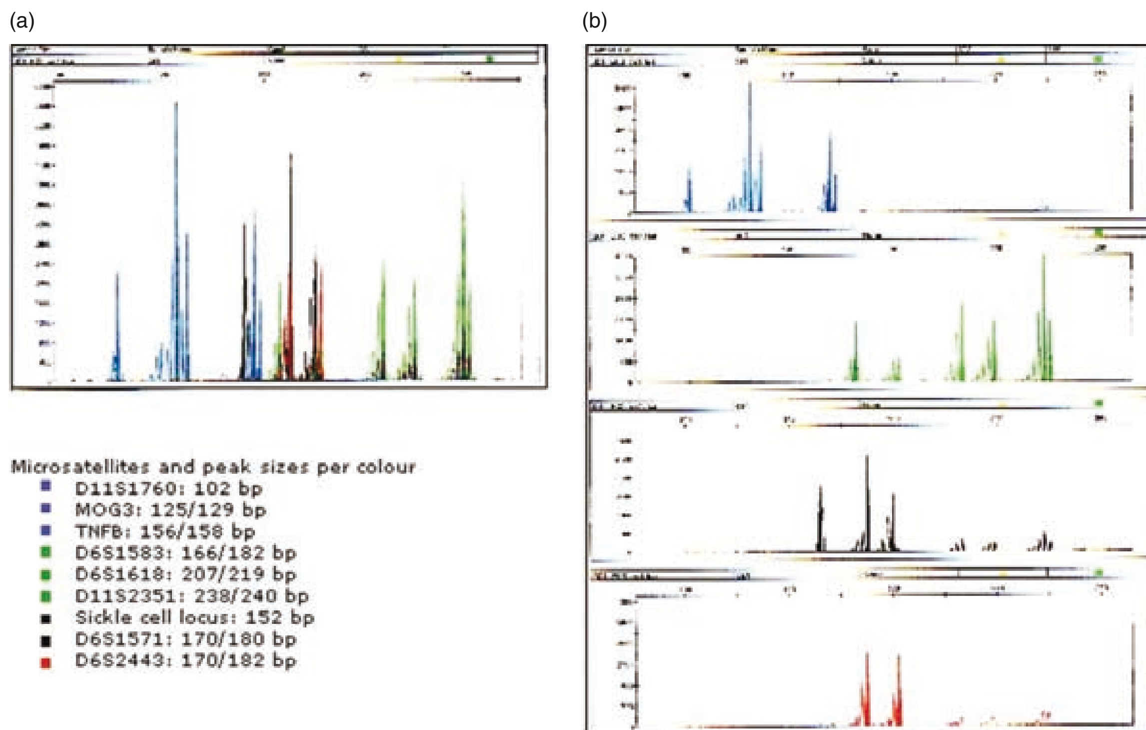

Figure 15.1 Example of a multiplex polymerase chain reaction (PCR) on one single cell. The analysis is for sickle cell anemia and human leukocyte antigen (HLA) typing. The sickle cell locus is one single peak before SNP analysis and D11S1760 (homozygous) and D11S2351 (heterogygous) are microsatellites on chromosome 11 linked to the β-globin gene. The MOG3, TNFB, D6S1583, D6S1618, D6S1571, and D6S2443 microsatellite markers are on chromosome 6 spread over the HLA region. (a) A composite image of the peaks of all loci; (b) the peaks according to the color of the labeling.

Multiplex PCR: the "gold standard" for PGD

How does it work?

The simplest configuration for multiplex PCR combines a mutation (e.g. the sickle cell mutation in the β-globin gene) together with one linked marker (e.g. D11S1760 and D11S2351 microsatellites linked to the β-globin gene, see Figure 15.1). Ideally, both parents carry the same mutation and, moreover, they are both heterozygous for the microsatellite, and they carry four different alleles (Figure 15.1). Multiplex PCR, first, increases accuracy, because two loci are analyzed for one chromosome. If one locus fails to amplify, diagnosis may still be obtained from the second locus. Second, when allele-drop out occurs in any of the configurations, this is detected: every possible mutation genotype (homozygous normal, homozygous affected, heterozygous) can only occur together with one microsatellite genotype (Dreesen *et al.,* 2000; Goossens *et al.,* 2003). Third, as explained above, it detects contamination (Figure 15.2).

Microsatellites have been used extensively in PGD for monogenic disorders because they are polymorphic (and thus offer a good chance of finding two different haplotypes in the couple), straightforward to detect, and amenable to amplification at the single-cell level (Fiorentino *et al.,* 2006). Several microsatellites may be analyzed in one assay, which can cover a whole area of one chromosome (e.g. the human leukocyte antigen (HLA) region) (Van de Velde *et al.*, 2004) or a large gene (e.g. NF1) (Spits *et al.*, 2005). The advantage of developing a whole battery of markers within a region of interest is that the test may be used for more than one family requesting PGD for a particular disease. This would avoid the tedious and time-consuming development of single-cell assays for each individual family (Spits *et al.*, 2005). However, in practice this has turned out more complicated than expected. First, in order to be able to use linked markers (of any kind) family members have to be available for analysis. This presents a problem if the couple did not conceive before and do not wish to reveal to their family that they are requesting PGD, or if the mutation

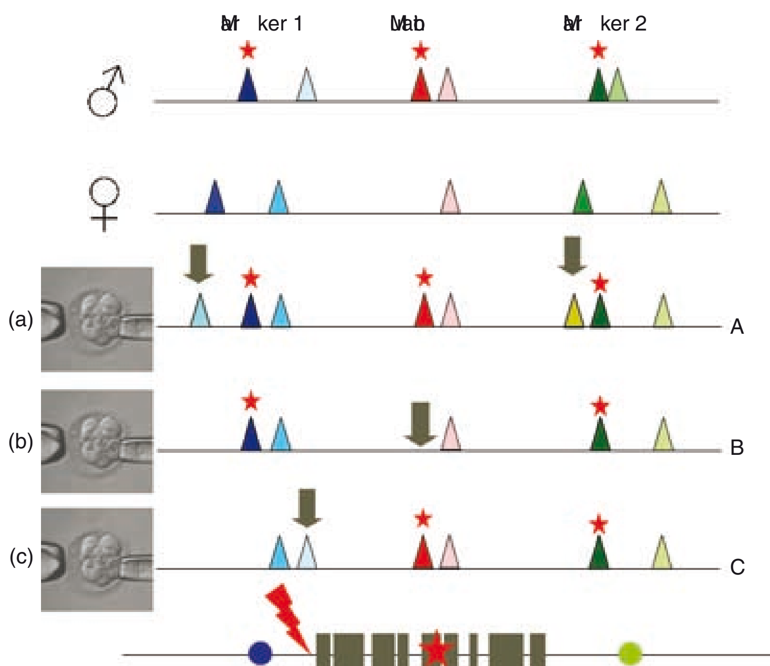

Figure 15.2 The principle of multiplex polymerase chain reaction (PCR) in PGD. The top two diagrams represent the genotypes of the parents. In this example, the husband carries a mutation (red peak), linked to the dark blue allele of marker one and the dark green allele of marker 2. (a) An occurrence of contamination: the alleles marked with an arrow are contaminating the markers as evidenced by the presence of three instead of two peaks. Note that the mutation analysis looks normal. (b) An occurrence of allele drop-out (ADO): the arrow shows the missing mutated allele in the mutation analysis. (c) An occurrence of recombination: both the mutation and marker 2 show that the embryo is affected, while marker 1 shows that it is unaffected. A recombination occurred between the mutation and marker 1.

is novel in the proband. In these cases, using linked markers exclusively is ruled out and mutation analysis will always have to be carried out. Possibly, mutation analysis with a linked marker can be proposed to the couple but then preferably phase should be established before PGD. This can be performed by sperm analysis if the husband is affected, or ultimately even by polar body analysis if the wife is affected. Second, in some cases it has proved very difficult to find microsatellites for which the family was completely informative. Either not many microsatellites are available (e.g. for DM1, Fragile X syndrome), or certain haplotypes are in linkage disequilibrium with the mutation, or the heterozygosity of the markers is not high. Finally, the exact location of the mutation (if known) has to be taken into account. If linked markers are to be used only, then at least two of these should flank the mutation. Using two markers on the same side of the mutation is dangerous because a possible recombination between the mutation and the linked markers will not be detected (Kakourou *et al.*, 2007). If this type of diagnosis is carried out, a linked marker with a low recombination fraction should be chosen, and a confirmatory prenatal diagnosis using mutation analysis should always be proposed if the patient is pregnant.

The development of a single-cell multiplex PCR

Very little can be found in the literature on how exactly an assay at the single-cell level, including several amplicons, is developed.

The first step is the family analysis. If the mutation was characterized elsewhere, it should first be confirmed in-house (Thornhill *et al.*, 2005). At least one misdiagnosis has been caused by incorrect information being sent by the referring center (Harper *et al.*, 2008). Blood samples from relevant family members have to be collected. Informative markers have to be identified in the couple and the phase has to be established using DNA from offspring (affected children, fetal material obtained after termination of pregnancy) or family members (Figure 15.3 and Figure 15.4).

The second step is the choice of strategy depending on the results of the family analysis. If the couple carries a frequent monogenic disease for which multiplex PCR tests were developed earlier, it is possible that they are informative for the markers used in this test and the couple can come through for PGD. If not, a new test, combining, for example, the mutation and one or more markers, has to be developed. At this point, the type

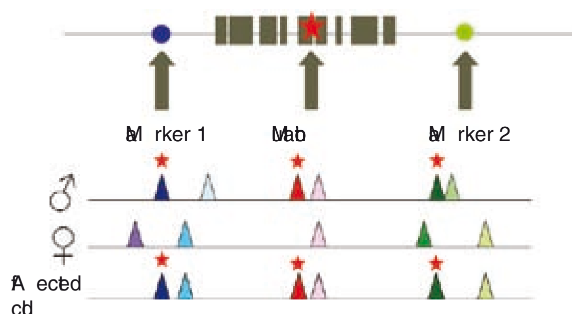

Figure 15.3 Segregation analysis in a family where the husband and his child are affected with an autosomal dominant disease. Comparison of their genotypes shows that the mutation co-segregates with the dark blue peak in marker 1 and the dark green peak in marker 2.

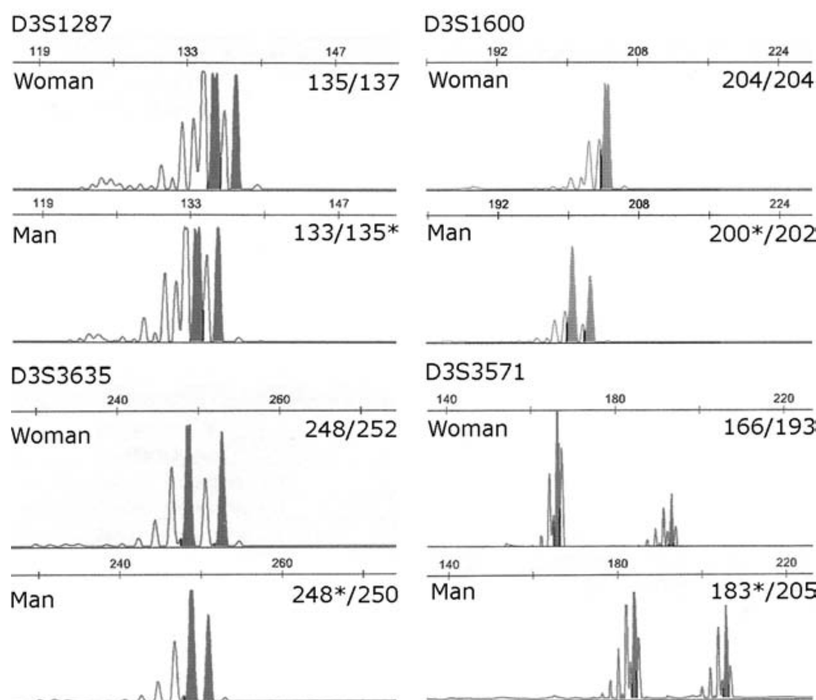

Figure 15.4 Informativity testing for a couple where the husband carries SCA7. The alleles marked with an arrow segregate with the disease.

of PCR fragment analysis is also chosen. For microsatellites, this is usually straightforward fragment length analysis. Single nucleotide changes may be detected using restriction enzymes, or alternatively minisequencing has been proposed recently (Fiorentino *et al.*, 2006).

Minisequencing

Minisequencing is a method in which one fluorescent dideoxynucleotide is inserted at the site of the mutation. As every ddNTP is labeled with a different fluorochrome (ddATP is green, ddCTP is black, ddGTP is blue, and ddTTP is red), homozygotes will show only one fragment of a certain color, and heterozygotes will show two fragments of different color.

Real-time PCR

Real-time PCR to distinguish homozygotes from heterozygotes has also been used (Vrettou *et al.*, 2004). In contrast to PCR where the amplicons are analyzed at the end of the PCR reaction, in real-time PCR the emergence of the different fragments is monitored during the PCR reaction. It will thus be possible to see whether only one type of amplicon emerges (homozygotes) or two (heterozygotes).

Double ARMS

Another elegant method for single nucleotide changes analysis was described by Moutou *et al.* (2007) for PGD for familial adenomatous polyposis coli (FAP): the double ARMS (allele-specific amplification) technique

uses specific primers for the mutation and the wild-type allele.

The third step is to test primers for the different amplicons on relevant DNA samples, first on DNA amounts routinely used in genetic diagnosis (e.g. 100 ng) and later on dilutions of DNA approaching the amount of DNA found in a single cell (e.g. 100 pg or less). Fine-tuning of the relative amounts of primer to be used in the reaction is carried out at this level. This is necessary because not all primers show the same amplification efficiency, and the labeling of the primers may differ from batch to batch. If it turns out that it is not possible to amplify all primers in the same PCR reaction, a second round with separate reaction mixes has to be set up. Usually, ten PCR cycles are carried out with all the primer sets, after which a second round of PCR is carried out for all PCR primers separately using PCR product from the first PCR round as a template. This has the advantage that the optimal conditions (primer concentration, annealing temperature) for each primer pair may be used in the second round.

Finally, the PCR is tested on single cells. These should preferably be heterozygous for all alleles tested, so that ADO may be assessed. Several cell types can be used, for example lymphocytes, buccal cells, skin fibroblasts, amniocytes. The lysis buffer to be used is either alkaline lysis buffer or proteinase K and SDS. At this level, more fine-tuning is usually required, in order to have PCR results that may be interpreted unambiguously.

The European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium guidelines recommend that at least 50 single cells and 50 blanks are tested to have an accurate idea of the ADO and contamination levels. Only if the results on these 50 cells are satisfactory, that is, the amplification efficiency is above 90 percent, the ADO rate per locus is less than 5 percent, and the contamination rate is also below 5 percent, is the test ready for clinical application (Thornhill *et al.*, 2005).

Preimplantation genetic haplotyping: major breakthrough or hype?

Methods of whole-genome amplification (WGA) could overcome the difficulty of insufficient DNA, providing sufficient template for numerous genetic analyses, such as array comparative genomic hybridization (array-CGH), microsatellite analysis, or single nucleotide polymorphism (SNP) analysis. The general applicability of WGA methods such as degenerated oligonucleotide primed (DOP) PCR (DOP-PCR) (Telenius *et al.*, 1992) and primer extension (PEP) PCR (Zhang *et al.*, 1992) is limited by nonspecific amplification artifacts, incomplete coverage of loci, and the small size of the DNA products.

Recently, a non-PCR-based isothermal method, termed multiple displacement amplification (MDA), has been applied to small DNA samples, leading to the synthesis of DNA with limited sequence representation bias (Dean *et al.*, 2002). This method is based on random hexamer priming of denatured DNA followed by strand-displacement synthesis at constant temperature, resulting in DNA products of high molecular weight. As DNA is synthesized by strand displacement, a gradually increasing number of priming events occur, forming a network of hyper-branched DNA structures. The reaction may be catalyzed by the ϕ29 DNA polymerase that possesses a proofreading activity resulting in low error rates (Figure 15.5).

The small-scale experimental set-up of the first reports validating single-cell WGA provided only limited information on the accuracy and reliability (Handyside *et al.*, 2004; Hellani *et al.*, 2004; Hellani *et al.*, 2005; Jiang *et al.*, 2005).

More extensive studies were recently published (Renwick *et al.*, 2006; Renwick *et al.*, 2007; Spits *et al.*, 2006a; Spits *et al.*, 2006b). Several variables have been tested (e.g. lysis buffer, denaturation prior to incubation, total incubation time) for the single-cell MDA and have shown that ADO rates for different loci (CF, FMR1, Marfan) can vary between 0 percent and 60 percent, with an average of 26 percent (Spits *et al.*, 2006a). Renwick *et al.* (2006) presented the first successful PGD cycles for cystic fibrosis and Duchenne muscular dystrophy, and later also reported a more extensive validation of single-cell WGA. These authors analyzed 64 polymorphic markers, including a set of 16 markers on chromosomes 13, 18, and 21 routinely used for aneuploidy screening in prenatal samples, in 49 blastomeres obtained from eight different embryos. The average ADO rate was 28 percent, ranging from 12 percent to 49 percent. If PGH were to be used for aneuploidy screening, because of the high levels of ADO, they recommend that seven microsatellite markers be included for the reliable detection of trisomy to reach a 95 percent confidence level. Renwick *et al.* (2006) go on to suggest that "Unfortunately, the ADO associated with the current MDA method of whole genome amplification will always reduce the power of detection of trisomy by PCR analysis. The data strongly suggest that this technology in its current form is not optimum for detection of chromosomal aneuploidy in pre-implantation embryos."

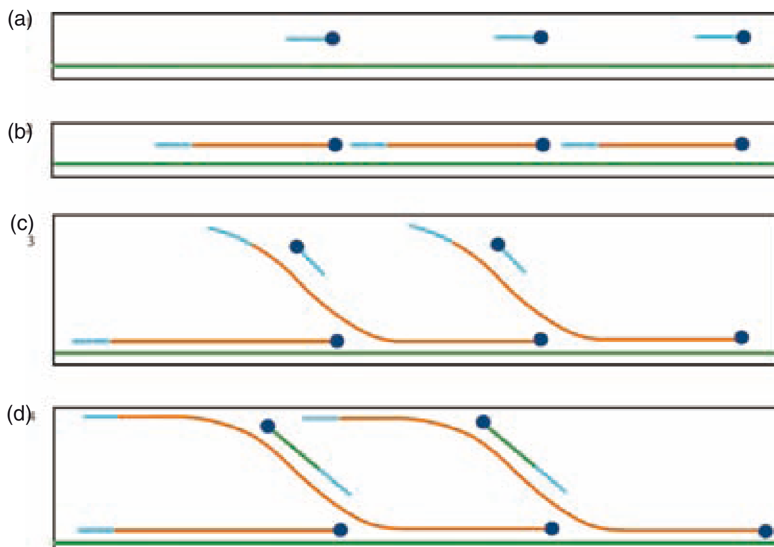

Figure 15.5 The principle of multiple displacement amplification (MDA): (a) the random hexamers (represented by a blue line) bind to the denatured DNA (represented by a green line); (b) the Φ DNA polymerase (represented by a blue circle) extends the primers until it reaches newly synthesized double-stranded DNA (represented by an orange line); (c) the enzyme proceeds to displace the strand and continues the polymerization, while primers bind to the newly synthesized DNA; (d) polymerization starts on the new strands, forming a hyperbranched structure. From Spits *et al.* (2006b). Reprinted by permission from Macmillan Publishers Ltd. Copyright 2006.

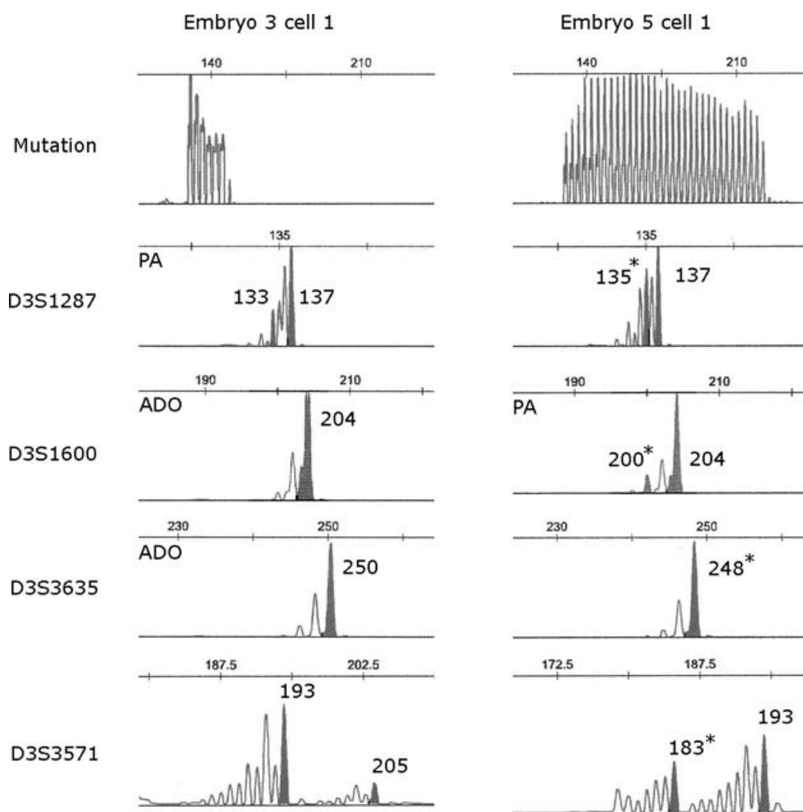

Figure 15.6 A preimplantation genetic diagnosis (PGD) result of the couple tested in Figure 15.4. The mutation of SCA7 was analyzed, along with four informative markers. These were analyzed after multiple displacement amplification (MDA). Embryo 3 is an unaffected embryo. During PGD, allele drop-out (ADO) occurred in D3S1600 and D3S3635. Embryo 5 is an affected embryo; the alleles co-segregating with the mutation are marked with an asterisk. ADO occurred in D3S3635.

The situation is different for the diagnosis of monogenic disorders or "preimplantation genetic haplotyping" (PGH), as these authors have coined PGD following MDA. We currently also use PGH for selected situations, such as when multiplex PCR turns out to be especially problematic, or if multiple loci have to be analyzed (e.g. patients carrying Marfan syndrome and Huntington disease, unpublished results). Our current experience with PGH is that it effectively shortens the time needed for the development of the

PGD, especially in complex cases, but that it puts a high burden on the PGD team during the cycle itself, because a large panel of markers and mutations have to be analyzed within the timeframe before blastocyst transfer (Figure 15.6). PGH has especially been advocated to facilitate transport PGD: at the IVF center, the biopsy would be performed immediately followed by the MDA. The amplified sample would then be sent to a genetic diagnosis center for further analysis (Handyside *et al.*, 2004). Whether the added time needed for the transport of the sample would ultimately leave enough time for a diagnosis to be reached before transfer of the embryos remains to be determined.

Conclusion

PGD for monogenic diseases is a demanding technique that requires highly skilled molecular biologists and a fully equipped molecular biology laboratory. Moreover, close collaboration with clinical geneticist and fertility specialists is also necessary.

However, technical breakthroughs in DNA diagnostics are readily adapted for PGD and help to make single-cell PCR more accurate and fast.

PGD for monogenic diseases may now be considered routine and a valid alternative to prenatal diagnosis, provided that the laboratory has the required know-how.

Acknowledgments

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Chapter 16

Quality control and quality assurance in preimplantation genetic diagnosis

Alan Thornhill and Sjoerd Repping

Key points

- Reliability and accuracy in any diagnostics laboratory depend on stringent quality control (QC) and quality assurance (QA) measures.
- The unique nature of embryonic test material necessitates a particular emphasis on the application of the appropriate single-cell specific test with a high degree of accuracy and reliability.
- Unlike many other routine tests performed on peripheral blood and other tissues, preimplantation genetic diagnosis (PGD) is, at present, essentially a one-shot test with no possibility of a retest following test failure or inconclusive results.
- PGD is a multidisciplinary treatment option that requires unambiguous written policies and procedures and clear lines of responsibility and communication to minimize errors.
- The quality system comprises a number of different components, including organization, personnel, facilities and safety, equipment, process control, process validation, documentation, adverse events, assessments, service and satisfaction, and process improvement.
- PGD is largely unregulated by any accrediting agency at present, but laboratories conform to ISO 15189. The introduction of standardization, proficiency testing, and external quality assessment procedures among centers offering PGD is in accordance with other forms of molecular and cytogenetic testing and would ensure the highest quality of care for all patients.

Introduction

Over the past decade, there has been an increasing awareness of the interplay between assisted reproduction and genetics. In part this has been driven by the knowledge that a great deal of infertility has a genetic component. In addition, the combination of sensitive genetic testing and assisted reproductive techniques has made it possible to diagnose genetic disorders or chromosomal abnormalities before embryo implantation. This growing understanding has led a number of professional bodies to develop policy documents, guidelines, and opinions on how to best implement genetic testing of embryos in an assisted reproduction setting. Generally, such documents direct policy describing current access to the testing (Soini *et al.*, 2006; ESHG/ESHRE, 2006) and for whom it should be available (PGDIS, 2004) but contain little practical detail (HFEA, 2007; Practice Committee of the SART/Practice Committee of the ASRM, 2007) to aid in the delivery of a standardized, high-quality clinical service. One previously published set of guidelines (Thornhill *et al.*, 2005) gives practical guidance based on consensus and published work, and the purpose of the current chapter is to expand upon the quality aspects outlined previously. All diagnostic laboratories should conform to ISO 15189 (an international laboratory standard specific for medical laboratories).

In the context of PGD, the quality system exists to ensure the quality of a service that is the delivery of accurate and reliable genetic test results from human preimplantation embryos. These results are used to help patients and their healthcare providers decide which embryos to select for transfer to the uterus in the hope of initiating a healthy, disease-free pregnancy. The quality system comprises a number of different components. In this chapter, for the sake of simplicity, these components are classified as follows: organization, personnel, facilities and safety, equipment, process control, process validation,

Table 16.1 General quality control/quality assurance measures for single-cell diagnostic work

Quality system component	Measure taken
Organization	Define interdisciplinary responsibilities in a written policy and relevant standard operating procedures
Personnel	Comprehensive training and compliance with written standard operating procedures
	Ensure all staff have appropriate qualifications
	Ensure staff wear appropriate personal protective equipment (gloves, etc.) for critical steps
Facilities and safety	Provide optimal laboratory facilities (including air quality, floor plan, workflow design)
Equipment	Identify all critical equipment for use in preimplantation genetic diagnosis (PGD)
	Implement routine calibration and preventative maintenance of all critical equipment (includes biopsy equipment and all diagnostic instruments)
	Implement installation validation for new equipment prior to clinical use
Process control	Implement witnessing for all critical procedural steps (in vitro fertilization (IVF) and diagnostic)
	Register of batches of culture media, diagnostic reagents, and materials to allow full traceability
	Implement comprehensive labeling standard operating procedures to avoid sample mix-up (especially when dealing with multiple samples (embryos) for the same patient)
	Batch test all diagnostic reagents prior to a clinical case
Documentation	Comprehensive, version-controlled standard operating procedures and forms
	Comply with written record retention policy
Adverse events	Encourage event reporting in a "no-blame" environment according to policy
Assessments	Participate in internal and external quality assurance programs
	Undergo periodic inspections from regulatory bodies, accreditation and/or certification agencies

documentation, adverse events, assessments, service and satisfaction, and process improvement (Table 16.1). For the purposes of this chapter we refer to PGD as a process in combination with in vitro fertilization (IVF), involving the removal of one or two cells from an embryo and the subsequent testing of those cells for specific genetic disorders, characteristics, or chromosome number with a view to targeted embryo selection.

In PGD, a diagnosis has to be made using only one or two cells. To ensure the highest standards of analytic reliability and accuracy for such single-cell analyses, both general and single-cell specific quality control (QC) and quality assurance (QA) measures must be taken.

As with any diagnostic procedure, there are generally three phases to consider: pre-analytic, analytic, and postanalytic. The focus of this chapter considers the individual components of the quality system with respect to these three phases of testing (see Table 16.2, Table 16.3, and Table 16.4), rather than discusses in any detail the various methods for design and optimization of specific PGD assays (which are described elsewhere). Finally, rather than providing exhaustive lists, we have used limited specific examples to illustrate some quality principles.

Components of the quality system

Organization

Pre-analytic

PGD is a multidisciplinary treatment that requires a well-defined organization scheme that includes all lines of responsibility across each of the different processes involved (ESHRE PGD Consortium, 1999; Geraedts *et al.*, 2001). In addition, it is of critical importance to set up clear lines of communication via email and through regular face-to-face meetings or teleconferencing, particularly if PGD is carried out in a transport or satellite setting.

Analytic

Timing of the biopsy and subsequent transfer of the cell to the diagnostic laboratory should be well communicated. It should be clear which test should be performed and under what conditions. All these details should be included in a test request form presented to the diagnostic laboratory prior to the start of the procedure.

Postanalytic

The organization scheme must include who is responsible for conveying the diagnostic result to the embryology laboratory, who is responsible for the final

Table 16.2 Pre-analytic quality control/quality assurance measures for preimplantation genetic diagnosis

Quality system component	Measure taken	FISH-specific	PCR-specific
Organization	Determine lines of responsibility and accountability between participating disciplines and individuals	Translocation case: collection of suitable blood and sperm samples for test development (as applicable)	Single-gene defect case: ordering/ collection of appropriate blood/ DNA samples from proband and family members to ensure accuracy during test development
Personnel	Genetics consultation	For cases involving aneuploidy risk* or chromosomal rearrangements	All single-gene disorders
Process control	Karyotype couple	Exclude confounding chromosomal abnormality	
Process validation	Ensure appropriate test offered	FISH testing of couples' lymphocytes to rule out polymorphisms	PCR test of couples' DNA/single cells to rule out polymorphisms
Documentation	Verification of original diagnosis	Review official cytogenetics report	Review official molecular genetics report
Process validation	Create validation plan	Identify acceptable accuracy and efficiency prior to beginning validation work	
		Determine minimum number of single cells analyzed for assay development	
		Determine acceptable cell type for appropriate validation	
	Determine test accuracy and reliability	Use lymphocytes of known genotype (normal and abnormal)	Analyze DNA/cells from homozygous normal and affected individuals Analyze heterozygous single cells to establish allele drop-out/amplification rates
	Develop standard operating procedures for scoring to ensure consistent result interpretation	Establish criteria for signal intensity, background	Establish cut-off values for failed amplifications/contamination in fluorescent PCR
	Perform "dry-runs" in case conditions	Fix individual blastomeres onto slides	Tube individual blastomeres/cells
Service and satisfaction	Provide information for patients/clients	Up-to-date and comprehensive pregnancy, live birth, and misdiagnosis rates Full costs, written information explaining procedures and associated risks and benefits	

FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction.
*Genetics consultation optional for "routine" aneuploidy screening cases.

selection of the embryo(s) for transfer, and who is responsible for the embryo transfer itself. The results from the diagnostic test should be summarized and authorized to produce a final report that is sent to the clinician requesting the PGD. A similar embryology development summary report would accompany the diagnostic report and be placed together in the patient file for the scrutiny of the treating clinician.

Personnel

Pre-analytic

For single-gene disorders, a genetic counselor and molecular biologist experienced in pedigree and linkage analysis should determine what samples are needed for reliable and accurate diagnosis. For structural chromosome abnormalities, a cytogeneticist should determine what probes are needed for appropriate chromosome-specificity to enable reliable and accurate diagnosis using fluorescence *in situ* hybridization (FISH).

Analytic

All personnel undertaking single-cell-based diagnosis should be suitably qualified and trained to written standard operating procedures. If the possibility of accreditation exists, personnel should possess or be working toward that accreditation. Training for single-cell diagnostic assays should be at least to the

Table 16.3 Analytic quality control/quality assurance measures for preimplantation genetic diagnosis

Quality system component	Measure taken	FISH–specific	PCR–specific
Organization	Work supervised by qualified senior staff	State registered clinical scientists, MRC Path. Registration (UK)	
Personnel	Work only carried out by appropriately qualified and trained personnel	Cytogenetic experience preferable	Molecular genetic experience preferable
Facilities and safety	Ensure facilities are suitable for single-cell diagnostic work	Humidity (cell fixation) Dark room (fluorescence microscopy)	Clean room availability (preparation of single-cell reagents)
Equipment	Dedicated equipment for single cell diagnostic use (if applicable)	Post-hybridization washes (avoid contamination with other cells)	Micropipette devices (single-cell use only)
Process control	Ensure appropriate sample labeling Use of controls for clinical assay Back-up procedures in the event of diagnostic failure Double witnessing critical procedures	Printed slide labels Lymphocyte control (+) Incorporate rescue FISH probe panel	Printed tube labels DNA (+) and blanks (-) controls Rerun PCR products
Documentation	Version-controlled worksheets for all sample specific laboratory processes	Cell fixation worksheet (inc. position, description, identity of nucleus on slide) Chain of custody component	Tube loading worksheet – (inc. labeling, tube contents, gel loading sequence, etc.)
Adverse events	Record deviations from standard operating procedure; assay; equipment failures; diagnostic errors	Additional post-hybridization Wash or use of unvalidated probe set	Investigate contamination, PCR failures, and misdiagnoses of non-transferred embryos
Assessments	Audit test turn-around times (for timely embryo selection/transfer)	Audit blastomere FISH efficiency and accuracy	Audit blastomere amplification efficiency and accuracy
Service and satisfaction	Survey staff, collaborating clinics and patients to assess quality of service	Reports assessed on intelligibility, timeliness, and accuracy	
Process Improvement	Combine data from audits, inspections and/or adverse events to validate new standard operating procedures or modify existing standard operating proceduress	High false-positive rate with Multi-Vysion probe set led to use of a "rescue" probe panel involving probes for different regions of the same chromosome	Example: suboptimal PCR amplification efficiencies could lead to the adoption of a two-cell biopsy policy where the first cell appears anucleate

Table 16.4 Postanalytic quality control/quality assurance measures for preimplantation genetic diagnosis

Quality system component	Measure taken
Organization	Determine lines of responsibility and accountability between participating disciplines and individuals
Personnel	Appropriately qualified staff to sign off PGD report Appropriately qualified staff to discuss PGD results with patient
Documentation	Provide standardized written report to patient/client Further specific information available on request to clients Record retention policy to ensure audit trail for all relevant documents and records
Adverse events	Routine reporting of misdiagnoses and adverse events
Assessments	Assess blastomeres from non-transferred embryos to confirm single-cell diagnosis Confirm PGD result by amniocentesis/CVS/cord blood Regular collection and analysis of outcome data Reporting of outcome data to regulatory body and/or ESHRE PGD Consortium
Service and satisfaction	Ensure timeliness of reporting based on agreed diagnostic test turn-around time Reports assessed on intelligibility, timeliness, and accuracy

standard required for routine testing in a clinical diagnostic laboratory, although it is clear that the range of techniques and platforms may be limited for PGD.

All personnel involved in clinical PGD must follow an approved documented training program that includes both theory and hands-on training covering all aspects of single-cell work. Personnel should be trained in current procedures by an experienced member of staff and should demonstrate competency before being allowed to perform clinical work without supervision. In general, IVF cycles are managed by a clinical team led by a gynecologist with the support of a genetic counselor or nursing staff specializing in PGD. IVF, embryo culture, and biopsy procedures should be performed by suitably trained and competent embryologists. Diagnostic testing should be performed by technical staff with supervision from a senior technician or laboratory manager. Diagnostic reports should only be signed off by an appropriately qualified senior staff member or laboratory director.

Postanalytic

Ideally, test results (particularly those for cases involving chromosome translocations and single-gene disorders) should be interpreted and explained by a genetic counselor or clinical geneticist. In practice, the final decision regarding embryo selection for transfer to the uterus will lie with the patient, the embryologist, and the clinician (who bears ultimate responsibility for the embryo transfer procedure).

Facilities and safety

As with any diagnostic test, there is a requirement to provide appropriate facilities for the specific type of work performed. However, a number of other special conditions apply for single-cell diagnostics. For example, many IVF laboratories find it less intrusive to engage in micromanipulation activities (including embryo biopsy and blastomere preparation) in a room separate from the main laboratory in which routine procedures are performed thus leading to high traffic and the potential for interruption or accidents.

There are no specific safety requirements for PGD for personnel over and above those that should already be in place for routine IVF and laboratory diagnostic work. From the perspective of the embryo, risk assessments and audits should be performed to estimate possible harm to the embryo during embryo biopsy procedures, and appropriate steps should be taken to minimize potential damage where possible.

Witnessing and correct labeling are essential in clinical PGD to avoid sample mix-up at any stage of the process. Critical procedures (e.g. transfer of embryos between dishes) have to be double-witnessed by a second person (HFEA, 2007) and a rigorous protocol for labeling slides or microcentrifuge tubes should be implemented.

Pre-analytic

With regard to FISH procedures, all probe vials should be tested before clinical application, to confirm that they contain the correct chromosome-specific probe labeled with the correct fluorochrome, and to assess that signal specificity, brightness, and discreteness are within acceptable parameters as per written procedures. This can be done alongside the positive control used for each clinical case to ensure the testing is done under clinical conditions. Prior to each FISH procedure denaturation, hybridization, and wash temperatures should be verified. Temperature ranges should be validated in individual laboratories and instruments calibrated periodically to ensure accuracy.

Analytic

PCR tests

Workflow design is particularly important for single-cell PCR and should incorporate the following to minimize the omnipresent threat of contamination:

- Physical separation of pre-PCR, PCR, and post-PCR laboratories, and the biopsy laboratory should be located so as to minimize the risk of contamination of PCR samples.
- There should be a physical separation of the area for setting up the second round of PCR, and of both the primary PCR set-up area and the area where secondary products are analyzed when performing two rounds of PCR.
- PCR reagents and consumables should be restricted for use to specific work areas to minimize contamination.
- In addition to the physical space and layout, personnel should attempt to perform unidirectional workflow to avoid contamination.
- If using whole-genome amplification prior to specific sequence detection, the areas of critical importance with respect to contamination control are the laboratory in which single cells are prepared and the room in which reaction mixes for cell lysis and whole-genome amplification are prepared.

FISH tests

If the process of blastomere spreading and fixation involves the use of potentially embryo-toxic fumes (e.g. methanol or acetic acid) it should be performed in a separate laboratory or within a fume hood or safety cabinet. In addition, temperature and humidity may need to be monitored and adjusted to facilitate optimal cell spreading. Fortunately, contamination from the laboratory environment is comparatively much less of a risk for FISH-based tests than for polymerase chain reaction (PCR).

Postanalytic

Great attention should be paid to the transfer of the diagnostic results to the embryology laboratory to avoid any chance of sample mix-up.

Adverse events

In general, adverse events may be the drivers for positive change and process improvement in PGD. The somewhat intimidating term "adverse events" may cover intentional deviations from standard operating procedures to unintentional deviations with little or no consequence (e.g. collecting a patient blood sample for test validation in the wrong tube – an error which can be easily corrected by taking a further blood sample) to those of catastrophic proportions (e.g. transferring an abnormal embryo as a result of misdiagnosis, which results in the birth of an affected child). A simple and consistent recording system should be implemented with an emphasis on creating a "no-blame" culture to encourage adverse event reporting. Records should be maintained and reviewed for corrective action taken, preventative action planned, implemented, and monitored. Any change implemented should be reflected in updated written procedures. Examples are provided to illustrate possible events in each of the diagnostic phases.

Pre-analytic

An example of a common problem encountered in the pre-analytic phase of PGD involves the collection of parental blood samples in tubes designed for DNA extraction when preparation for FISH testing is required. Such errors should be recorded and preventative action taken to ensure smooth patient flow and avoid delays in treatment.

Analytic

It is important to record deviations from the standard operating procedures to determine if the procedure was performed in a sufficiently compliant way and to record those errors occurring more than once. Common errors usually indicate ambiguous standard operating procedures, which should then be modified. If changes to standard operating procedures are made (e.g. a change of FISH method) this should be registered in the central standard operating procedure and this change should be read and signed off as read and understood by all personnel.

Postanalytic

Misdiagnosis may be ascertained by confirmation of diagnosis on nontransferred embryos, prenatal testing, and analysis at birth. Any misdiagnosis should be recorded and reported (internally and, if appropriate, externally). A thorough investigation should be instigated on discovering a misdiagnosis to prevent any future avoidable errors. Misdiagnosis rates should be calculated for each type of assay (PCR or FISH) and for all assays from a particular clinic. Misdiagnosis rates include those in which affected pregnancies arose and post-transfer QC assays were discordant with the biopsy result. Misdiagnosis rates, along with pregnancy rates, should be made available to all prospective patients upon request. The European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium currently collects and reports specific misdiagnosis cases and rates in an effort to identify areas for PGD process improvement (Gianaroli *et al*., 2000; Sermon *et al*., 2007; Wilton et al., 2009).

Process control: routine quality control

Routine QC is the bedrock of any successful laboratory and is covered in more detail elsewhere (Thornhill & Snow, 2002). For a brief overview of general QC measures for single-cell diagnostic work see Table 16.1 above.

For accurate and reliable single-cell diagnostic work there should be a particular emphasis on equipment and reagents. All solutions for PCR should be split into single-use aliquots. All plastic ware used in PCR should be certified DNA- and DNase-free. All reagents should be isolated for single-cell use only. All batches of reagents should be recorded and may be linked back to specific assays (allowing full traceability). All new and old batches of reagents should overlap and be subject to strict quality control and inventory. Wherever possible, all solutions should be purchased ready to use and should be of "molecular biology" grade or equivalent.

Alternatively, solutions prepared "in house" should be subject to strict QC.

To minimize contamination in PCR-based tests, work practice controls must be in place as follows: appropriate protective clothing for single-cell PCR work should be worn at all times, and workflow should be unidirectional as far as possible, as described previously (Thornhill & Snow, 2002).

The existence of, and adherence to, clearly written standard operating procedures are essential to avoid ambiguity. For example, the clinical testing protocol should include explicit instructions regarding which embryos are acceptable for biopsy, how many cells will be removed from each embryo, summarized results from the validated assay, scoring criteria, and reporting procedures, as well as a framework for counseling patients when delivering of diagnostic results. Deviations from standard operating procedures should be recorded, and if frequent deviations occur the laboratory should consider changing the written procedures.

Process validation

Process validation takes place primarily in the preanalytic phase of diagnostic testing. In view of the cost of PGD treatment, and the unique nature and origin of the test material, the additional cost and inconvenience to the patient of precycle screening to ensure appropriateness of testing is justified. Before commencing a PGD cycle, it is vital to verify the DNA diagnosis using peripheral blood from the couple. Furthermore, it is prudent to apply the specific PGD test to DNA or single cells from the particular couple to discover any unexpected test results that could render future blastomere results questionable (e.g., a polymorphism which may exist under a primer used in the single-cell assay but not in the routine laboratory assay) (Thornhill *et al.*, 2002).

FISH-based tests

If using a probe set previously shown to have a very low polymorphism rate (e.g. AneuVysion X, Y, 18) it is not necessary to provide precycle testing on a couples' lymphocytes. If, however, probes for polymorphic targets are used, such as DYZ1 (Yq12), precycle testing of the male partner's interphase nuclei should be considered. It is recommended that testing with the D15Z1 probe is performed on lymphocytes of both reproductive partners before treatment (since it cross-hybridizes to one chromosome 14 in around 15 percent of cases) (Shim *et al.*, 2003; Cockwell *et al.*, 2007). Other reported polymorphisms include the 16qh- polymorphism which, if undetected, could lead to false-positives (monosomies) and false-negatives (trisomies) (Colls *et al.*, 2004; Chatzimeletiou *et al.*, 2006). In such cases, availability of validated alternative chromosome-specific probe sets is advisable. For probe mixes containing subtelomere probes and locus-specific probes with known polymorphism and cross-hybridization, preliminary work should be done using cells from both reproductive partners. When sexing for X-linked diseases the X and Y probes should be included in the first round of FISH to maximize their efficiency.

For cases involving structural chromosome abnormalities it is advisable to always test lymphocytes from the couple undergoing treatment with the specific probe set planned for clinical use. The probe set should at least contain probes able to detect all the expected forms of the rearrangement with chromosome imbalance. However, where suitable probes are not available, probe mixes that cannot detect some unbalanced forms of a rearrangement may be used providing the imbalance has been assessed to be non-viable in a recognizable pregnancy or to have a very low prevalence (Thornhill *et al.*, 2005).

Diagnosis based on a single mononucleate cell is acceptable for chromosome rearrangements providing there are at least two informative probes for the chromosome imbalance associated with unbalanced forms of the rearrangement that are considered likely to be prevalent or viable in a recognizable pregnancy. Diagnosis based on concordant results from two mononucleate cells is recommended where there is only one informative probe available.

For situations in which probe sets yield an unacceptably high rate of inconclusive results, it is advisable to validate a rescue panel of probes for use in a subsequent round of hybridization to confirm or reject initial findings. In this way, the rate of false-positives and the overall FISH error rate may be reduced (Colls *et al.*, 2007).

FISH validation

Preliminary work for each different probe set should include analysis of both metaphase spreads and interphase nuclei. At least ten metaphase spreads should be examined to ensure that the probes hybridize specifically to the correct chromosomes and to assess chromosome polymorphism and signal cross-hybridization, and, with respect to a chromosome rearrangement carrier, that the probes hybridize as expected to the different segments of the rearrangement. At least

100 interphase nuclei should be scored using appropriate scoring criteria that should include an assessment of signal specificity, brightness, and discreteness (Thornhill *et al*., 2005).

Since the analytical performance in blastomeres frequently approaches that of lymphocytes, it is not always necessary to test each new probe set on blastomeres before use in a clinical PGD cycle. Rehybridization procedures on single blastomeres in a clinical test environment require prior validation. The use of commercial probes is recommended since they generally come with manufacturer's QC and validation. However, the use of home-made probes may be acceptable with appropriate QC/QA and validation.

Scoring criteria (published or "in-house") should be determined ahead of time and should be adhered to as per written procedure with any deviations from procedure noted. Signals should be analyzed by two independent observers and discrepancies adjudicated by a third observer. If no agreement is reached the embryo should not be transferred, that is, it should be given the diagnosis of no result, uninterpretable or inconclusive. No probe should be passed for clinical use unless it meets an individual laboratory's predetermined and documented minimum score for efficiency, intensity, specificity, and minimum background.

Use of intra-assay controls-FISH

The use of positive and negative controls for PGD is contentious since suitable positive controls are not readily available for most chromosome rearrangements and negative controls only serve to confirm that the correct probes are in the mix. Controls should be performed wherever possible but their limitations in the context of single-cell diagnosis involving a different cell type should be considered. For example, a positive lymphocyte control included for all PGD FISH cases can verify the suitability of the probe and the conditions required for a specific clinical run, but provides little or no information regarding the nuclear preparation and quality.

Since at least three acceptable methods of spreading and fixing single blastomeres have been described (Thornhill *et al*., 2005), it is important to validate the method of choice in your own laboratory taking into account reproducibility and ease of use, post-fix nucleus location, quality of FISH, and so on.

PCR-based tests

Before offering a single-cell PGD clinical assay, confirmatory testing of the specific mutation on DNA (by use of standard tests) or review of the documented testing at another institution should be performed by a suitably qualified professional. Confirmatory testing of the clinical PGD assay on parental and proband blood DNA is important since the single-cell-specific PGD assay could be non-informative owing to PCR failure as a result of polymorphisms present in the population (Thornhill *et al*., 2002).

If using a generic haplotyping approach based on linkage rather than mutation detection (Renwick *et al*., 2006) it should not be necessary to perform a single-cell-specific test on patient DNA to detect the mutation. Instead, blood from relevant individuals is tested in advance to assess informativity for a panel of linked markers in the gene of interest.

Before beginning validation work toward a clinical test, a validation plan should be developed. This plan should define acceptable cell types, their numbers and genotype (e.g. heterozygous cells for assessing allele drop-out (ADO)), and negative controls for testing for contamination. The plan should state acceptable amplification efficiency, contamination, and ADO rates. As an example, many laboratories consider a contamination rate of less than 5 percent satisfactory (preferably zero), ADO rates at <10 percent, and amplification efficiency of at least 90 percent (Thornhill *et al*., 2005). A number of design processes and precautionary measures apply to the single-cell PCR procedure itself relating to amplification failure, contamination, and ADO, and have been described at length elsewhere (Thornhill & Snow, 2002). From a quality system perspective the procedures designed to optimize single-cell PCR after appropriate validation testing should be strictly followed and routine audits should be performed to assess assay quality.

Assay validation should be performed on appropriate DNA samples (including affected (autosomal dominant), carrier (autosomal recessive, X-linked diseases), and unaffected samples for the mutation to be tested, or heterozygous for appropriate polymorphisms if markers only are to be used) before moving on to validation in single cells. This should be followed by assessment of single cells or low template DNA equivalents from the same genotypes. Some centers run a large series of blanks periodically to assess baseline levels of contamination (Lewis *et al*., 2001).

Equipment

Diagnostic centers should identify critical equipment and ensure that back-up plans are in place in the event of failure of this equipment, especially in light of the time constraints in PGD. Critical equipment relating to PGD in the IVF laboratory would, for instance, include

the micromanipulation apparatus needed to perform embryo biopsy. Safeguards against equipment failure include a second duplicate micromanipulator with biopsy capability or an emergency agreement with another center in the case of failure. An example of critical equipment in the diagnostic laboratory is the FISH microscope or DNA analyzer. In either case, measures should be taken to ensure adequate cover in the case of failure.

All equipment should be identified and placed on a preventative maintenance schedule. In many cases, this will also include a service contract. New equipment should undergo stringent installation validation and calibration prior to clinical use. Note that duplicate equipment is preferably identical, allowing reproducibility of results and more cost-effective service contracts. Calibration and preventative maintenance are particularly critical in single-cell diagnostics since the margin for error is often extremely small and the opportunity for repeat testing is limited.

Details of essential equipment within the IVF laboratory to support the delivery of a PGD service have been described previously (Thornhill & Snow, 2002; PGDIS, 2004; Thornhill *et al.*, 2005). Owing to the time constraints imposed by single-blastomere diagnostic testing, there is a need for IVF laboratories to support extended culture (often to the blastocyst stage) and to provide robust cryopreservation services to support the cryopreservation and storage of surplus genetically "normal" embryos following preimplantation testing. Furthermore, laboratories must be equipped to perform intracytoplasmic sperm injection (ICSI) if undertaking single-gene PGD with PCR to avoid possible contamination with cumulus cells and sperm (Thornhill *et al.*, 2005).

Documents and records

The cornerstone of a robust quality management system is good documentation. Since PGD involves a multidisciplinary team, the potential exists for a wide range of standard forms, records and standard operating procedures documenting the many and various processes involved. Before performing any procedures, patients should have the opportunity to read relevant patient information sheets. All activities should be performed according to written standard operating procedures and recorded whether they relate to patients or laboratory processes. Standard version-controlled forms should be devised for use in each of the relevant processes and completed according to the standard operating procedure. Standard consent forms should be completed before performing any patient-related procedure. The laboratory should have a record retention policy that is at least as stringent as the minimum requirement dictated by local laws or regulatory policy (where applicable). In addition, in some countries, physical samples may need to be retained along with records. Typical documents associated with a PGD cycle using FISH (in addition to routine IVF documentation) might include: PGD patient information sheet; PGD patient consent form; PGD intention to treat form; diagnostic test request form; embryo biopsy standard operating procedure; cell preparation standard operating procedure; slide transport standard operating procedure; biopsy worksheet; cell preparation worksheet; record of sample chain of custody; blastomere FISH standard operating procedure; FISH method and results worksheet; FISH image record; FISH report; record of embryo selection and transfer; record of analysis of untransferred embryos; PGD invoice (for accounting purposes).

Assessments

Internal audits

For FISH-based tests spreading efficiency, FISH efficiency, and "no result" rates should be continuously monitored and reported internally. For PCR-based tests contamination, ADO rates, and amplification efficiency rates should be continuously monitored and reported internally.

Generally, where possible, confirmation of the diagnosis should be performed on all embryos not selected for transfer or cryopreservation to provide quality assurance as well as accurate and up-to-date misdiagnosis rates to prospective PGD patients. If confirmatory testing is not possible, clinics should make special efforts to follow up with the parents after prenatal testing or birth.

Follow-up data should be kept along with the cycle data, and should be sent to the ESHRE PGD Consortium or another data-collecting entity, for use in comparing clinics and methods of diagnosis.

Where possible, the IVF laboratory should compare PGD pregnancy rates and matched non-PGD (i.e., routine IVF) pregnancy rates within that center.

External quality assessment (proficiency testing)

Providing or participating in appropriate external quality assessment schemes (EQAS) is difficult when considering single-cell tests. In contrast to DNA extracted in large quantities from peripheral blood, single-cell

samples cannot be aliquoted, retested, or shared between large networks of laboratories. However, these limitations should not prevent effort being made to identify areas in which PGD providers can engage in the process of EQAS.

To date there has been no formal mechanism in place for external quality assessment (proficiency testing) for PGD diagnostic laboratories (Corvelyn *et al*., 2008). However, the ESHRE PGD Consortium recently implemented a voluntary system for both FISH image analysis and molecular testing from DNA samples to address this problem, with the aim of providing proficiency testing at least annually. Such schemes are already well established for both cytogenetic (Hastings *et al*., 2007) and molecular diagnostic tests (Simoni *et al*., 1999; Dequecker *et al*., 2001). In the near future, further use of whole-genome amplification techniques on single cells prior to diagnosis (Schowalter *et al*., 2007) to produce large quantities of DNA for sample exchange between laboratories may facilitate the routine implementation of EQAS for single-gene disorder PGD.

Competency assessment of testing personnel

Competency assessment prior to, during clinical testing, and periodically thereafter will require evaluation of skills in each of the laboratory tasks using criteria developed within each laboratory.

Process improvement

Events and assessments can all lead to process improvement. A major drive for process improvement is the registration of adverse events. A yearly analysis should focus on reoccurring errors to find aspects that require improvement. In addition, the scientific literature should be monitored to identify potential technological advancements that could improve the process by increasing sensitivity and specificity. Other drives for improvement include changes that reduce costs or time with respect to pre-analytic, analytic, and postanalytic phases of PGD.

Service and satisfaction

In the IVF sector, as in many other areas of medicine, more and more routine blood tests (e.g. FSH, estradiol, and so on) are being outsourced to external laboratories. Commissioning centers expect standardized, interpretable timely reports in conjunction with an underlying presumption of diagnostic accuracy. While PGD tests themselves are more sophisticated and more sensitive to error than routine blood tests, it is essential for diagnostic centers to maintain a patient focus in terms of service delivery in addition to diagnostic accuracy. Moreover, recognizing that both patients and the IVF clinical team are "clients" of the diagnostic team helps to support this ethos. Client satisfaction may be measured in the form of surveys with a focus on timeliness, accuracy, and interpretability of reports. Patient and clinical feedback should be sought wherever possible. The potential for poorer, more infrequent communication between diagnostic laboratories and satellite IVF centers should be managed pro-actively by strict adherence to standard operating procedures and regular communication.

Transport PGD

Satellite or transport PGD is the term used to represent IVF services supplied at one center and diagnostic services specific to PGD performed elsewhere and by unrelated entities. For example, transport PGD for chromosomal translocation testing involves transportation of slides containing fixed blastomeres from the IVF center to the diagnostic center. Transport PGD for PCR assays involves transportation of tubes containing lysed cells from the IVF center to the diagnostic center for the purpose of testing for single-gene sequences using PCR-based methods.

Special additional measures may be required to ensure smooth continuity of service in the case of transport PGD (see Table 16.5). Prior to performing any clinical diagnostic testing, the IVF center and the diagnostic center should agree on a validation plan to ensure accuracy and reliability. This plan will incorporate cell preparation, sample labeling, test requisitioning, sample transport, and reporting. The diagnostic center should validate the sample transportation protocols to ensure that transport of cells does not compromise amplification efficiency or FISH hybridization.

Transport PGD should require stricter QC or QA than "in-house" operations, with appropriate documentation including extremely detailed written procedures for sample transportation, result reporting, turn-around times, and so on.

Centers sending out cells and tubes should be trained in the biopsy, tubing, or fixation procedure according to the diagnostic center's procedures or after pre-approval of differences by the diagnostic center. If this is not possible, the satellite center should arrange to have a suitably qualified and trained embryologist to perform the biopsy and cell preparation.

Table 16.5 Special considerations for "transport" preimplantation genetic diagnosis

Quality system component	Measure taken
Organization	Establish clear lines of responsibility for each part of the process and ensure coherent lines of communication between centers
Personnel	Ensure staff at IVF center are suitably qualified and trained
Process control	• Transportation of sample standard operating procedure required • Adherence to embryo biopsy/cell preparation standard operating procedures at IVF center
Process validation	Perform sufficient number of dry-runs prior to clinical case
Documentation	Ensure all relevant contracts, standard operating procedures, and third-party agreements are in place before starting clinical work
Adverse events	Encourage IVF center to report incidents and other non-conformances
Assessments	• Inspection and assessment of IVF center – ensure biopsy and blastomere preparation procedures are suitable • Provide regular feedback of outcome data, spreading efficiency, etc.
Service and satisfaction	• Monitor timeliness of reporting based on agreed diagnostic test turn-around time • Provide up-to-date diagnostic information for patients and clinicians
Process improvement	Initiate meetings/conference calls to discuss test changes, results, adverse events, etc.

Conclusion

Although PGD has been performed for more than 15 years, PGD testing is largely unregulated by accreditation agencies and regulatory bodies at present, with some exceptions (ESHRE PGD Consortium Steering Committee, 1999; Corvelyn *et al*., 2008). It is clear that the application of single-cell-specific tests requires a high degree of accuracy and reliability. This requires an appropriate quality system to be in place that ensures proper quality control and assurance. The introduction of standardized methods, proficiency testing, and external quality assessment procedures among centers offering PGD (whether on-site or at a satellite laboratory) is in accordance with other forms of cytogenetic (Hastings *et al*., 2007) and molecular testing (Simoni *et al*., 1999; Dequecker *et al*., 2001) and should improve the standards of testing (Kettelhut *et al*., 2003; Ibarreta *et al*., 2004; McGovern *et al*., 2007) to ensure the highest quality of care for all patients.

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Section 3
Chapter
17

Ethics and the future

Preimplantation genetic testing: normative reflections

Guido de Wert

Key points

- Although it is widely acknowledged that assisted reproductive technologies (ART) may contribute substantially to human well-being, it is at the same time realized that the "reproductive revolution" generates a wave of moral questions concerning the responsibilities of prospective parents, the doctors and scientists involved, and society as a whole.
- Preimplantation genetic diagnosis (PGD) is at the center of this revolution. Possible categorical moral objections to PGD concern the biopsy, the selection of embryos, the burdens and risks of the preceding in vitro fertilization (IVF) procedure, the slippery slope, and the link with embryo research. These objections, however, are not convincing.
- The main ethical and regulatory issues to be addressed concern: controversial cases of PGD which fit the medical model *stricto sensu*, such as PGD of late-onset disorders and of lower-penetrance mutations; the paradigm case of PGD beyond the medical model, i.e. sex selection for social reasons; so-called "intermediate" cases of PGD, especially PGD/human leukocyte antigen (HLA)-typing.
- Finally, the normative aspects of multiplex genetic testing (simultaneously testing for many genetic characteristics) will briefly be discussed.

Introduction

No doubt, assisted reproductive technologies (ART) may contribute substantially to human well-being. At the same time, however, the "reproductive revolution" raises a wave of moral questions concerning the responsibilities of prospective parents, the scientists involved, and society as a whole. This holds true particularly for preimplantation genetic diagnosis (PGD), which aims at a selective transfer. PGD was primarily developed in response to requests for help from people at high risk of passing on a serious genetic disorder to their children. In recent years, the number of PGD cycles, as well as the number of centers involved in PGD, has increased year by year (Sermon *et al.*, 2007). More recently, clinical trials concerning preimplantation genetic screening (PGS) for aneuploidy have been introduced in some centers. This chapter considers ethical and legal aspects of preimplantation genetic testing, which includes both PGD and (various types of) PGS. While preimplantation genetic testing may be performed pre- and post-conceptionally, I will concentrate on the latter.

Preimplantation genetic diagnosis

Here, I will first comment briefly on some of the objections presented by critics of PGD. Second, some of the moral conditions for clinical PGD will be addressed. Third, I will focus on some of the main ethical issues regarding PGD, more in particular on the debate about controversial indications.

Categorical objections?

Critics have at least five moral (deontological or consequentialist) objections. Are these objections overriding or convincing?

Unjustified selection

Objections to the selection of embryos inherent in PGD come from different perspectives. A first objection holds that selecting embryos is intrinsically wrong given the "sanctity of human life;" the embryo should be respected as a human person, that is, the embryo has an absolute right to life. The German Embryo Protection Act, for example, is founded on the principle that embryos in vitro are wholly worthy of respect and nothing should be done to them which is not for their own maintenance (Gesetz, 1990). This (minority)

view is problematic for several reasons (Hursthouse, 1987). First and foremost, the "sanctity of life" doctrine is based on shaky premises. It is certainly correct to say that the embryo belongs to the human species, but it does not follow that it has the same status as "one of us," as human persons. The basis of personhood and moral status of humans is controversial, but preimplantation embryos lack any of the properties that might reasonably be claimed to confer personhood. One relevant point concerns the absence of so-called developmental individuation: until the embryonic cells are differentiated and organized to become the primitive streak, there is no individual in any sense of the word, biological, legal, or moral. The view that an embryo has the same status as an actual person because it has the *potential* to become an actual person is difficult to accept. The dominant view, both in society and in ethics, then, is that the embryo has a relatively "low" moral status. In view of its potential and/or symbolic value, the embryo deserves some respect and regulation is commonly used to ensure that embryos are not destroyed for trivial reasons.

Furthermore, the minority view is at odds with various accepted practices like in vitro fertilization (IVF) (where the disposal or loss of spare embryos is rather common), the prescription and use of intrauterine contraceptive devices (IUDs) (which have a double working mechanism, including the prevention of implantation of fertilized oocytes) and prenatal diagnosis. There is a strong consensus that regular prenatal diagnosis and selective abortion can be morally justified as a means to prevent serious suffering for the future child and/or the parents/family. PGD, then, can be justified as well: the German, Italian, and Swiss approach of accepting prenatal diagnosis and selective abortion while prohibiting a selective transfer is inconsistent (Gesetz, 1990; Buggio, 2005).

Second, from a so-called "disability rights" perspective, it is objected that a selective transfer of only "healthy" embryos, like selective abortion, is at odds with the rights and interests of disabled people. These practices, according to the critics, discriminate against the disabled, and imply a denial of their equal worth and even of their right to exist. The presumed logic of this so-called "expressivist" argument is questionable (Chadwick, 1987). After all, it is not only affected embryos and fetuses that are disposed of or aborted. Healthy embryos and fetuses are regularly disposed of or aborted too, for example if they constitute a threat to the welfare of the woman or couple. But it does not follow that any newborn or adult that is a threat to someone's welfare is less worthy of respect. The crucial boundary here is that between embryos/fetuses, on the one hand, and children/adults, on the other, not between disabled and healthy. Another objection is the "loss of support" argument, i.e. the charge that as the use of genetic technologies reduces the number of persons suffering from genetic disorders, public support for the disabled will dwindle (Buchanan *et al.*, 2000). Needless to say, this objection would also apply to *pre*-conception PGD (polar body biopsy) – and practices like folic acid supplementation, aiming at the prevention of neural tube defects. There clearly is a societal obligation to maintain – and even to improve – support for disabled people, but it is a *non sequitur* to argue that this generates a claim on the part of those individuals that society must ensure that their numbers do not decrease. It is unwarranted to construe a conflict between, on the one hand, the needs and rights of prospective parents who want to prevent the birth of a disabled child and, on the other, the interests of disabled people.

To endorse the "anti-embryo-selection" ethos would have bizarre implications. Prospective parents and physicians would be morally obliged to accept or transfer all embryos available, irrespective of their "quality." This is difficult to accept in view of the parental and professional responsibility to consider the welfare of the future child. This policy could also have dangerous consequences for the health of the prospective mother (see below). In other words: depending on the consequences, it is not the non-transfer, but the transfer of defective zygotes or embryos which is problematic from a moral point of view.

It is also noteworthy that affected spare embryos may be used in embryo research, including stem cell research (Mateizel *et al.*, 2006). This is allowed in many countries, and is ethically justified in view of the relatively low moral status of the preimplantation embryo and on the condition that this research use meets the principles of proportionality and necessity/subsidiarity.

To biopsy or not to biopsy

Another objection to (invasive, post-conception) PGD concerns the biopsy: "The individual blastomeres of the embryo have the same developmental potential as the embryo 'as a whole' – these cells are totipotent. Therefore, isolating a blastomere involves the creation of a second embryo. As this will be destroyed, the

biopsy is inherently wrong." For this reason, among others, post-conceptional PGD is prohibited by the German Embryo Protection Act (Gesetz, 1990). This objection is weak. First, it is unknown as to whether individual blastomeres isolated at the 6–10-cell stage are still totipotent. Though some of these cells may be able to develop into a blastocyst, these cells probably lack the potential to develop into a child because of a strongly reduced "inner cell mass" (personal communication, Dr. Hilde Van der Velde). Second, even if it were proven that (invasive) PGD involves the creation and destruction of a second embryo, we could still justify this technique, in view of the relatively "low" moral status of a preimplantation embryo, and the "pro's" of PGD in comparison with a selective abortion (see below). Finally, the objection does certainly not apply to a trophectoderm biopsy at the *blastocyst* stage (Kokkali *et al*., 2007).

Disproportionally burdensome?

It is a major advantage of PGD that it may avoid traumatic (repeated) selective abortions. At the same time, however, PGD carries significant disadvantages. First, PGD necessitates IVF or intracytoplasmic sperm injection (ICSI), which is not the most pleasant way to "make" babies. In turn, IVF/ICSI give rise to various risks for women (such as infection, ovarian hyperstimulation syndrome, multifetal pregnancies, and so on), and offer only a moderate chance of success in terms of their "take-home baby rate." Second, the safety of the biopsy (or sequence of biopsies) as well as the reliability of some of the tests used remains to be proven. In view of these disadvantages, some paternalistic critics conclude that PGD is "too burdensome, whereas there are reasonable alternatives." These critics ignore, however, that balancing the advantages and disadvantages of the respective options is highly subjective. For some couples, the so-called "reasonable alternatives", more in particular prenatal diagnosis and selective abortion, are a priori unacceptable. For couples carrying specific balanced translocations, PGD may avoid repeated miscarriages of fetuses carrying unbalanced translocations. Also for *infertile* couples at high risk of conceiving a disabled child, who opt for IVF/ICSI as an infertility treatment, PGD may be far more attractive than a non-selective transfer, followed by prenatal diagnosis and, possibly, termination of pregnancy. Interestingly, according to the European Society for Human Reproduction and Embryology (ESHRE) data collection VI, nearly 50 percent of PGD cycles were performed in couples having both fertility and genetic problems (Sermon *et al*., 2007).

These "anti-paternalistic" reflections underscore the importance of the concept of informed choice (see below).

Slippery slope

The "slippery slope" argument comes in various types. A first one is that PGD will result in embryo selection for less serious or even minor defects. But what is less serious? What is (too) minor? It is likely that there will always be substantial disagreement on these points. Some critics argue, for example, that late-onset disorders *per se* are of less concern, but that position is debatable. The prospect of PGD for less-serious or minor defects is, in fact, an argument for debate about where to draw the line, and may hardly be seen as an overriding objection to PGD. In any event, the present risks, burdens, costs, and moderate take-home baby rates of IVF or PGD function as barriers against trivial use.

A second variant is that PGD will eventually allow prospective parents to design what they consider to be the "perfect" child, whether that involves the selection of intelligence, beauty, heterosexuality, or any number of other characteristics. The feasibility of this type of selection is strongly exaggarated in the media and in science fiction: in reality, the preferred characteristics are rather complex, determined by many different genes and environmental factors. More embryos may be needed to obtain the desired genetic combinations than one woman can produce in a lifetime. The complexity of the preferred phenotypes makes the prospect of the "designer baby" highly unlikely (Human Genetics Commission, 2006). Whether this prospect would become more realistic if it were to become possible to create large numbers of embryos by fertilizing large numbers of oocytes obtained by in vitro maturation (IVM) is yet to be seen. There may be a few exceptions to this general technical difficulty. However, this is an argument for debate about drawing the line and adequate regulation, and not an argument to prohibit PGD altogether.

Embryo research

A final objection does not so much concern PGD as such, but the development of new applications of PGD in so far as these may require preclinical research with human (preimplantation) embryos, aiming at investigating the safety, reliability, and effectiveness of the experimental procedure. A good example may be PGD of mitochondrial disorders, especially for mutations which do not meet the criteria proposed by Poulton and Turnbull (Poulton & Turnbull, 2000; Bredenoord *et al*., 2008). In order to investigate the possible value

of PGD in these cases, female carriers of the particular mutation might be asked to donate oocytes to be fertilized for "preclinical" PGD. Scientists could then study the distribution of defective mitochondria during early embryo development.

Critics argue that embryo research unjustifiably treats embryos solely as a means. Again, the dominant view (also in ethics) holds that preimplantation embryos have a lower moral status (see earlier) and that research with these embryos may be justified within some ethical contraints. As we all know, the "European Convention on Human Rights and Biomedicine" allows the research use of spare embryos, but prohibits the creation of embryos solely for research purposes (Council of Europe, 1996), and this position is echoed in the legislation adopted by a number of European states. We may question, however, whether there is a fundamental moral difference between using spare embryos in research and the generation of embryos for research purposes. After all, the embryo is used instrumentally in both cases, and the moral status of the embryos is identical.

The main issue may well be the protection of the autonomy and health interests of the oocyte donor(s) (Gerrand, 1993; Olsthoorn-Heim *et al*., 2006; Pennings *et al*., 2007). After all, women may have to undergo hormone stimulation and invasive medical procedures, which carry some known and unknown medical risks. Furthermore, there is a risk of more or less subtle pressure and even exploitation. While these concerns are clearly relevant, they can hardly justify an absolute ban on creating embryos for research purposes. The interests of donors may be sufficiently protected by imposing protective conditions, such as a requirement that medical risks be minimized, and by limiting the numbers of hormone treatments as well as the dosage of hormones given to candidate donors. Of course, a valid and informed consent presumes adequate and balanced information and measures to safeguard voluntariness – think of the provision of counseling by independent experts. This condition is also relevant if female carriers who opt for experimental PGD would be solicited to provide oocytes for preclinical safety studies. While these women may eventually benefit from the new technique, their dependence (being patients) makes them vulnerable.

I conclude that there are no convincing, categorical objections to PGD. At the same time, PGD raises some complex questions, which need scrutiny and debate.

Conditions

In this paragraph, I will briefly comment on some of the relevant conditions for PGD.

Informed consent and counseling

PGD requires free and informed consent. It is of utmost importance that the couple understands the (practical) benefits and disadvantages of PGD in comparison with the other options, such as regular prenatal diagnosis and assisted reproduction using donor gametes.

It is important to discern the information component and the consent component of informed consent (Beauchamp & Childress, 2001). General information which ought to be provided concerns, among other things, the nature of PGD and the alternative reproductive options, the likelihood of success, the kinds of decisions that may have to be made at a later stage in the treament (e.g. regarding the – selective – transfer and regarding surplus embryos), and the known and unknown risks of the procedure(s), both for the woman and for the prospective child. Given the aim of informed consent, namely, to enable individuals to make informed choices, the specific informational needs of the patient should be considered: disclosure of information ought to be tailored to every individual applicant.

With regard to the consent component, so-called "radical" feminists maintain that women cannot give truly voluntary consent for infertility treatments, because their choices are conditioned by "the patriarchal power structure and a pronatalist ideology." It is argued that if women were not conditioned to think that motherhood is their supreme fulfillment, they would not submit themselves to the risks and burdens of ART (Sherwin, 1987). So-called "liberal" feminists, however, rightly argue that to insist that the desire for children is merely a social construction is as untrue as insisting that women never freely choose to have an abortion (Steinbock, 1996). The challenge is to provide each couple or woman with an opportunity to have access to desired reproductive services within a framework that is sensitive to their needs and values, and minimizes the potential for coercion. As we will see later, possible tensions may arise between the preferences and values of the applicants, on the one hand, and the interests of the prospective child, on the other. Should traditional nondirectiveness, then, be the guiding principle?

The interests of the child

One of the vexing issues regarding medically assisted reproduction concerns the scope and implications of the responsibility of doctors involved: do they also have a professional responsibility to consider the interests of the future child and, if so, how should this responsibility be fulfilled?

Some argue that any such scrutiny of reproductive decision-making constitutes an unacceptable violation of individual reproductive autonomy. It is observed that people who are able to conceive "naturally" are not scrutinized in this way and do not need a "license to procreate." This criticism is, however, debatable. As a matter of fact, the potential child will not exist without the involvement of a medical team in his/her coming into existence. That team, therefore, has a role-specific responsibility concerning the interests of the future child. Those who object that the refusal to give unconditional access to "high-risk" patients amounts to an invasion of their right to reproduce, may well wrongly interpret this liberty right as a positive claim right. Indeed, people's right to reproduce is the right to procreate without interference from the state or a third party. Infertile patients do not have an unqualified "positive" right to reproduce, that is, a right to assistance in reproduction, which would imply an unqualified duty of the physician to provide ART.

There is no consensus on how to make this professional responsibility to consider the welfare of the child operational. Three guiding principles are distinguished (De Wert, 1998a; Pennings, 1999):

- The "minimal risk" or "maximum welfare" standard: doctors should refuse to give access to ART if there is *any* risk to the well-being of the prospective child. This standard is simply too strong – the large majority of applicants should be excluded from assisted reproduction, while (especially) ICSI would be a priori unacceptable.
- The "minimum threshold," more in particular the "wrongful life" standard: assisted reproduction should be refused if there is a serious risk that the life of the child would be so miserable to him that no one would want to live such a life – it would be better not to exist. This is an extremely low standard, which leaves very little room for contraindications for assisted reproduction based on the welfare of the child – in fact, the doctor would be obliged to make decisions which many of us would consider to be counterintuitive and irresponsible. The fact that future children might have lives that were on balance worthwhile cannot function as an excuse for imposing grievous suffering and deprivation on them.
- The "high risk of serious harm" or "reasonable welfare" standard; this is an intermediary between the other ones, which avoids their counterintuitive implications.

While a further debate about these guiding principles is important, it would be naïve to assume that consensus about the "best guiding principle" will result in consensus about each individual case. Each of the principles needs interpretation (what is high risk, what is serious, and so on) – and may invite controversy if applied to individual cases (Arras, 1990).

Traditionally, the debate about the welfare of the child focused on possible psychosocial risks, but medical risks are equally relevant. As we will see, the professional responsibility to consider the welfare of the child is an important topic in the context of PGD.

Indications

One of the "classical" questions concerning prenatal diagnosis is also relevant with regard to PGD: what genetic tests should be performed? Should a line be drawn, and if so where? Should one stick to the traditional "medical model," i.e. the view that PGD, like prenatal testing, should be restricted to the identification of disabilities and disorders, allowing the prevention of the birth of affected children?

Some commentators argue that this model is too restrictive, as it limits the freedom of prospective parents to test and select for or against "non-medical" traits. This model should, therefore, be replaced by a "parental autonomy" (or the "designer") model (Robertson, 1992). As we will see, there is an in-between, third, category: PGD for "intermediate" cases.

The medical model

The dominant moral justification for selective abortion as well as a selective transfer is the prevention of severe suffering related to genetic defects and disorders – not just the suffering of the future affected child but also suffering of the parents (family). But how severe does a defect or disorder have to be in order to qualify for prenatal testing or PGD? (For people carrying specific balanced translocations, the rationale of PGD may be to prevent additional miscarriages of fetuses carrying an unbalanced translocation.)

Some commentators have proposed making an exhaustive, detailed list containing all the acceptable

indications. This approach is problematic, because, among other reasons, it pays insufficient attention to the personal circumstances and values of the future parents. Although the point of view of the future parents is ethically important, respect for autonomy is not unqualified. PGD should not be performed for defects or conditions which cannot reasonably be considered to be a serious threat to the well-being of the future child and/or the family. The question therefore arises "Where precisely to draw the line?" This is, of course, difficult to answer. It is regularly suggested that three relevant factors should be considered simultaneously (De Wert, 1990; Council on Ethical and Judicial Affairs, 1994; Steinbock, 2002):

- the severity of the defect or disorder, determined partly by the availability of treatment
- the age of onset of the disorder
- the penetrance of the genetic defect (the probability that the genotype will affect the phenotype).

Clearly, prenatal testing for a defect which *(nearly) always* results in a *serious childhood* disorder differs in a morally relevant way from prenatal testing for a defect which *may sometimes* result in a *mild* disorder *late* in life.

Let me now focus on PGD for three types of disorders.

Dominant, "mid-life" onset, untreatable disorders

Huntington disease is the paradigm case for this category. Critics argue that a child carrying the Huntington disease mutation will have "many decades of good and unimpaired living. Moreover, the parents are not affected in the way they would be were the disease of early onset." And, so they argue, we should acknowledge the moral ambiguity of the quest for "perfect" babies ("perfectionism") (Post, 1991).

Obviously, it would be a misguided effort to fight against all contingencies of human life. The question is, however, whether carriers of Huntington disease, who have a high (50 percent) risk of transmitting the mutation to their children, have the moral right to prevent this by making use of PGD. In view of the fact that Huntington disease is a severe disease, and that the penetrance of the mutation is complete, the answer may well be affirmative. It is insensitive, if not an insult, to (dis)qualify preventive measures as symptomatic of "genetic perfectionism." Of course, carriers of Huntington disease will usually be healthy for three to four decades. However, the prospect of their eventual fate imposes an extremely severe burden.

The request of a carrier of Huntington disease to access IVF may *in itself* pose a dilemma for doctors involved. After all, a carrier's competence as a parent will probably be lost steadily, increasing the burdens on the other parent. Furthermore, the child may be confronted with the extreme suffering and death of the affected parent at a young age. However, these child welfare considerations do not constitute an overriding argument against medical assistance in the reproduction of Huntington disease carriers. It is well-known that most children of Huntington disease families are able to cope reasonably well (Forrest Keenan *et al.*, 2007). Relevant variables include the coping skills of the parent not at risk of Huntington disease, the quality of the family network, and the age of onset of the disorder. In many cases the child will be an adolescent or even an adult when the parent who carries the mutation becomes symptomatic. Even if the prospective father or mother is already *symptomatic*, it may well be justified to assist in individual cases. Specific risk factors to be considered include possible role inversion, where children carry out tasks and responsibilities for an affected parent which would normally be undertaken by an adult, and illness of the other parent (Forrest Keenan *et al.*, 2007).

There is a strong consensus that PGD of Huntington disease can be morally justified. As similar cases should be handled in the same way, PGD for early-onset, dominant, Alzheimer disease, hereditary Pick disease, and autosomal dominant types of amyotrophic lateral sclerosis, for example, are acceptable as well.

Some applications of PGD for late-onset disorders aim at preventing the transmittance of the mutation while protecting the asymptomatic applicant's right not to know his own genetic status. One of the possible strategies is "non-disclosure" PGD: couples are offered PGD without ever being informed of the test results (Schulman *et al.*, 1996). They will be told only that embryos were tested and that only apparently disease-free embryos were replaced. Hence, people at risk would derive no information about their own genetic status, while PGD, if performed accurately, could reduce the risk for their progeny to zero. However, this procedure raises troubling issues (Braude *et al.*, 1998; De Wert, 1999). Let us suppose that the first PGD cycle does not identify any affected embryo. The statistical

risk of the parent at risk may become close to zero. To tell the applicant this good news would constitute an indirect breach of other at-risk applicants' right not to know; after all, they may draw their conclusion if they do not receive this good news. The problem, then, becomes whether the doctor involved should offer a second (and a third, fourth, etc.) IVF/PGD cycle. Another problem arises, if there are no embryos available for transfer, either because all embryos carry Huntington disease, or for other reasons, like IVF failure. The client at risk might, rightly or wrongly, infer that he is a carrier. Should one consider a *sham* transfer? For these reasons, most IVF/PGD clinics are highly sceptical about non-disclosure PGD.

Lower-penetrance mutations for disorders which may be preventable and/or treatable

A relevant example of this category is hereditary breast/ovarian cancer (HBOC). PGD for relevant breast cancer gene mutations (BRCA-1 and -2) is even more controversial than PGD for Huntington disease, because the penetrance of these mutations is incomplete and preventive interventions, including prophylactic surgery, may effectively reduce morbidity and mortality in carriers (Editorial, 2006). Again, we should resist premature conclusions (Delhanty, 2006; Niermeijer *et al.*, 2006). First, although these mutations have an incomplete penetrance, the risks are still (very) high; in seriously affected families, female carriers of a BRCA-1 or -2 mutation may have a lifetime risk for breast cancer of 50–80 percent, while their risk for ovarian cancer is 20–50 percent. Why should the penetrance need to be almost complete in order to qualify for PGD? This criterion would be inconsistent, as sex selection for X-linked disorders is widely accepted even though the risk for boys of having the mutation and developing the disease is "only" 50 percent. Second, morally relevant questions concern the effectiveness of available preventive and/or therapeutic measures, and the burdens involved (De Wert, 1998b). Unfortunately, the effectiveness of medical surveillance is currently far from optimal. Though the effectiveness of prophylactic bilateral mastectomy appears to be high, longer follow-up studies of more carriers are necessary to definitely establish the protective value of this procedure (Meijers-Heijboer *et al.*, 2001). Last, but not least, prophylactic surgery may have major implications for women's quality of life. An analogy might help. Take the theoretical case of a family affected with testicular cancer, caused by a highly penetrant mutation; would we recommend prospective parents who request PGD to have their future son castrated?

The fear of prospective parents ("at risk") that their future children may inherit a BRCA gene mutation is far from unreasonable. The Human Fertilisation and Embryology Authority (HFEA) rightly decided to allow PGD of HBOC (HFEA, 2006). According to a recent study from the UK, the majority of BRCA gene mutation carriers endorse the HFEA decision, even though they would not opt for PGD themselves (Mernon *et al.*, 2007).

Similar questions (will) arise regarding other types of hereditary cancer (Offit *et al.*, 2006; Niermeijer *et al.*, 2006; Spits *et al.*, 2007). Although complete penetrance is too strict a criterion for access to IVF/PGD, it is reasonable to define a minimum penetrance. One of the questions to be addressed is whether it makes a difference if the applicants are fertile or need assisted reproduction anyway. Would it be justified to be more permissive in the latter situation? This question is especially relevant as PGD may be an attractive option for patients suffering from hereditary cancer who opt for "fertility insurance" by means of cryopreserving "reproductive material" (Ethics Committee of the ASRM, 2005; Donnez *et al.*, 2006).

PGD for mitochondrial disorders?

Part of the mitochondrial disorders is caused by a mutation in an mtDNA-encoded gene. For many mtDNA mutations, it is difficult to provide an accurate prediction of the phenotype. Poulton and Turnbull (2000) formulated the following criteria to evaluate the possibility of prenatal diagnosis and PGD for mtDNA mutations: there should be: a close correlation between the level of mutant load and disease severity; a uniform distribution of mutant mtDNA in all tissues; and no change in mutant load over time. The question as to whether these criteria are flexible guidelines or strict rules is a real clinical dilemma. People sometimes request PGD of an mtDNA mutation which does not fit these criteria. A good example is m.3243A>G. A couple may opt for PGD "just" in order to *lower* the risk of conceiving a severely affected child, knowing that the technique cannot guarantee that the child will be free from the disease (Bredenoord *et al.*, 2008). Is it a priori unacceptable to assist because PGD should aim at giving certainty? Or may PGD be acceptable if people insist on it after adequate counseling and accept the residual risk, which might be further reduced by prenatal diagnosis? What other conditions have be to imposed in view of the professional's responsibility to

consider the welfare of the child? What about the cut-off point regarding the mutant load of embryos to be transferred?

It is of utmost importance to give balanced information about possible alternative reproductive options, including the use of donated oocytes. In the future, it might become possible to eliminate defective mitochondria by means of somatic cell nuclear transfer into a "healthy" enucleated oocyte. The ethics of this approach is beyond the scope of this chapter (De Wert, 2000).

The parental autonomy model

Some commentators argue that prospective parents should be free to use PGD for the selection of any offspring characteristic they prefer, as selection for medical as well as non-medical purposes is part of reproductive freedom (Robertson, 1992). In the context of PGD, this view might receive somewhat broader support as many people consider embryos to have a lower moral status than fetuses. The paradigm case for the parental autonomy model is PGD for social (non-medical) sex selection, but clearly, other applications, which may raise partly specific moral problems, will emerge.

Sex selection for non-medical reasons

There seems to be a strong consensus in European countries that this type of sex selection is not acceptable. In fact, it is prohibited in many countries. Objections are, among others, that children should be accepted and loved unconditionally, that non-medical sex selection might set the scene for "designer babies," that it may reinforce sexist views and gender stereotypes, and that it may distort the sex-ratio. Conversely, critics of a prohibition argue that this procedure is not necessarily sexist, and that reproductive freedom should be respected if there is no evidence of foreseeable harm from allowing people freedom to choose. They argue that concerns that boys would be favored if selection was permitted, and that, as a consequence, the sex-ratio would be distorted, could easily be circumvented by allowing non-medical sex selection only for "family balancing." Adhering to this condition may also lessen the risk of sexism. Both the UK Parliamentary Science and Technology Select Committee and the Ethics Committee of the American Society of Reproductive Medicine (ASRM) have concluded that there is insufficient empirical evidence to support the prohibition of non-medical sex selection. The ESHRE Task Force on Ethics and Law has not been able to reach a unanimous decision in this regard (Shenfield *et al.*, 2003).

The Health Council of the Netherlands suggested some sort of a compromise, namely to allow sex selection for "family balancing" when PGD is necessary for medical reasons anyway and no additional testing is needed (Health Council, 1995, 2006). For many people, the latter will probably be more ethically acceptable than IVF/PGD specifically for non-medical sex selection. Is the sex of the child so important that it is proportional to perform and undergo IVF/PGD for non-medical reasons – and if so: why precisely? Isn't the request indicative of undue stereotyping? The ethical debate might be enriched and better informed if counselors could provide information about specific motivations of individual applicants. If we could reasonably conclude that applicants may, in individual cases, have a strong and ethically acceptable interest in IVF/PGD for non-medical sex-selection, it should only be done for balancing, and it should not be collectively funded (Pennings, 2002). It would be unacceptable if clinics were to become overloaded by performing IVF/PGD for family balancing and had to postpone clients with a medical indication for PGD.

If doctors take the Health Council principle "that doctors should not perform any additional actions for the purpose of (non-medical) sex selection" seriously, there is little room left for such selection, since doctors should give priority to embryological criteria in deciding which embryos to transfer. Clearly, to start a new IVF cycle while there are still (high-quality) embryos of the non-preferred sex available for transfer would blur the distinction between non-medical sex selection in the context of medically indicated IVF/PGD, on one hand, and IVF/PGD just for non-medical sex selection, on the other. To modify the transfer policy for family balancing would raise problems of fairness in the context of medically indicated IVF/PGD as well.

"Superbabies?"

To a large extent, the prospect of the so-called "designer baby" may well be unrealistic, considering the complexity of the preferred characteristics such as IQ. However, there may be some exceptional cases where "enhancing" by means of PGD could become realistic. To select for "athletic genotypes" may be one of these. According to Bouchard *et al.* (1997), it is likely that in the years to come probes will be used to identify the carriers of DNA sequence variations desirable for sports performance. It has been suggested that nothing will prevent parents or coaches from using genetic probes in children for the purpose of identifying

talented individuals. As Rankinen *et al*. state, "There is also the possibility that athletic-minded and overambitious parents may…advocate embryo selection based on specific athletic probes to allow parents to fulfill their dreams. On the surface this scenario may appear like science fiction. It is not." (Rankinen *et al*., 2001).

If the scenario of PGD-mediated designing may not be altogether unrealistic, a proactive ethical reflection is needed. Relevant questions include the goals of medicine, the moral quality and weight of the couple's reproductive interests, the proportionality of destroying embryos for designing purposes, and, last but not least, possible adverse effects (harms) for the child and society at large (President's Council on Bioethics, 2003). Here I will make just a few remarks about the latter.

Habermas (2003) categorically opposes genetic designing, as this would transform our (children's) self-understanding; we could no longer see ourselves as the authors of our own life histories and recognize one another as autonomous persons. We agree that too much genetic intervention may make us feel mere objects of our parents. Feinberg (1980), writing about how to respect children's autonomy, rightly stressed the importance of the "right to an open future." The best way for parents to respect autonomy is to give the child as much input as possible at every stage. Most people prefer to have a high degree of self-creation and independence, but is the importance of independence from parents an absolute value? Glover (2006) recently asserted that several values are at stake: "For a richer life or for greater power to shape ourselves some loss of independence may be a price worth paying." He argues that it may be morally justified to cross the medical boundary (i.e. leave the medical model) and eliminate (both medical and) *non-medical obstacles to human flourishing*. However, how do we then define (children's) flourishing and identify the non-medical obstacles to flourishing? Are our intellectual limits among these, as Glover seems to suggest? To enhance intelligence by means of PGD seems, however, to be unrealistic. Using PGD to conceive a child with an athletic genotype may be more realistic – but would this be a paradigm case of "removing an obstacle to flourishing" or should we worry about parents who may be so obsessively interested in the child having a specific excellence that they may prevent it from living its own life and developing its own identity – thereby *causing* an obstacle to flourishing?

Apart from ethical questions regarding the implications of genetically designing children for these children's welfare and autonomy or open future, we should also consider the possible adverse effects for society at large. What about the dangers of uniformity and diminishing human variety if many prospective parents would make similar choices? What about increasing genetic competition, resulting in a "rat race?"

Assuming that some PGD-mediated designing (although very limited) might become technically possible in the future, the normative issues involved need proactive scrutiny.

"Dysgenic" PGD?

PGD may also allow prospective parents to select in favor of disabilities. The best-known example concerns hereditary deafness. In cases of non-syndromic, monogenetic deafness, various situations may be discerned in view of its genetic heterogeneity: some deaf couples can conceive deaf children only; others can beget hearing children only; while a third group can conceive both. The reproductive genetic risk is 50–75 percent in the latter group. In these couples, PGD may be possible in individual cases, if the causative mutation is known. What about a deaf couple's request of PGD in order to selectively transfer *affected* embryos (De Wert, 1999; Davis, 2001)?

The couple may point to psychosocial and developmental risks of *hearing* children growing up with (two) deaf parents. Concerns include that (young) hearing children will have difficulties in understanding the implications of their parents' disability and related behavior, that deaf parents will have only limited access to the experiences of hearing children, and that there is a risk of role inversion. Furthermore, they may argue that "deafness is not a handicap, but just a variant on the specter of normalcy." After all, deaf people have their own rich culture and their own (non-verbal) language. We may reasonably doubt, however, whether the "just a variant" view is tenable; after all, outside the micro-cosmos of the deaf subculture deafness *is* a disability which causes a variety of serious and lifetime challenges. Though deaf people still can (and usually do) live a reasonable happy life, selection for deafness seems to be at odds with the professional responsibility of the reproductive doctor. The couple's relational concerns should be tackled by educational support and advice, not by "dysgenic" PGD.

Interestingly, ongoing technology development may at the same time complicate and solve the current moral puzzle. Should parents choose a cochlear implant for a deaf child? Until now, cochlear implants

have been controversial, among other things, because their success is patchy. However, when the perfect version of the cochlear implant would become available in the future, parents will harm a child they leave deaf. To select for a deaf child, then, becomes self-defeating (Glover, 2006).

Intermediate cases

Some applications of PGD do not fit into the medical model *stricto sensu*, as (part of) the testing is not linked to possible health problems of the future child, whereas there still is a link to the medical model in the wider sense, in that the testing may be relevant for the health of a "third party." These cases illustrate that the dichotomy between the medical model, on one hand, and the parental autonomy model, on the other, is simplistic. I will focus on two cases to illustrate this third, intermediate, category (De Wert, 2005).

PGD/HLA-testing

As we all know, PGD/HLA-testing is controversial (Pennings & De Wert, 2003). Critics argue that this procedure involves the instrumentalization of the "donor" child (in fact a misnomer, as the parents donate the cells), that it carries disproportionate (psychosocial) risks for the child, that healthy embryos which cannot be matched may be destroyed, and the slippery slope argument: "this is but a first step toward the designer baby." We may, however, seriously question whether these objections are convincing. What matters first and foremost is whether the parents will value the child only as a transplant source or whether they will also love the child for itself. There is no evidence whatsoever to suggest that the parents involved would be bad parents. The objection in terms of the status of the preimplantation embryo is weak in view of both the dominant view that this status is relatively low and the disaster hanging over the heads of the parents and the terminally ill child. Finally, the slippery slope argument disregards the "therapeutic intent" of PGD/HLA-testing.

Regulations in Europe differ. France and the Netherlands, for example, permit PGD/HLA-testing on the condition that PGD is *primarily* undertaken to avoid a serious disorder in the future child (HLA testing is "only additional"), while the UK and Belgium permit PGD solely in order to provide a compatible "donor" as well.

Obviously, PGD/HLA-testing is not an easy way out of the parents' dilemma. For various reasons, including the "take-home baby rate" of only 10 percent (Fiorentino *et al.*, 2005) and the fact that parents may feel pressurized to opt for this procedure while they cannot afford another child, it is imperative to develop alternative strategies. One future option might be PGD/HLA-testing *type 2* in order to select matched embryos from which embryonic stem cells could be derived to produce hematopoietic stem cells for cell therapy (De Wert, 2005; De Wert *et al.*, 2007). If effective and safe, the type 2 case is, *prima facie*, an even better option from a medical, psychosocial, and ethical point of view – assuming that to create embryos for "instrumental use" may be justified.

Sex-selection for "mixed" reasons

Let us, to illustrate the second intermediate case, take the example of a male patient suffering from an X-linked disorder, e.g. hemophilia. It is well-known that some hemophiliac patients prefer to conceive boys only, because sons will not carry the mutation, *whereas all daughters would be obligate carriers of hemophilia.* What if this patient were to request PGD in order to select for male embryos: would this be an acceptable indication for PGD?

Adhering to the medical model *stricto sensu*, the answer should be negative as none of this male's children will be affected with hemophilia: there is no medical indication for PGD/sex-selection. This reasoning, however, does not do justice to the problem at hand. The reason for sex-selection is a "mixed" one. On one hand, the reason is non-medical, as the future daughters would not be affected with hemophilia. On the other, however, the reason is a medical one. After all, it concerns these possible daughters' reproductive dilemmas regarding the health interests of the next (third) generation. Considering their dramatic family history, some couples find it of utmost importance to stop the transmission of the causative mutation – just in order to spare their children the agony of burdensome reproductive choices. PGD for this reason is, so I would argue, legitimate.

Intermezzo: autonomy or heteronomy?

In the context of regular prenatal diagnosis, the principle of respect for autonomy is of utmost importance. This means, first, that women at higher genetic risk should be free to decide whether or not to make use of prenatal testing: the doctor should not put pressure on them to opt for testing. Second, in the case of a "positive"

result of prenatal diagnosis, the prospective parents should be free to decide whether or not to terminate the pregnancy: the doctor should support them, whatever decision they make. Does the same moral framework apply to IVF/PGD? Should "IVF-doctors" refrain from putting applicants under pressure to opt for PGD, and are prospective parents free to decide whether or not to accept (potentially) affected embryos for transfer? In order to clarify the present issue, I will first comment on the locus of decision making concerning PGD, and second on the locus of decision making concerning the transfer (De Wert, 1999).

With regard to the first topic, I will focus on the treatment of male subfertility. In view of the higher levels of chromosomal and other genetic anomalies in subfertile males, the availability of genetic counseling and the (routine or selective) offering of genetic testing are prerequisites for clinical ICSI. Should a man carry a balanced chromosome translocation involving a high risk for the future child, or should both partners be carriers of cystic fibrosis, then at least some centers will only offer ICSI if the couple intends to make use of PGD offered for "avoidance" (Silber *et al*., 1995). This example illustrates that, in some cases, the offer of PGD is a "*coercive*" offer – an offer that patients may hardly refuse in view of the adverse consequences for access to ICSI. Critics hold that a "coercive offer" clashes with the principle of respect for the applicants' autonomy: the doctor should give unconditional support, whatever they may choose. These critics ignore, however, that doctors offering ART have their *own* responsibility to avoid serious harm to the future children. The real issue, then, is not *whether* it is acceptable to "coercively" offer PGD to infertile couples at high genetic risk who apply for ART (i.e. to give access to those couples only *on the condition* that they opt for PGD), but *when* this is acceptable, which criteria should be used. In general, the greater the magnitude and probability of harm to the future child, the less morally justifiable it is to conceive children or to assist in reproduction. With regard to the present case, I tend to conclude that when couples at high risk of conceiving a cystic fibrosis child apply for ICSI, doctors may justifiably make access dependent upon the couple's willingness to make use of PGD. As discussed above, interference with reproductive autonomy in such circumstances may be justified where it is necessary to avoid a high risk of serious harm.

The second issue regards "post-PGD" decision-making authority. When there are no other "definitely healthy" embryos available for the transfer, couples may insist that embryos be transferred in case of an inconclusive result of PGD and/or when they consider the (residual) genetic risk for the future child to be acceptable. It is sometimes argued that the choice as to whether or not to have the embryo transferred must always lie with the potential mother. In view of the physician's *own* professional responsibility to prevent serious harm to the prospective child, a (partial) shift with regard to the "locus of decision-making" seems to be inevitable. However, the question arises again: what standards should be used in overruling the preferences and autonomy of the couple? When would physicians cross the boundary between a legitimate concern for the welfare of the prospective child, on one hand, and a dubious "preventive perfectionism," on the other?

In view of the potential dissent with regard to (un) acceptable "harm:probability ratios," it is imperative that the center's transfer policy be discussed with the couple in advance of PGD. Applicants should understand the Janus-faced character of PGD: while PGD may increase their reproductive options, it does not necessarily maximize their reproductive freedom.

Preimplantation genetic screening

Different types of screening IVF embryos raise different normative issues. I will now comment briefly on a standard type of screening (screening for triploidy), an experimental one (screening for aneuploidy), and a possible future approach (comprehensive genetic screening).

PGS for triploidy

It is "good IVF practice" to check the number of pronuclei. Tripronuclear zygotes, which may be the result of two sperm penetrating the oocyte, will not be transferred. These zygotes will fail to implant or result in a spontaneous abortion or in the birth of a nonviable child. Furthermore, these zygotes could, if the maternal pronucleus would be extruded, generate a hydatidiform mole, which may develop into a tumor in the recipient. There is no doubt, this type of screening is the least controversial type of reproductive genetic screening: indeed, *not* to screen for triploidy would be morally unacceptable. At the same time, this screening raises some intriguing conceptual and normative issues regarding the status of normal and tripronuclear zygotes. Does "embryonic life" start when the sperm penetrates the oocyte or later, at so-called syngamy?

Are pre-syngamy zygotes embryos or "pre-embryos?" Are triploid embryos truly embryos given that they cannot develop into a viable child? Do these embryos have the same moral value as embryos that may result in a viable baby?

Interestingly, a recent study suggests that tripronuclear zygotes may be used for somatic cell nuclear transfer (SCNT) (Egli *et al*., 2007). If so, this would be very welcome considering the problems involved in obtaining fresh oocytes for scientific SCNT (Olsthoorn-Heim *et al*., 2006; Colman & Burley, 2007; Pennings *et al*., 2007).

PGS for aneuploidy

Although PGD has been introduced primarily as an alternative option for couples at high risk of transmitting a genetic defect, it may also be routinely applied in the context of regular IVF. According to the ESHRE PGD Consortium, the number of PGD cycles performed for aneuploidy screening has increased considerably (Sermon *et al.*, 2007). Most numerical chromosomal abnormalities are not compatible with life. Theoretically, aneuploidy screening could increase the success rate of IVF and facilitate the transfer of one single embryo, thus reducing the chance of a multiple pregnancy. This screening could be particularly useful in case of advanced maternal age, recurrent miscarriage, and recurrent implantation failure. To date, many studies have addressed the impact of aneuploidy screening in different groups of patients, yet its effectiveness has not been proven (Staessen *et al.,* 2004; Shahine & Cedars, 2006; Health Council, 2006; Donoso *et al*., 2007). A recent study even suggested that this type of screening significantly reduced the rates of ongoing pregnancies and live births after IVF in women of advanced maternal age (Mastenbroek *et al*., 2007).

What about the ethics of PGS for aneuploidy? First, there are no valid categorical objections to this type of screening. After all, its primary aim, i.e. to increase the success rate of IVF, is unproblematic if not commendable, whereas the means, namely to exclude embryos affected with serious chromosomal aberrations (which often lack viability) from transfer, is clearly morally acceptable. Proponents of *polar body* aneuploidy screening claim that this strategy has a *moral* advantage, as (first) polar body diagnosis aims at a selective fertilization of normal oocytes, thus avoiding embryo-selection. Some of the leading investigators, however, have recommended that both the first and the second polar bodies should be studied. Whereas they qualify this strategy as *pre*-conception testing, this combined biopsy in fact constitutes a shift toward "*intra-*" conception screening; after all, the second polar body appears during the fertilization process. Again, what is the moral status of the presyngamy zygote? Does it really make sense to make a fundamental ethical difference between "intra-" and post-conception PGS?

Second, PGS for aneuploidy is still experimental and should be scrutinized in the context of randomized controlled clinical trials, aimed at a systematic evaluation of its presumed advantages and possible disadvantages (Harper *et al*., 2008). There are serious questions – if not increasing doubts – regarding the effectiveness of this screening. It is surprising that some proponents of this screening can uncritically claim that its effectiveness has already been proved and that aneuploidy screening is now just a matter of good clinical practice. Clearly, this is at odds not only with the interests of IVF patients, but also with professionals' responsibility to avoid futile interventions and to enable society to distribute scarce resources available for healthcare in a just and evidence-based way. Recently ESHRE's PGS Task Force argued that polar body biopsy and 24-chromosome analysis would in theory be the best option (Geraedts, 2009).

Participation in research on aneuploidy screening presumes informed consent. Again, there are some questions concerning the "locus of decision making." Who has decision-making authority with regard to the actual use of such screening in individual cases: the doctor or the couple/woman? As long as the screening is experimental, the answer is self-evident: it is for the woman/couple to decide whether or not to participate in the trial. Who has decision-making authority concerning the disposal of chromosomally "abnormal" embryos? What if a couple insist on transferring an embryo with, for instance, an XXY or XYY karyotype, when there is no "normal" embryo available for transfer? Wouldn't a doctor's categorical refusal to transfer embryos with these mild aberrations be symptomatic of the disputable "minimal risk" or "maximum welfare" standard?

Comprehensive genetic screening: "the transparent embyro"

New technologies (DNA microarrays, the so-called DNA chip, DNA sequencing, and so on) may permit genetic screening for nearly all chromosomal aberrations and many of the common Mendelian disorders and susceptibilities for complex disorders at once. This approach may seem to be ideal, as we could both

select the most viable embryo for transfer (increasing the take-home baby rate) and optimally reduce the risk of having an affected child. However, an unmitigated enthusiasm for such comprehensive preimplantation screening is naïve, as this screening raises some complex issues, especially if the aim would be to detect as many anomalies and genetic risk factors as possible (De Wert, 2008). To begin with, there are problems surrounding informed consent. Considering the complexity of comprehensive screening, truly *informed* consent would hardly be possible. Second, we must seriously question whether this really promotes reproductive autonomy as people may regularly be confronted with rather complex, if not impossible, trade-offs. The same may hold true for physicians who have to decide which embryos to transfer. Third, if profiling would include susceptibility screening for multifactorial *later-onset* disorders, it may well involve an interference with the future child's right not to know, that is, the right of the child to later decide for him- or herself whether or not be tested for genetic susceptibilities. This could of course be prevented by not transferring embryos carrying susceptibility for a multifactorial disorder but then, as we are all "fellow mutants," there will often be no embryo suitable for transfer. Fourth, comprehensive preimplantation screening may provide unexpected information about the genetic status of (one of) the prospective parents themselves, which may be at odds with their right not to know.

Some of these problems might be avoided by (wide) *preconception* screening of the prospective parents themselves, but the fundamental question would be the same: what is the primary goal of reproductive genetic screening? Is it to promote reproductive autonomy or to maximally prevent the conception or birth of affected children – and what are the relevant guiding principles? The prospect of comprehensive genetic screening has a heuristic value, as it enforces us to address this basic issue.

Conclusions

While preimplantation genetic testing is still controversial, it is, rightly, increasingly being accepted as a morally justified option. As we have seen, the presumed categorical objections to PGD are debatable. Nevertheless, preimplantation genetic testing, testing both within and beyond the medical model, raises lots of normative questions which need further analysis and discussion. In view of the dynamics of this field, it is crucially important to timely identify possible new developments and applications. This should contribute to a proactive normative reflection.

*This chapter borrows from De Wert's contribution to Fauser B *et al.*, eds., *Molecular Biology in Reproductive Medicine*. New York: Parthenon, 1999; pp. 645–65.

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Chapter 18

Preimplantation genetic diagnosis: the future

Leeanda Wilton

Key points

- The biopsy technique has remained relatively unchanged in 20 years. Alternative methods of biopsy may be sampling cells using lasers and optical tweezers.
- Metaphase comparative genome hybridization (CGH) has only been reported clinically for a small number of cycles.
- Array CGH may enable an "off the shelf" test for aneuploidy and monogenic disorders
- Microfluidic polymerase chain reaction (PCR) chips may be developed for preimplantation genetic diagnosis (PGD)
- Cryopreservation of biopsied embryos has been difficult but vitrification may improve the success rates

Introduction

There are two main challenges of preimplantation genetic diagnosis (PGD). These are: to sample sufficient genetic material from early embryos or oocytes without adversely affecting their viability and development; and to perform rapid and reliable genetic diagnoses on single or very few cells. These challenges continue to dominate efforts to improve PGD technology.

Most developments in PGD over the last ten years have been in the area of genetic testing, whereas there have been fewer innovations in embryonic cell biopsy.

Does embryo biopsy adversely affect embryo development?

Genetic diagnosis of an oocyte or early embryo is still achieved by sampling, or biopsying, cellular material, performing a test on the material, and inferring the genetic constitution of the oocyte or embryo. Breaching the zona pellucida (ZP) has been achieved either mechanically by gently tearing a hole using a fine glass needle, chemically using an acid solution, and, more recently, by using a laser beam to form a hole in the ZP. Micromanipulators are then used to remove cellular material from the embryo.

The first published techniques for removing cells from embryos involved cleavage-stage embryo biopsy (Wilton & Trounson, 1989; Kola & Wilton, 1991) of one or two cells. For PGD this is usually done on day 3 of embryo development when the embryo has somewhere between six and ten cells. Clearly, embryos can continue development and result in ongoing pregnancies after transfer as many live births have been reported using this approach (Verlinsky *et al.*, 2004; Harper *et al.*, 2008). Early animal studies demonstrated that cleavage-stage embryo biopsy did not affect implantation or fetal development (Wilton *et al.*, 1990). Biopsied human embryos were shown to develop normally to the blastocyst stage (Hardy *et al.*, 1990). However it is difficult to determine if sampling cells from cleavage-stage human embryos reduces viability and ongoing development past the blastocyst stage. Intuitively it would seem as though there must be some adverse effect but the only way to ascertain this would be to compare the outcome of transfer of biopsied embryos to those that have not been biopsied in a controlled trial. For obvious reasons this would not be possible for patients having PGD for high-risk genetic conditions such as monogenic disorders or chromosomal translocations. It might be possible to compare the outcomes for in vitro fertilization (IVF) patients having PGD for aneuploidy, but the selective advantage of knowing that the embryos were euploid for a number of chromosomes would have to be removed. Therefore we would have to compare the outcomes for biopsied (but not selected) embryos with non-biopsied embryos in a control group. This has not been done as most people would consider it unethical to biopsy the embryos for no reason.

Embryos also lose cells when they are thawed after cryopreservation and this has been used as a model to determine if cell loss affects embryo development

(Cohen *et al.*, 2007). It is unclear whether this is a valid extrapolation as there are obvious key differences. Cryopreservation is a chemical insult to embryos, whereas embryo biopsy is a physical insult. It is not known whether lysis of cells is the same as aspirating cells. It has been determined that the loss of implantation potential after cryopreservation is directly comparable to the amount of cell loss after thawing (Edgar *et al.*, 2000). That is, fully intact thawed embryos have the same implantation potential as fresh embryos. Moreover, cryopreserved embryos lose an average of 30 percent of blastomeres in thawing and 30 percent of their implantation potential after transfer (Edgar *et al.*, 2000).

If cell lysis after cryopreservation may be equated with cell biopsy for PGD then this would indicate that there may be a negative impact of cell removal on ongoing embryo development. For patients having PGD for high-risk conditions like chromosomal rearrangements or monogenic conditions there is currently no choice but to biopsy embryos. For infertile patients having PGD for aneuploidy testing, the potential negative effect of embryo biopsy must be weighed against any selective advantage of determining that the embryos are euploid for some chromosomes. There has been one study comparing the effect of biopsying one or two cells from cleavage-stage human embryos but this focused on the diagnostic success rather than the impact of cell removal on embryo growth (Michiels *et al.*, 2006).

Other techniques of sampling genetic material from early embryos and oocytes have been developed. In particular, some centers biopsy the first and/or second polar bodies from oocytes arguing that this approach samples only redundant material and not prospective embryonic material. One of the disadvantages of this approach is that the paternal genetic contribution to the embryo cannot be assessed. This precludes polar body biopsy from zygotes from male translocation carriers, or males affected with autosomal dominant conditions. It is well described that many aneuploidies that exist in early development arise post-zygotically during the first cleavage divisions (Voullaire *et al.*, 2000; Voullaire *et al.*, 2002; Wells & Delhanty, 2000; Mantzouratou *et al.*, 2007) and these would not be detected by polar body biopsy.

Biopsy of trophectodermal cells from blastocyst stage embryos would seem to counteract several problems of earlier stage biopsy. The trophectoderm cells do not contribute to the developing fetus, which is derived from the inner cell mass. Also, by biopsying the trophectoderm, several cells may be removed, which should make genetic diagnostic techniques more efficient. One of the earliest reports of blastocyst biopsy used a laser to remove the trophectoderm cells, which were subjected to fluorescent *in situ* hybridization (FISH) to detect aneuploidies (Veiga *et al.*, 1997). Despite apparent advantages of blastocyst biopsy few other groups have adopted the procedure clinically. Rather surprisingly, blastocyst biopsy seems to offer little, if any, improvement in the successful diagnosis rate compared with single-cell biopsy. The successful diagnosis rate of several trophectoderm cells after blastocyst biopsy is approximately 94 percent (McArthur *et al.*, 2005; Kokkali *et al.*, 2007) which is not different from the 93 percent reported by the European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium (Harper *et al.*, 2008) where almost 90 percent of cycles use cleavage-stage biopsy.

Despite the development of polar body and blastocyst biopsy it remains true that the most common way to obtain cellular material from an embryo is the original technique of cleavage-stage biopsy (Harper *et al.*, 2008). In the most recent publication from the ESHRE PGD Consortium (data VII) a total of 3,196 cycles of PGD were reported from 45 PGD centers around the world. Eighty-nine percent of these used cleavage-stage, 11 percent polar body, and 0.1 percent blastocyst biopsy, respectively (Harper *et al.*, 2008).

Embryo biopsy techniques have changed relatively little over the last 20 years since PGD was first developed, whereas there have been much more significant advances in single-cell diagnostic technology (see later). In particular, many genetic tests are extremely rapid, which is important in the context of PGD. All of the oocyte and embryo biopsy techniques described above are invasive, laborious, and require a relatively high degree of skill to perform well. It can take many months to train an already accomplished embryologist in the skills of embryo biopsy. One of the key requirements of PGD is a short turn-around time for results, and the laborious nature of embryo biopsy means that a significant amount of the testing time is taken up by the embryo biopsy. Embryo biopsy techniques have progressed little over many years and a significant step forward is needed.

An alternative method of sampling cells or polar bodies from early embryos might be by the combined use of lasers and optical tweezers. Lasers have

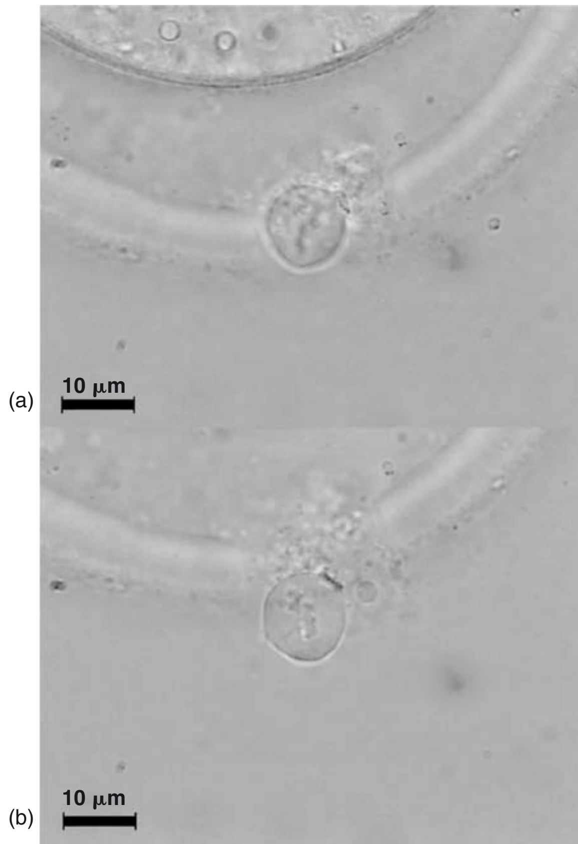

Figure 18.1 Laser-mediated extraction of the first polar body in a bovine oocyte. (a) After switching the laser trap on, the polar body is dragged through the laser-drilled hole out of the zona pellucida (ZP); (b) the polar body is almost dragged out of the ZP. Reproduced from Clement-Sengeweld *et al.* (2002). With kind permission from Springer Science and Business Media. Copyright 2002.

been used for some years to achieve zona drilling or assisted hatching (Blanchet *et al.*, 1992; Obruca *et al.*, 1994; Munné *et al.*, 1998) but these still required micromanipulation pipettes to stabilize the oocytes or embryos. Non-contact lasers remove the requirement for micromanipulators for assisted hatching (Schütze & Clement-Sengewald, 1994; Germond *et al.*, 1996), although a micropipette is still required to remove the polar body or cell (Antinori *et al.*, 1996). Optical tweezers have also been used for cell sorting (Ashkin *et al.*, 1987) and sperm manipulation (Colon *et al.*, 1992; Clement-Sengewald *et al.*, 1996). Laser pressure catapulting has been used to project small numbers of cells and individual chromosomes into microtubes (Schütze & Lahr, 1998; Schermelleh *et al.*, 1999; Langer *et al.*, 2005).

In a very innovative pilot study, the three techniques of cutting lasers, optical tweezers, and laser pressure catapulting were combined to sample polar bodies from bovine and a small number of human oocytes (Clement-Sengewald *et al.*, 2002).

In brief, a cutting laser was used to breach a hole in the ZP. The polar body was then trapped with optical tweezers and dragged through the hole in the zona (Figure 18.1). The polar body was placed on a polyethylene naphthalene membrane on a glass slide and a laser was used to cut the membrane around the polar body. The lid of a polymerase chain reaction (PCR) tube was placed over the polar body and the polar body was projected into the lid using laser pressure catapulting. This involves a single shot of the laser underneath the slide (Clement-Sengewald *et al.*, 2002). One of the significant advantages of this approach is that the laser is directed tangentially to the specimen, meaning that there is no pressure toward the oocyte. Consequently, micromanipulation pipettes are not required to hold the oocyte in place. This obviates the need for fragile glass pipettes and expensive micromanipulators, and the whole process of biopsy and movement of the biopsied material into the PCR tube may be completed without any physical contact with the specimen. The significant advantages of this approach include that expensive micromanipulators or glass micromanipulation pipettes are not required and the skill level is much less that that required for traditional micromanipulation. Moreover, the biopsy process is rapid, reportedly taking less than 40 seconds per polar body (Clement-Sengewald *et al.*, 2002). Human contact with the specimens is limited, which may be advantageous in minimizing contamination in PGD for monogenic conditions. There are no reports using this approach to remove blastomeres from cleavage-stage embryos in humans or any other species so it remains relatively untested at this stage. The impact on embryo development would need to be determined before it could be applied clinically and undoubtedly the laser system is expensive. Nevertheless, this approach holds much promise for simplifying laborious embryo biopsy techniques.

Of course, the ideal approach for genetic testing of embryos would be to completely avoid sampling cellular material from embryos. Significant advances have been made in recent years in live cell imaging techniques and it might be possible in the future to adapt these to identify genetic, in particular chromosomal, errors in human embryos. Schuh and Ellenberg (2007)

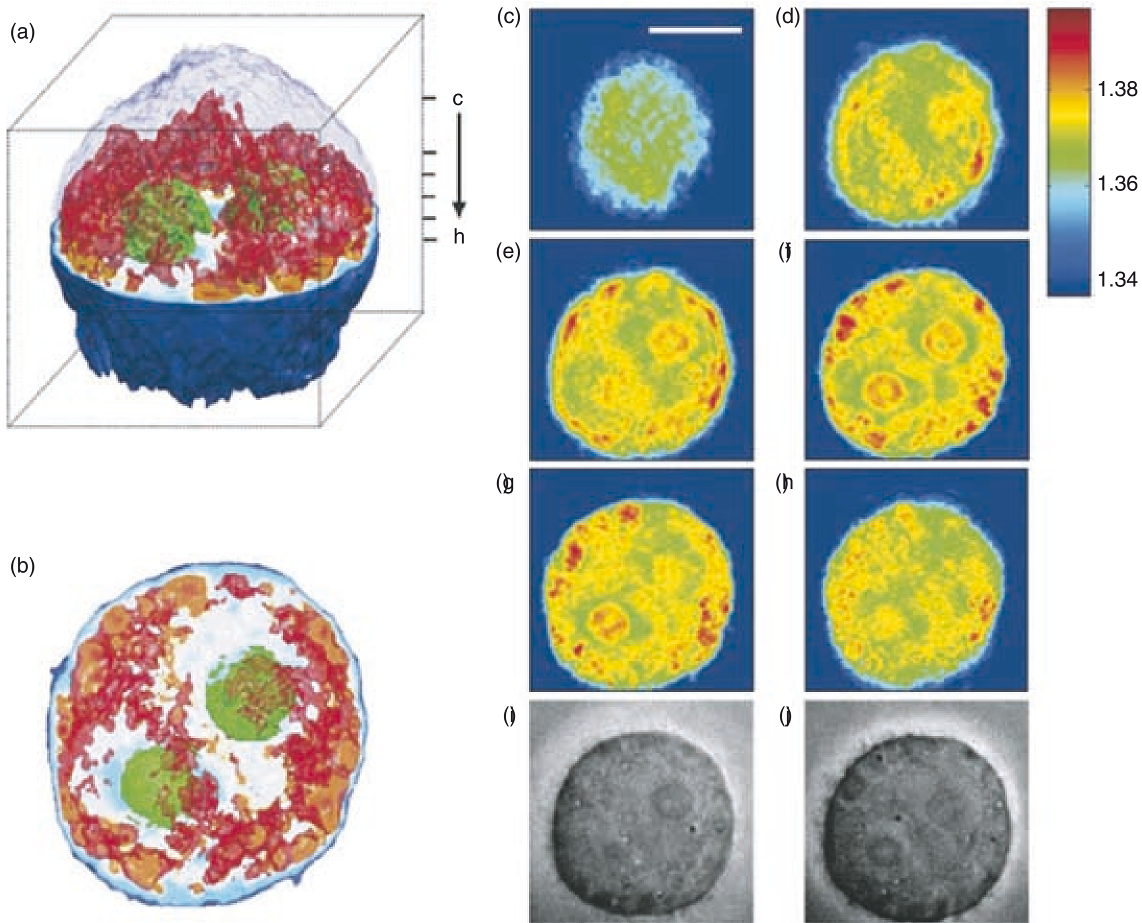

Figure 18.2 Refractive index tomogram of a HeLa cell. (a) A three-dimensional (3D) rendered image of a HeLa cell. The outermost layer of the upper hemisphere of the cell is omitted to visualize the inner structure. Nucleoli are colored green and parts of cytoplasm with refractive index higher than 1.36 are colored red. The side of the cube is 20 μm. (b) Top view of (a). (c–h) Slices of the tomogram at heights indicated in (a). Scale bar, 10 μm. Color bar, refractive index at λ = 633 nm. (i, j) Brightfield images for objective focus corresponding to (e) and (f), respectively. Reprinted by permission from Macmillan Publishers Ltd: *Nature Methods*, Choi *et al.* (2007), copyright 2007.

have used four-dimensional (4D) confocal fluorescence microscopy to observe spindle and chromosome dynamics in live mouse oocytes. Individual bivalent chromosomes could be observed as they aligned on the spindle. The resolution was not great enough to be able to distinguish and identify chromosomes but this might be possible in the future. Currently, fluorescent dyes and exposure to ultraviolet (UV) light are required to identify subcellular structures. These are likely to cause damage to the cells and so would not be suitable for live embryos that were for clinical use.

Three-dimensional (3D) mapping of cells can be achieved without the use of fluorescence dyes, although exposure to laser light is still required. Choi *et al.* (2007) report high-resolution 3D tomography of single cells and multicellular organisms by measuring the refractive index of living cells using phase contrast microscopy. Cellular organelles and the nucleus could be observed (Figure 18.2). It was not possible to identify smaller structures but future refinements of this type of technology hold promise for microscopic cell analysis. Potentially this could obviate the need for cell biopsy in PGD, which would be a significant advantage both in terms of minimizing the detrimental impact on embryo development and in ease of use.

Developments in genetic testing

The first technology used to detect chromosome errors in single cells biopsied from human embryos in clinical PGD was FISH (Griffin *et al.*, 1992). FISH techniques

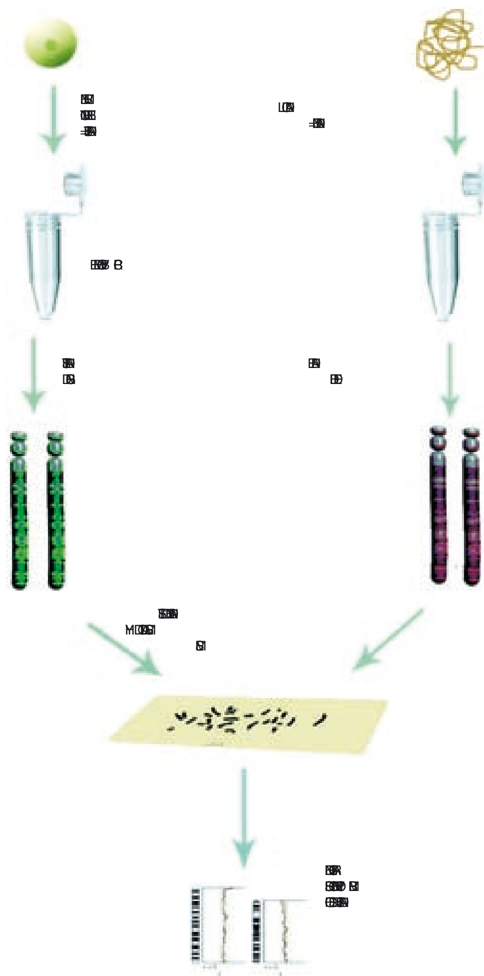

Figure 18.3 A schematic diagram of the single cell comparative genomic hybridization (CGH) protocol. Reproduced from Wilton (2005). With permission from Oxford University Press.

have improved since this time: hybridization times are shorter and more fluorochromes are available. It has become common practice to perform two rounds of FISH, meaning that more chromosomes can be identified in a single nucleus. This approach remains limited because still only five fluorochromes are available and repeated denaturation of the nucleus results in DNA degeneration and a reduced FISH efficiency (Bahçe *et al.*, 2000; Wilton, 2002). Only a limited number of chromosomes may be analyzed and most PGD laboratories offer testing for seven to nine chromosomes, commonly including X/Y, 13, 16, 18, 21, and 22.

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique which enables enumeration of all chromosomes in single interphase cells (Voullaire *et al.*, 1999; Wells *et al.*, 1999). CGH works by comparing known normal whole genomic DNA with the unknown DNA from the biopsied cell. The normal, or reference, DNA is labeled with a red fluorochrome and the unknown, or test, DNA is labeled with a green fluorochrome. Both are simultaneously hybridized to normal male metaphase chromosomes which act as a template for the hybridization (Figure 18.3). Computerized imaging is used to measure the red:green fluorescence ratio along the length of individual template chromosomes. If this ratio is 1:1, then the test cell is interpreted as being euploid for this particular chromosome. If the red:green fluorescence ratio for a chromosome is significantly less than or greater than 1 then the test cell is interpreted as having an excess or missing copy of that chromosome or chromosomal segment (Figure 18.4).

There have been very few reports of clinical application of metaphase CGH in PGD either on blastomeres after cleavage-stage embryo biopsy (Wilton *et al.*, 2001; Wilton *et al.*, 2003) or on polar bodies isolated from oocytes (Wells *et al.*, 2002). Metaphase CGH requires a high degree of skill in single-cell whole-genome amplification, FISH, and karyotyping. The hybridization takes a minimum of 48 hours and the analysis of the fluorescent metaphase chromosomes is laborious. Consequently it takes about four to five days to obtain results, which is longer than day three embryos can be kept in culture. There have been two approaches to overcome this. Embryos have been biopsied on day three and then cryopreserved until the CGH results are available (Wilton *et al.*, 2001; Wilton *et al.*, 2003). The exposure of embryos to cryopreservation is not ideal and it has been estimated that freezing and thawing results in a 30 percent loss of implantation potential (Edgar *et al.*, 2000), although this would only apply to the one or two embryos that would have been transferred fresh had CGH not been done. The other approach has been to apply metaphase CGH to polar bodies (Wells *et al.*, 2002). This allows enough time for CGH analyses to be completed without cryopreservation. However, CGH on polar bodies can only detect maternal meiotic abnormalities. It is well documented that many early embryonic chromosomal errors, including mosaicism, occur during post-zygotic cleavage divisions.

The application of metaphase CGH to human embryos and blastomeres has been very informative. Metaphase CGH has unequivocally demonstrated that errors of all chromosomes exist in early human

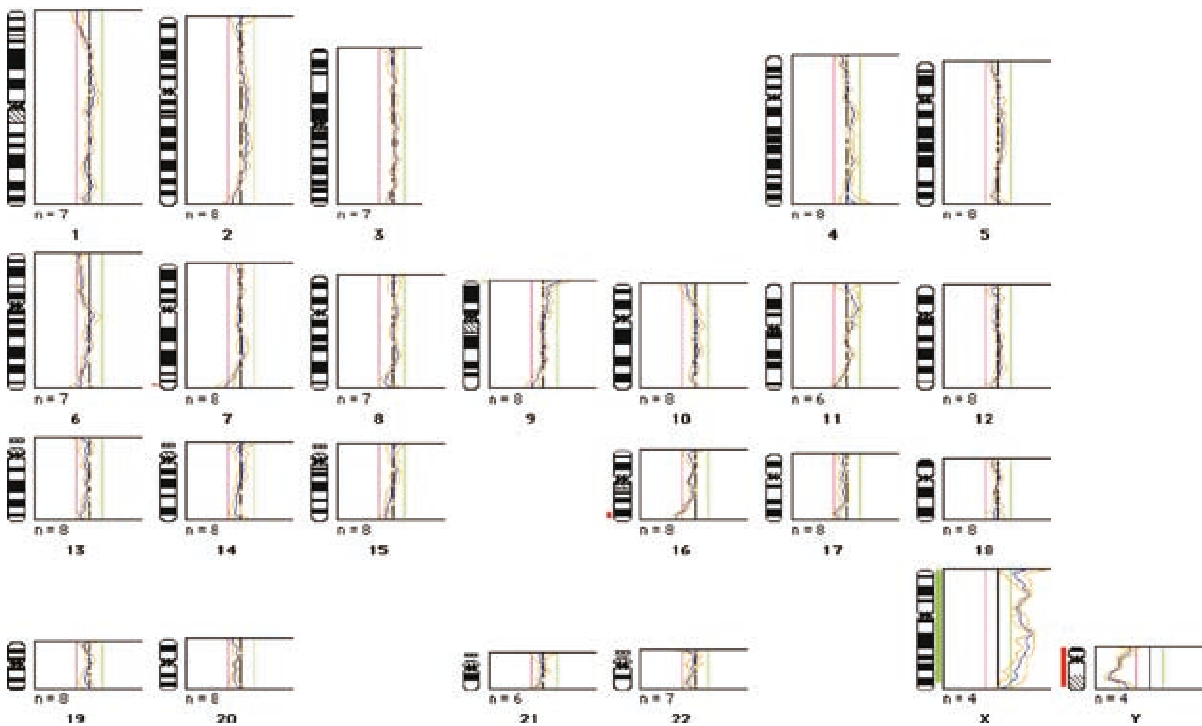

Figure 18.4 A comparative genomic hybridization (CGH) profile showing the green:red fluorescence ratio for all chromosomes. For any given chromosome, if the test DNA is euploid the green:red fluorescence ratio (shown as the blue line) will be 1.0. If the test DNA is missing a chromosome, then the ratio will decrease and shift to the left; if it has an extra chromosome the ratio will increase and shift to the right. This is shown here by the imbalance of the sex chromosomes. Compared to the male reference DNA, the test DNA is enhanced for the X chromosome and diminished for the Y chromosome, showing that it is female. Reproduced from Wilton (2005). With permission from Oxford University Press.

embryos. Some aneuploidies that have been found at measurable frequencies in embryos are rarely observed in spontaneous abortuses, indicating that lethality of these conditions (Voullaire *et al.*, 2000; Wilton *et al.*, 2003). These observations have highlighted a significant limitation of FISH testing for aneuploidy in that not all chromosomes can be tested and so there are certainly some embryos that are diagnosed as "normal" that actually harbor aneuploidy of chromosomes that were not tested (Wilton, 2005). It has been conservatively estimated that approximately 25 percent of aneuploid embryos are diagnosed as "nomal" by FISH for nine chromosomes because probes for the aneuploidy chromosomes were not included in the test (Wilton, 2005). Metaphase CGH studies have also identified partial aneuploidy where large segments of chromosomes are missing or in excess (Voullaire *et al.*, 2000; Wells & Delhanty, 2000; Wilton, 2005). These, too, would probably remain undetected using FISH, which can only enumerate the target sequences usually located around the centromere. Metaphase CGH provides information along the entire length of the chromosome. Despite the benefits of the additional information provided by metaphase CGH analyses, the technical challenges mean that it is not routinely used for aneuploidy detection in PGD.

An alternative to metaphase CGH for aneuploidy detection would be to use a microarray, rather than metaphase chromosomes, as the template for the *in situ* hybridization. Microarrays are a solid support, often a glass slide, spotted with known short sequences of DNA. The principle is the same as for metaphase CGH in that test DNA is compared to known normal reference DNA after both have been labeled with a different fluorochrome. The DNA sequences spotted on the array determine what may be detected. For example, an array spotted with a number of sequences along the length of each chromosome could be used to identify aneuploidy and whole chromosomal imbalance. With sufficient sequences along each chromosome microarray CGH could also be used to detect partial aneuploidy, including that caused by reciprocal translocations and

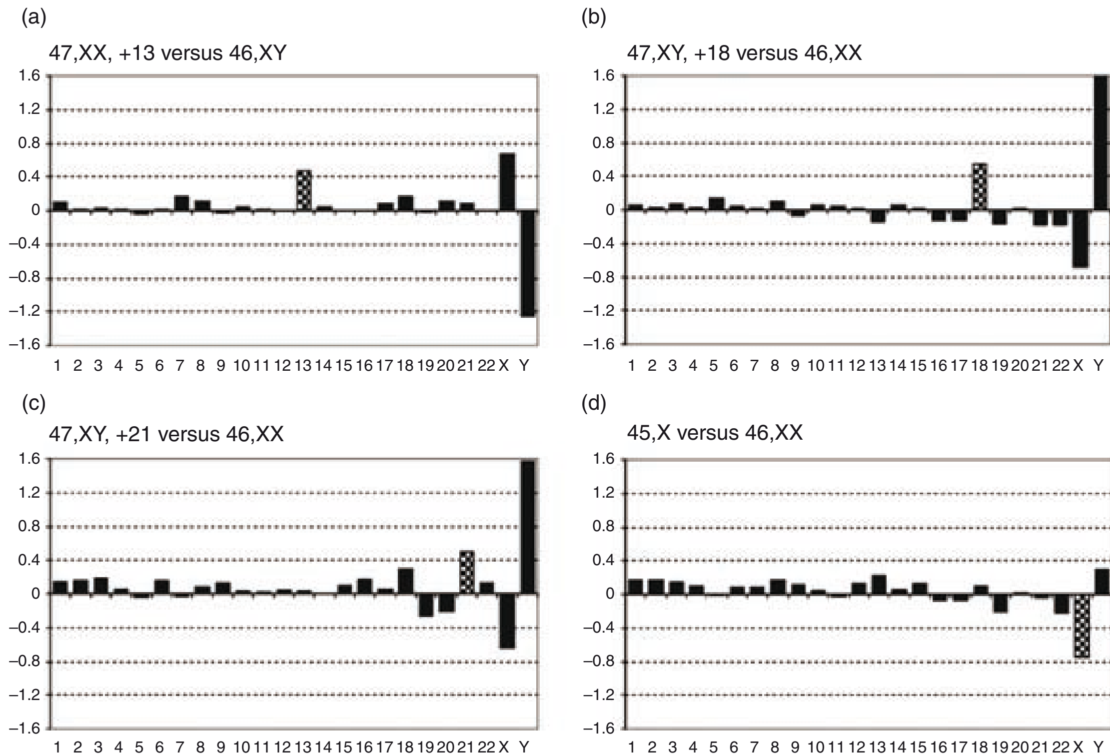

Figure 18.5 Examples of single-cell array comparative genomic hybridization (CGH) profiles performed on aneuploid cell lines. For each panel, the x-axis represents the 22 autosomes, followed by the X and Y chromosomes. The y-axis marks the $\log_2$ mean ratios of all spots of each chromosome. Following Φ29 DNA polymerase amplification, single cells containing trisomy 13 (a), 18 (b), and 21 (c) were hybridized versus non-amplified gDNA of the opposite sex, and monosomic X single cell (d) versus non-amplified XX gDNA. The checkered columns represent the abnormal chromosomes. Reproduced from Le Caignec *et al.* (2006). With kind permission from Joris R. Vermeesch and Oxford University Press.

chromosome rearrangements. This would have the significant advantage that individual feasibility testing and test development that are currently required to detect unbalanced segregants of reciprocal translocations using FISH would not be necessary. An "off the shelf" microarray could be used to diagnose the imbalance caused by any translocation. Microarrays can be spotted with many thousands of sequences, and, potentially, microarrays could be used to simultaneously detect complete and partial aneuploidy as well as common mutations that cause a number of diseases.

Microarrays are now commonly used to measure gene expression and genetic mutation and variation in many fields of molecular biology. As always in PGD, the challenge has been to refine these standard methodologies to the single-cell level. Significant advances have been made in recent years in obtaining genetic information from single cells using microarrays (Le Caignec *et al.*, 2006; Fiegler *et al.*, 2006). A microarray composed of over 4,000 clones has been used to detect the chromosome imbalance in single lymphocytes and fibroblasts known to be aneuploid for chromosomes 13, 18, 21, or X (Le Caignec *et al.*, 2006). In this same study, a small number of blastomeres from embryos previously diagnosed as aneuploid by FISH in a PGD program were subjected to microarray CGH. The microarray analysis confirmed the FISH diagnosis of XO in one blastomere and identified monosomy for chromosomes 1, 2, and 4 in three blastomeres from another embryo (Le Caignec *et al.*, 2006) (Figure 18.5). The resolution of detection was 34 Mb, similar to that obtained by conventional metaphase CGH analysis (Voullaire *et al.*, 1999). Higher resolution single-cell microarray analysis has also been reported using trisomy 21 and microdeleted (15q11–13) cells (Fiegler *et al.*, 2006) but has not yet been applied to blastomeres. The use of microarrays for single-cell diagnostics holds much promise but, to date, very few blastomeres have been analyzed and larger scale verification is required before the technology could be put into clinical practice.

It is likely that in the not too distant future microarrays will be used in PGD applications. There could be a "standard" microarray spotted with sequences that detected aneuploidy, chromosome imbalance caused by rearrangements, and many of the more common monogenic conditions and polymorphisms. Many of the diagnoses that are currently performed after individualized test development could be detected by an "off the shelf" microarray. In other applications microarray technology has been almost too successful. Single-nucleotide polymorphism (SNP) genotyping arrays and CGH-arrays have identified extensive copy number variation, which may encompass up to 12 percent of the human genome (Bejjani & Shaffer, 2006; Redon *et al*., 2006). It is difficult to determine which of these variations are functionally significant and associated with abnormality or disease against this wide-ranging background variation.

There have been other significant advances in the last two years in nucleic acid amplification and analysis, in particular using total analysis or "lab on a chip" systems (see review by Zhang & Xing, 2007) and in the future some of these technologies may be applicable to PGD. Microfluidic PCR chips have the advantage of very fast reaction times, primarily because the thermodynamic properties and small size of the chips mean that heating and cooling times are very rapid. Hashimoto *et al*. (2006) report PCR times of less than 20 minutes. Reaction volumes are small, which is ideal for single-cell applications and also minimizes reagent expense. Microfluidic PCR has recently been used to amplify multiple genes from single bacteria (Ottesen *et al*., 2006), an obvious parallel to the requirements of PGD. Additionally, amplification chips have been coupled to analysis and information chips such as a microarray (Hashimoto *et al*., 2006) that offers opportunities to perform DNA amplification and analysis in a single closed system. This would be advantageous to PGD by removing the multiple sample handling required by current technology and reducing the risk of sample mis-identification and mix-up.

Cryopreservation of biopsied embryos

Significant effort is put into identifying embryos that are genetically suitable for transfer for patients undergoing preimplantation genetic testing. Usually one or two embryos are transferred back to the patient in the PGD cycle and, as in routine IVF, any additional embryos are cryopreserved. It has been known for some years that standard techniques that successfully cryopreserve intact embryos give very poor outcomes when applied to biopsied embryos (Joris *et al*., 1999; Magli *et al*., 1999; Ciotti *et al*., 2000). In particular, individual blastomere survival is extremely poor with many cells lysing. Presumably this is because the relatively large hole in the ZP required for embryo biopsy allows toxic cryoprotectants unfettered access to the blastomeres and causes cell lysis.

Cryopreservation methods that result in significantly improved cell and embryo survival have been reported for biopsied embryos (Jericho *et al*., 2003). In this study, the sucrose concentration in the freezing solutions was increased and maternal serum replaced other protein sources in order to offer better membrane protection against cryoprotective chemicals. The success of this technique has since been repeated by other workers (Zheng *et al*., 2005). The disadvantage of this method is that maternal serum is required, and collection and preparation of autologous serum is laborious and time-consuming. Consequently, most laboratories still use routine cryopreservation techniques for biopsied embryos despite knowing that success rates are poor.

An alternative approach to embryo freezing is vitrification, which avoids the damaging effects of ice crystal formation (Kuwayama *et al*., 2005). There has been a single report of vitrification of biopsied cleavage-stage embryos and the outcomes were impressive, with 90 percent of blastomeres surviving and 80 percent of embryos with all blastomeres intact at thawing (Zheng *et al*., 2005). Vitrification may be the way forward for cryopreservation and storage of biopsied embryos, and further trial and application of this approach is urgently needed.

Can chromosome and/or genetic testing of embryos tell us anything about embryo viability?

Many years of testing embryos for aneuploidy have led to the accumulation of a wealth of information about the nature and extent of chromosome abnormalities in embryos.

Almost overwhelmingly, these aneuploidies are lethal to embryo development. If genetic anomalies could be correlated to particular morphological characteristics of embryos then, in the future, it might be possible to predict more accurately which embryos

were aneuploid without the need for invasive, expensive preimplantation biopsy and genetic testing.

Already there has been progress in this area. It has been known for some years that arrested or slowly developing embryos have a high frequency of aneuploidy and/or other chromosomal anomalies (Munné *et al.*, 1994; Munné *et al.*, 1998; Magli *et al.*, 2001; Magli *et al.*, 2007). Additionally, embryos with accelerated cleavage are more likely to be aneuploid (Harper *et al.*, 1994; Magli *et al.*, 1998; Magli *et al.*, 2001; Magli *et al.*, 2007). Other morphological characteristics known to be associated with chromosomal errors include pronuclear morphology (Kahraman *et al.*, 2002; Balaban *et al.*, 2004) and fragmentation (Magli *et al.*, 2001; Magli *et al.*, 2007). The association of cellular fragmentation with aneuploidy has recently been refined to ascertain that embryos with fragments scattered throughout have a higher frequency of aneuploidies than embryos where the fragments are confined to a smaller area of the perivitelline space (Magli *et al.*, 2007). As more associations between embryo morphology and chromosome abnormality are identified it may become possible to restrict embryo biopsy to those embryos that have some reasonable chance of being euploid.

As well as having normal growth and chromosome complement, a viable embryo must also have the appropriate gene activity. Recent studies examining the gene expression of early embryos have shown that, not surprisingly, particular expression patterns are linked to certain developmental stages (Wells *et al.*, 2005a) and morphological characteristics (Wells *et al.*, 2005b). It is most likely that embryos with aberrant expression of a number of key developmental genes have reduced viability. This may lead to the possibility of measuring gene expression in one or a few biopsied cells and selecting embryos on the basis of optimal gene expression patterns (Wells *et al.*, 2005a).

We can, but should we?

In this chapter I have outlined recent developments and future prospects for the diagnostic capabilities of PGD. It is clear that application of microarrays to clinical PGD is likely to happen in the near future. As described above, a microarray may be spotted with many thousands of sequences, making it possible to diagnose aneuploidy and single-gene conditions from a single chip. Theoretically, almost every genetic mutation could be diagnosed in early embryos. To date, PGD has predominantly been applied to diagnose severe disease. It is true that some applications of PGD, including late-onset diseases, aneuploidy testing for infertility, and HLA-matching, were controversial when first introduced but have become more accepted over time. Every day more genetic errors and polymorphisms are linked to diseases and non-disease traits and potentially it will be possible to diagnose all of these in early embryos. A significant challenge for PGD practitioners, ethicists, and other members of the community will be how to decide which applications of PGD are acceptable (see Chapter 17). Just because we can, does not necessarily mean we should.

Conclusion

There has been remarkable progress in the capabilities of PGD in the last five years. Most developments have been in the area of molecular testing and the future in this area is bright. Robust, reliable, and rapid single-cell microarray technology is tantalizingly close to clinical application. This will allow simultaneous detection of multiple genetic characteristics, including mutations, polymorphisms, aneuploidy of all chromosomes, chromosome imbalance caused by translocations, and cryptic rearrangements. It is anticipated that there will be "off the shelf" arrays, which will detect many common mutations and aneuploidies, obviating the need for laborious and expensive individualized test development that is currently standard practice.

In contrast, progress in embryo sampling techniques has been less impressive, and embryo biopsy techniques remain very invasive and slow. Non-contact techniques, such as laser optical tweezers and laser pressure catapulting, hold promise but require significant development and testing to ensure safety.

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