Section 1 Chapter 1

Background

Introduction to preimplantation genetic diagnosis

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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

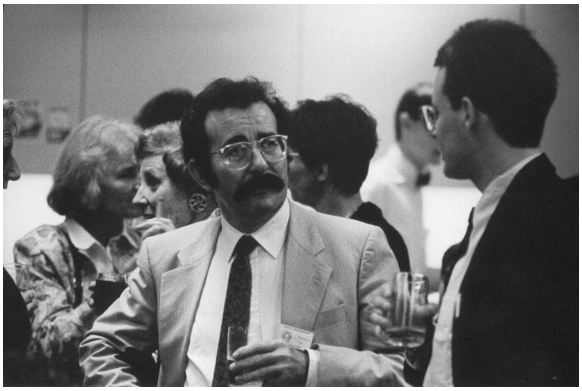

Figure 1.5 Robert Winston and Darren Griffin in Prague, 1990.

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.