PREIMPLANTATION DIAGNOSIS

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DEDICATION

This thesis is dedicated to my wife, Lingjia Wang whose unfailing support and everyday discussions have been most valuable throughout the duration of this study.

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DECLARATION

The experimental work described in this thesis was conducted under the meticulous supervision of Professor Colin D. Matthews and Dr. Robert F. Seamark in the Department of Obstetrics and Gynaecology, The University of Adelaide. The data presented is from original studies by the author. This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference had been made in the text. I give my consent to this copy of my thesis, when deposited in the University Library, to be available for photocopying and lending.

All experimental procedures had the approval of The Ethics of Human Research Committee of The Queen Elizabeth Hospital and the Animal Ethics Committee of The Queen Elizabeth Hospital.

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ABBREVIATIONS

bp	base pair
CF	Cystic fibrosis
DNA	deoxyribonucleic acid
hCG	Human chorionic gonadotrophin
HGPRT	hypoxanthine–guanine phosphoribosyl transferase
HPRT	hypoxanthine phosphoribosyl transferase
HTFM	human tubal fluid medium
IVF	in vitro fertilisation
Kb	kilobase
LDH	lactate dehydrogenase
MEM	Minimum Essential Medium
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PMSG	pregnant mare serum gonadotrophin
RNA	ribonucleic acid

THESIS SUMMARY

The study aimed to develop reliable procedures for determining the genetic status of embryos derived by IVF procedures prior to implantation. This may be a preferred route for prenatal diagnosis as it allows pregnancy to be established using only acceptable embryos, thus avoiding the trauma and risks of artificial termination at later stages of pregnancy.

To achieve this aim, procedures were initially developed using readily available murine embryos which could then be adapted with only minor modification to the human. Early studies of single cell embryo biopsy in the mouse (series 1-8) resulted in a similar rate of blastocyst formation, but a lower rate of hatching of biopsied versus control embryos. This difference was eliminated in subsequent experiences (series 9-13) and safety of the procedure confirmed by showing that embryo transfer of hatching blastocysts resulted in equivalent rates of implantation and fetal development for both biopsied and non-biopsied control groups. These experiments highlight the use of the hatching rate of embryos in culture as a means of providing a simple and reliable guide to evaluating the technical skill of single cell embryo biopsy.

As a basis for the genetic analysis of biopsied cells, procedures based on the polymerase chain reaction (PCR) were developed to amplify fragments of the mouse testis specific gene sequence (pYMT2/B) on the Y chromosome and the ovary specific gene (ZP3) sequence on chromosome 5. Using these oligonucleotides, PCR amplification, allowed

41 male and female mice blood samples to be sexed accurately. The reliability of the procedure was further demonstrated by showing that it was possible to biopsy 164 single cells from 8-cell mouse embryos and successfully amplifying the DNA on every occasion (100%) by PCR. The accuracy of the procedures was further confirmed by establishing that all of the 39 mice born following biopsy and transfer had been sexed correctly. These mice were examined extensively for signs of abnormality. Birthweights, macroscopic, x-ray and histopathological examination together with haematological and biochemical analyses all confirmed essential normality.

As a preliminary to the extension of the procedure to human studies, a PCR protocol was established for primers derived from the motif of human testis-determining gene (SRY) on the Y chromosome and the autosomal gene (ZP3). PCR analysis was performed on blood DNA from 21 men and 20 women and on 20 single lymphocytes using this protocol and on all occasions, the correct sex of origin was confirmed. It was also shown that the PCR procedures developed could be successfully adapted to determine the genetic sex of sperms. Using the modified procedure, 653 of 671 motile human spermatozoa (97.3%) were sexed with 355 (54.4%) determined as Y bearing and 298 (45.6%) as likely X bearing. Interestingly, the length, perimeter and area of the sperm heads and the length of the sperm necks and tails in X bearing human spermatozoa were found to be significantly larger than those of Y bearing spermatozoa.

As a further preliminary test of the reliability and the accuracy of the procedure prior to application to studies with human embryos, PCR

amplification of the targeted SRY and control (ZP3) genes was performed on blood DNA from further 120 men and women, and on 38 single lymphocytes. All results confirmed the correct sex of origin (100%). The sex of 21 single embryo cells biopsied from 4–8 cell human polyspermic embryos was then determined and apart from two degenerate embryo cells (recognised at biopsy) which produced negative results, 19 single embryo cells demonstrated 100% PCR amplification. Eleven of the embryos (57.9%) were determined to be 'male' and 8 (42.1%) 'female'.

Using novel oligonucleotide sequences, the PCR amplification technique was then applied to the diagnosis of the common three point mutation (ΔF508) present in cystic fibrosis, the most common of the non-sex-linked genetic diseases in the Caucasian population. Using a direct PCR amplification method, the results of 22 blood samples and 174 single lymphocytes from homozygous normal, heterozygous and homozygous abnormal subjects for the ΔF508 mutation showed both 100% PCR amplification and the correct diagnosis. Ten human embryos and another 11 selected nucleated blastomeres following single cell embryo biopsy also showed 100% PCR amplification rate and allowed a confident diagnosis.

The study confirms the feasibility of prenatal diagnosis on human preimplantation embryos and describes a protocol allowing precise diagnosis of a single gene defect in a single biopsied cell. The clinical availability of such procedures offers new prospects for couples with sex-linked chromosomal genetic diseases, cystic fibrosis, and, with the ongoing development of suitable techniques, for other clinically important inherent single gene defects.

CHAPTER ONE

PREIMPLANTATION EMBRYO AND GAMETE DIAGNOSIS

LITERATURE REVIEW:

1.1   Introduction

Every pregnant couple hopes their baby will be normal and healthy. Unfortunately about 1 per cent of the babies born are affected by a severe genetic or chromosomal disability (McLaren, 1987). The risk of transmitting a particular genetic disease to a child is high if both parents carry the same recessive single gene defect (one in four of their children will be affected), or if one parent carries a dominant gene defect (one in two children will be affected) (Monk, 1990a). Some of these babies will die at birth or within the neonatal period, others may expect to live for 2 or 3 years (as in Tay Sachs disease), for 10 or 12 years (Lesch Nyhan syndrome) or for 20 years or more but with rapidly increasing disability (Duchenne muscular dystrophy). Some conditions are obvious at birth (Down's syndrome); others like Huntington's disease, do not become evident until middle age. Many diseases are severe and result in much suffering, both for the child and for the immediate and extended family. Only now are the first attempts being made to cure genetic disorders by specific somatic therapy (Friedmann, 1989; Felgner and Rhodes, 1991; Miller, 1992). To date the commonly utilised treatments are subjective and only prolong life, and they are often painful and traumatic themselves.

In many cases, the potential parents will be aware of a risk of the genetic transmission of a disease, either because they have already given birth to an affected child or they have a family history of the disease, with both partners having been found to be carriers of the same defect. At the present time, a couple at risk of giving birth to an unhealthy baby may be offered chorionic villus sampling at 8 weeks or amniocentesis at around 14–16 weeks of pregnancy. Both procedures carry risks of the accidental interruption of pregnancy of a normal fetus. If the fetus proves to be affected, the couple has to consider the option of terminating the 'wanted' pregnancy by therapeutic abortion, a procedure traumatic for the mother, the family and the medical staff. While some couples may experience this trauma several times in their attempts to have a healthy baby, for other couples, therapeutic abortion even for a genetic indication is unacceptable to their philosophy of life.

Preimplantation diagnosis is a new form of prenatal diagnosis. Since only those embryos free of the risk of expressing the disease would be transferred to the uterus, the need to consider therapeutic abortion is eliminated. Preimplantation diagnosis offers therefore an important new option in clinical medicine.

Preimplantation diagnosis may utilise spermatazoa, oocyte (polar body), blastomere or trophectoderm material. The analytical methods useful include metabolic analysis, chromosomal analysis, in situ hybridization and the polymerase chain reaction (PCR) amplification.

In recent years, the DNA basis of many genetic diseases, including some X–linked diseases, cystic fibrosis and thalassaemia has become known and has made preimplantation diagnosis feasible using the modern techniques of molecular biology.

Accompanying developments in preimplantation diagnosis, have been advances in adjuvant techniques such as embryo cryopreservation, embryo co-culture and techniques of uterine lavage. Some of these advances offer additional options to the field of preimplantation diagnosis. The safety of preimplantation diagnosis particularly embryo biopsy is a matter of concern and has been the subject of scientific and lay debate. This thesis surveys the development of preimplantation diagnosis, critically reviews the procedures involved and establishes practical procedures to be used in human preimplantation diagnosis.

1.2   Historical development

1.2.1   Animal research

The development of preimplantation diagnosis has depended on the concept of totipotency of the early embryo cells (Driesch, 1894; Morgan, 1895; Nicholas and Hall, 1942). The ability of early cells of the embryo to individually form complete animals has allowed for the potential for normal embryo development after blastomere removal.

The isolation of individual blastomeres was first demonstrated in fishes and amphibians (Holtfreter, 1929, 1931; Spemann, 1938; Hörstadius, 1939). At that time, the prevailing view seemed that totipotency was unlikely in eutherian mammals but this view was not confirmed by subsequent work. Viable embryos were evident in vivo six days after isolating and transferring single blastomeres separated from the 2-cell rat embryos using either direct or indirect methods of cell isolation (Nochlas and Hall, 1942). Live young were born after transferring the '2-cell' embryos (with only one of the blastomeres

remaining intact) in both the rabbit (Seidel, 1952, 1960) and the mouse (Tarkowski, 1959a,b).

The first successful demonstration of the potential of preimplantation diagnosis (resulting in 18 living births) utilised rabbit blastocysts and trophectoderm biopsy of 200-300 cells and using methods of sex chromatin detection (Gardner and Edwards, 1968).

By 1921, the diploid chromosome number in the human was known to be 46 and the sex chromosomes designated X and Y (Painter, 1923). Sex chromatin (Barr body) had been recognised (Cajal, 1909; Barr, 1939) and later was detected in living cell monolayers using phase-contrast microscopy (DeMars, 1962). However, the sex chromatin approach is unsuitable for preimplantation diagnosis of the human blastocyst. The human blastocyst implants much earlier in the developmental sequence, at a stage when the embryo contains only one hundred cells, thus much less trophectodermal material would be available for sex chromatin detection. In contrast, in many other species including the rabbit, sheep and cow, the implantation conceptus can contain thousands of cells, the great majority of cells being trophectoderm.

Subsequently chromosomal analysis was recognised to be a superior and more reliable method when compared with sex chromatin analysis particularly when only small amounts of material were available. As a consequence the research impetus moved in this direction. Chromosome preparations were made from single mouse oocytes (Tarkowski, 1966) and later with the mouse blastocyst (Tarkowski et al., 1970; Graham, 1970). When a single pronucleus was removed for analysis, most of the now haploid mouse embryos developed initially but then arrested at the morula stage

(Modlinski, 1975) thus a link was forged between chromosome influence and embryo development. Karyotyping was also used for mouse half blastocyts (with 33% efficiency) (Epstein, 1972; Epstein et al., 1978), for second polar body of the mouse egg (Modlinksi and McLaren, 1980), and later for the single blastomere isolated from a 4-cell mouse embryo (Severova and Dyban, 1984). Karotypic analysis of the human oocyte and the 8 cell human embryo was demonstrated in 1983 (Angell et al., 1983).

During this period, micromanipulation techniques of embryo biopsy advanced further to earlier stages of embryo development. The mouse egg could be immobilized by a mouth controlled suction pipette and the pronucleus could be removed by use of a micropipette regulated through a screw-type syringe (Modlinski, 1975). This formed the basis of the modern technique of single cell embryo biopsy. The development of the technique of second polar body biopsy in the mouse (Modlinski and McLaren, 1980), the injection of single or multiple sheep blastomeres into foreign zona pellucida (Willadsen, 1980) and bovine morula biopsy and bisection (Lambeth et al., 1983) all associated with pregnancy and birth had strong positive influences on the general acceptance of embryo biopsy. The developmental potential of isolated blastomeres from 4- and 8-cell mouse embryos (Tarkowski and Wroblewska, 1967; Rossant, 1976; O'Brien et al., 1984) and from sheep embryos (Willadsen, 1981) to form pregnancies was established, but with low rates of success.

The measurement of the mouse embryo X-linked enzyme-hypoxanthine-guanine phosphoribosyl transferase (HPRT) activity by a non-invasive biochemical method, provided the first demonstration that the X chromosome was active during oogenesis (Epstein, 1969) and allowed embryo sexing since the amount of HPRT derived from the female embryo

was twice that of the male embryo (Epstein, 1972; Epstein et al., 1978; Monk and Harper, 1978).

By about mid 1980's, the potential existed for preimplantation diagnosis to be performed in the human by means of embryo biopsy, however the human embryo demanded very special consideration particularly the safety and ethical aspects as well as a need for new levels of precision if it were to be used for clinical work.

1.2.2   Human research

Four advances are identified as being crucial to the development of modern preimplantation diagnosis in the human: firstly, the establishment of in vitro fertilization (IVF) and embryo transfer techniques in human in 1978 by Edwards and his colleagues (Edwards et al., 1980); secondly, the application and refinement of micromanipulation techniques; thirdly, developments in DNA analysis of many inherited diseases; and fourthly, the advent of the polymerase chain reaction by Mullis in 1983 (Mullis et al., 1984; Mullis, 1990) and its application to genetic medicine (Saiki et al., 1985).

The feasibility of human preimplantation diagnosis was considered in 1985 by McLaren (1985) in a review which examined several different procedures in detail including embryo biopsy at the blastocyst stage and biopsy of the second polar body. It was recognised that there would be many ethical concerns when the human embryo was to be the subject of research.

By 1984, 32 cases had been documented of the natural occurrence of human chimeras (chimera being an individual containing genetically different cell populations derived from more than one zygote) (LeDouarin and McLaren, 1984; Silver, 1990). One major concern with manipulating the human embryo was that 'genetic engineering' would produce artificial chimeras or even human monsters. The difference between true genetic engineering and embryo research for preimplantation diagnosis was clouded. The Parliamentary Assembly of the Council of Europe held joint hearings to discuss questions relating to human embryo research (Council of Europe 1986a,b). The recommendations from the meeting recognized that 'recent progress in the life sciences and medicine, in particular animal and human embryology, has opened up remarkable new scientific diagnostic and therapeutic prospects' (Council of Europe, 1987). The report also recommended the prohibition of undesirable uses of embryos such as the production of chimeras or the deliberate creation of identical twins. In November 1986, a group of about 30 scientists and clinicians met at the CIBA Foundation in London to discuss the feasibility of detecting genetic or chromosomal abnormalities before implantation in order to develop an alternative form of prenatal diagnosis which would circumvent the need for therapeutic abortion, at least while no means of specific corrective therapy was available (Whittingham and Penketh, 1987).

Since 1987, preimplantation diagnosis research techniques utilising human oocytes, spermatozoa and embryos together with the birth of a few human babies following embryo biopsy have been reported (Handyside et al., 1990) however misdiagnosis has also occurred (Soussis et al., 1991; Winston et al., 1991) and the thrust to perform preimplantation diagnosis in patients has been of concern to a number of scientists and clinicians since

premature application to clinical medicine risked a major setback to the progress (Trounson, 1992).

1.3   The choice of genetic material and methods of sampling

Preimplantation diagnosis can be performed using spermatozoa, oocytes (polar body) or embryos at various stages (Edwards and Hollands, 1988). Due to the limited dimensions and amount of the starting material it has been necessary to fully utilise the advances in microscopy, micro-instrumentation and the techniques of molecular biology to reach the current clinical situation. The various substrates and methods of sampling for preimplantation diagnosis together with their advantages and disadvantages are now discussed.

1.3.1   Spermatazoa

Pre-selection of the gender of the offspring has long captured man's attention (Gledhill, 1988). Efforts to find methods to separate X and Y bearing sperm have been hampered by the lack of a satisfactory method of detection of each gender spermatozoa. The confident separation of X and Y bearing spermatozoa would offer an interesting but very challenging approach to preimplantation diagnosis particularly to sex-linked diseases. Although several methods purport to separate (or enrich one fraction) gender spermatozoa in the human, either no good proof exists of the results or the method leaves spermatozoa in a state where fertilisation cannot be anticipated.

Fluorescent labelling of the Y chromosome using quinacrine (Barlow and Vosa, 1970; Sumner et al., 1971) has been successfully used to analyse the sex ratio in sperms. However, the proportion of sperm with single spots following quinacrine fluorescence have commonly been lower than the expected 50%. In addition, a small fraction of sperm showing two bright fluorescent spots may not be YY-bearing, but may be Y-bearing sperm (Sumner and Robinson, 1976; Beatty, 1977). In addition several studies have utilized karotyping (after fusion with zona free hamster eggs) (Yanagimachi et al., 1976) to analyse the sex ratio and frequency of chromosome abnormalities in sperm.

More recently fluorescent in situ hybridisation (FISH) has succeeded in sexing human sperms with 96% accuracy (Han et al., 1992). The amplification and analysis of DNA sequences in single human sperm by PCR has been demonstrated (Li et al., 1988; Cui et al., 1989). The use of PCR to diagnose cystic fibrosis allele ΔF508 in single sperms has also been reported (Liu et al., 1992). However the sperm which has been analysed by PCR amplification cannot be used for fertilization.

Several methods have addressed the separation of X and Y sperms, including electrophoresis (Kaneko et al., 1983, 1984a), flow fractionation (Bhattacharya, 1977), Sephadex gel filtration (Steeno and Adimoelja, 1974), albumin centrifugation (Ericsson et al., 1973), Percoll density gradients (Kaneko et al., 1984b), and flow cytometry (Garner et al., 1983). However until recently no reliable 'assay' has existed to confirm these methods. The most convincing report is of live rabbit births from X and Y sperm separated by cell sorting (Johnson et al., 1989).

1.3.2 The Oocyte (Polar Body)

The clinical practice of IVF have made oocytes more available for research. Chromosome analysis of multipronuclear human oocytes fertilized in vitro (Rudak et al., 1984) and of oocytes lacking signs of fertilization or of cleavage (Veiga et al., 1987; Van Blerkom and Henry, 1988; Wramsby, 1988; Macas et al., 1990) has allowed better understanding of the mechanism of normal and abnormal fertilization. The normal oocyte contains only the X chromosome so it is not useful for separation of gender. However, a number of genetic diseases are transmitted via the oocyte, thus the detection of mutant DNA in the oocyte would be valuable if the oocyte which remained could be fertilized. Neither of the two major techniques currently in use to detect mutant DNA namely PCR and in situ hybridization, leave the oocyte in a satisfactory viable condition.

Both the *first* polar body (which exists before fertilization) and the *second* polar body (extruded at fertilization) contain hereditary material (DNA) potentially available for preimplantation diagnosis (Penketh and McLaren, 1987).

While removal of the _first_ polar body has been reported for preimplantation diagnosis (Verlinsky et al., 1990; Strom et al., 1990; Gordon and Gang, 1990; Monk and Holding, 1990), this method may not provide precise information about the oocyte due to the problem of telomeric crossover (Arnheim et al., 1990; Handyside et al., 1992). Biopsy of the first polar body is not optimal for preimplantation diagnosis for additional reasons, namely: (1) since paternal alleles cannot be analysed (Verlinsky et al., 1992), no precise information of the embryo can be anticipated; (2) the need for double biopsy (polar body biopsy combined with later embryo biopsy) for precise diagnosis

may adversely effect the safety of the biopsied embryo (Trounson, 1992); and (3) the polar body is often degenerate and may not be informative.

Although the _second_ polar body has been targeted for biopsy in order to study the chromosomes (following microinjection into fertilized egg) (Modlinski and McLaren, 1980), this approach is unlikely to be of assistance in preimplantation diagnosis, since again crossover of telomeric loci can occur with a high frequency during meiosis 1 (McLaren, 1985; Penketh and McLaren, 1987).

1.3.3   The Embryo

The cleaved diploid embryo which contains more than two cells is an important entity for preimplantation diagnosis. The totipotential nature of most early blastomeres allows for individual cells to be removed without adversely affecting the further development of the embryo and blastomere biopsy is therefore the fundamental technique for preimplantation diagnosis. Improvements of the embryo biopsy technique using minimally invasive procedures has assisted the safety of embryonic development.

Embryo biopsy at 2-cell and morula stages

Embryo biopsy can be experimentally performed at any embryo stage from the 2-cell to the blastocyst.

The potential of blastomeres isolated at the 2-cell stage to develop were studied in the rat (Nicholas and Hall, 1942), rabbit (Seidel, 1952, 1960) and the mouse (Tarkowski, 1959a, b; Mulnard, 1965a, b). Although the majority of the remaining one cell (half) embryos developed into normal (but

smaller) blastocysts, some blastocysts resulted in purely trophoblastic vesicles devoid of the inner cell mass (Nijs et al., 1988). Following embryo biopsy at the 2-cell stage, the rate of 'half-embryo' development was also significantly lower than the non-biopsied controls (Rands 1986; Nijs et al., 1988; Nichols and Gardner, 1989). Thus, the 2-cell stage is probably best avoided for preimplantation diagnosis.

For different reasons, the morula is also not optimal for biopsy. Changes in DNA methylation are closely related to the mechanism of gene expression. From the morula to the blastocyst, there is a progressive loss of methylation during development, which allows for the beginning of differential gene expression (Monk, 1990b). Some cells at the morula stage have already acquired direction and will differentiate into cells of the inner cell mass, while other cells are destined for the trophectoderm. If cells already destined to differentiate into the inner cell mass are removed, fetal abnormality and/or fetal demise may be a real risk. Embryo biopsy at the morula stage has also been shown to be detrimental to the rate of implantation, fetal viability and birthweight (Krzyminska et al., 1990).

Single cell embryo biopsy at 4 to 16 cell stage

Two methodological approaches have been utilized.

The original *puncture* method employed an embryo held by one pipette before a second pipette was used to create an aperture about one-quarter of the surface area of the embryo, (by suction of a part of the zona into the pipette). A third needle pipette was then placed through the zona operture and the blastomere removed (Nicholas and Hall, 1942). Later, a simple *direct* puncture of the mouse zona pellucida at the 4- or 8-cell stage with a sharp

needle was shown not to adversely effect the viability of the biopsied embryos (Wilton and Trounson, 1986, 1989), and additionally the single biopsied blastomeres were able to be proliferated in vitro to enable preimplantation diagnosis. Cryopreservation of the biopsied mouse embryo was also shown to be successful (Wilton et al., 1989; Krzyminska and O'Neill, 1991).

Another early method to obtain isolated blastomeres had involved completely dissolving the zona of the rat embryo by acid Ringer's solution (Defrise, 1937; Hall 1935, 1936; Nicholas and Hall, 1942). More recently pronase (Mintz, 1962; Tarkowski and Wroblewska, 1967) or Tyrode's solution (Wood, 1975; Nicolson et al., 1975) have replaced acid Ringer's solution. The modern technique of *Zona drilling* of the oocyte using a very localised application of acid Tyrode's solution has been shown to assist fertilization using small numbers of spermatozoa (Gordon and Talansky, 1986; Depypere et al., 1988) and this technique has recently been used for human blastomere biopsy (Handyside et al., 1989).

Following zona drilling, blastomeres can either be removed with direct suction or can be extruded with external pressure on the embryo. In the mouse, direct aspiration has provided better results (Krzyminska et al., 1990; Takeuchi et al., 1992). No differences were found in the survival or developmental rates of embryos between an aspiration or a displacement technique (where a pipette was inserted through a second puncture site to inject medium and displace a blastomere out from the first puncture hole) (Roudebush et al., 1990). However, caution has been advised since zona drilling can dramatically alter the pH or the physiology of the embryo cells (Nijs et al., 1988; Gordon and Gang, 1990; Johnson et al., 1990; Hartshorne, 1992)

and certainly has been shown to be detrimental to the oocyte (Payne et al., 1991).

Although single cell biopsy performed at the 4 to 16 cell embryo stage allows good viability, a lower rate of blastocyst formation (Krzyminska et al., 1990) and implantation rate (Wilton and Trounson, 1989; Krzyminska et al., 1990) of biopsied embryos at the 4-cell stage have been reported. Single cell embryo biopsy at the 4-cell stage may be more risky than at the 8-cell or 16-cell stage, because if an additional cell of the embryo lyses after single cell embryo biopsy at 4-cell stage, the result will be an 'half-embryo' with a poorer outlook. Embryo biopsy at the 8-cell stage may therefore be the optimal choice for preimplantation diagnosis. It may well be that the 16 cell embryo will also be optimal for the precision of diagnosis and the safer development of the biopsied embryos when more experience is gained.

Trophoectoderm biopsy (blastocyst biopsy)

Before the modern operative techniques of single cell embryo biopsy and the advent of the polymerase chain reaction (PCR), most opinions suggested that trophectoderm biopsy performed at the blastocyst stage was the best choice (McLaren, 1985; Edwards, 1986; Whittingham and Penketh, 1987).

Successful trophectoderm biopsy has been reported in the mouse (Monk et al., 1988; Gomez et al., 1990), in the marmoset (Summers et al., 1988) and in the human (Dokras et al., 1990; Muggleton-Harris and Findlay, 1991). However, when trophoectoderm cells were biopsied in the mouse, a significantly lower rate of fetal development (Monk et al., 1988), a lower pregnancy rate at day 6.5 (Gomez et al., 1990) and a lower number of embryos which reached the egg cylinder stage (Nijs and Steirteghem, 1990) were reported. These results have been explained by the loss of the ability of some blastocyts to re-expand in culture (Edwards and Hollands, 1988) with the resultant loss of ability to hatch and implant.

Trophectoderm biopsy might also impair the chances of implantation and normal growth by reducing the number of cells able to produce essential enzymes for implantation, (Perona and Wassarman, 1986), or able to synthesize essential glycoproteins necessary for the adhesion of the embryo to the uterus (Psychoyos and Martel, 1985). In man, the polar trophoblast appears to be mesometrial during early implantation (Enders and Schlafke, 1979) so the mural trophoblast does not seem to be critical for the adhesion of the embryo to the uterus. Instead, the mural trophoblast appears to form the lining between the embryo and the uterine cavity, (until overgrown by the decidua), so that removing 10 or 20 cells might be less traumatic than a similar procedure in other species (Edwards, 1987).

One of the advantages of trophectoderm biopsy is that more embryo cells are available for the diagnosis by gene expression. The recent appreciation of 'imprinting' of some genetic characteristics in early embryos (Reik et al., 1987; Monk, 1990b) could however complicate the detection and

precision of gene expression from trophectoderm material. In early embryo development, it is likely that paternally derived genes mostly control the development of the placental tissues whilst maternally derived genes play a more important role in development of the embryo proper. Gene expression of trophectoderm cells may not therefore precisely reflect gene expression from the cells of inner cell mass. To what degree imprinting would affect preimplantation diagnosis remains to be identified. Diagnostic methods depending on the gene itself (DNA) would not be influenced (Edwards and Hollands, 1988) by a trophectoderm source.

Growing the human embryo to blastocyst has not been reliable thus far, and this fact together with the improvements of single cell biopsy technique at the 8 cell stage have for the moment relegated trophectoderm biopsy to the background. Thus, the use of trophectoderm biopsy for preimplantation diagnosis in clinical practice may be limited, except in some conditions when single cell embryo biopsy at 8 to 16 cell stage would not be appropriate for the diagnosis.

Since the frequency of blastocyst formation is relatively low in human IVF culture conditions, a second alternative would be the collection of blastocysts derived in vivo by uterine lavage of either natural cycles or following ovarian hyperstimulation (Critser, 1992). Uterine lavage was first reported in 1972 in order to obtain oocytes and unimplanted embryos from the human uterus (Croxatto et al., 1972; Buster et al., 1985). With preimplantation diagnosis in mind, modified instruments for uterine lavage have been used (Sauer et al., 1988; Brambati and Tului, 1990; Formigli et al., 1990), but the embryo recovery rate was only about 40% (Sauer et al., 1988; Formigli et al.,

1990) and retained pregnancies, pelvic infection (Buster and Carson, 1989) and discomfort (Brambati and Tului, 1990) were reported. The retained pregnancies may also be ectopic. Although four different regimes of superovulation have been used, the blastocyst yield has not been increased (Formigli et al., 1990; Carson et al., 1991). It seems therefore that uterine lavage would not provide any special benefits for preimplantation diagnosis rather it would risk more complications.

1.4   Choice of methodology for Diagnosis

All diagnostic methods of the preimplantation embryo require adequate sensitivity, reliability and above all, precision.

1.4.1   Metabolic Techniques

These include detection of the metabolic activity and gene expression in the early embryo.

Metabolic activity of the early embryo

Glucose and pyruvate *uptake* measurements in mouse (Gardner and Leese, 1986, 1987) and in human embryos (Leese et al., 1986; Wales et al., 1987; Hardy et al., 1989a) have been described in order to access the safety of embryo biopsy. However, these techniques are difficult to set up as a routine and very limited information exists with respect to other physiological markers of the early embryo.

Physiological activities of the early embryo result in the *release* of a variety of materials, including CO_2, H_2O, lactate, nucleotides, enzymes, hormones, other proteins, together with platelet activating factor and early pregnancy factors. Lactic acid can be detected from human embryos at about the 6-cell stage (Wales et al., 1987). Lactate dehydrogenase (LDH) is present at a very high concentration in early embryos (Biggers and Stern, 1973), and differences in the rate of appearance in the culture medium might correlate with embryo viability (Biggers and Stern, 1973).

Human embryo-derived platelet-activating factor (PAF) has been detected by quantitative bioassay in the early embryo (Collier et al., 1990). Histamine releasing factor has also been measured from the medium in which 2-cell human embryos were cultured (Cocchiara et al., 1986, 1987). While these release measurements are not relevant to the detection of genetic disease at the present time, they do illustrate the potential to recognize abnormal embryos and perhaps to recognise inherited genetic disease in the future.

Gene expression in the early embryo

Gene expression specific to the early human embryo first occurs between the four and eight cell stages of preimplantation development (Tesarik et al., 1986; Braude et al., 1988). Chorionic gonadotrophin-β mRNA is expressed in human 8-cell (day 3) embryos (Bonduelle et al., 1988) and hCG can be detected in the culture medium of day 7 embryos (Fishel et al., 1984; Hay and Lopata, 1988).

β-N-Acetylhexosaminidase is a ubiquitous lysomal enzyme but a mutation in the protein product leads to Tay-Sachs disease. The activity of

this enzyme can now be analysed at the late 2-cell human embryo (Sermon et al., 1991). Table 1. illustrates the current state of the knowledge of gene expression in both animal and human species.

Table 1

Expressed Gene	Species	Embryo Stage	Author	Year
Glucose phosphate isomerase	Human	Egg Early embryo	West et al.	1989
Insulin receptor and insulin-like growth factor I receptor	Chicken	Blastoderm (day 1)	Scavo et al.	1991
Globin	Chicken	Oocyte Early embryo	Imaizumi- Scherrer et al.	1989
Beta-galactosidase	Ovine Porcine	2-cell	Murnane et al.	1990
Interleukin-6	Ovine Porcine	Late embryo	Mathialagan et al.	1992
Metallothionein	Mouse	4- & 8-cell	Andrews et al.	1991
Adenosine deaminase	Mouse	Early embryo	Benson & Monk	1988
Beta-Glucuronidase	Mouse	Morula	Van Blerk et al.	1991

The Lesch-Nyhan syndrome has been investigated with a view to identifying a gene product of the early embryo. The Lesch-Nyhan syndrome is an X-linked disease, affecting only males, caused by an inherited deficiency in the level of activity of HPRT (Lesch and Nyhan, 1964; Seegmiller et al., 1967). The use of two mouse HPRT deficient models (Hooper et al., 1987; Kuehn et al., 1987) has made the demonstration of preimplantation diagnosis in this human disease theoretically possible. Transgenic chimeric male mice can lack HPRT activity. Theoretically, therefore the ratio of the dose of expressed X-linked gene in a single embryo cell of the normal female: chimeric female: normal male: chimeric male would be 2:1:1:0 respectively. The HPRT bioassay of single embryo cells confirmed the chimeric male mice to be HPRT negative (Monk et al., 1987), the normal female and normal male confirmed about the 2:1 ratio (Monk and Handyside, 1988; Monk et al., 1990) and the chimeric female and chimeric male were about 1:0 ratio (Monk et al.,. 1988). Thus the distinction between the normal female, chimeric female, normal male and chimeric male was possible (Monk et al., 1990). Unfortunately, most of the experiments showed some misdiagnoses due to the overlapping dosage results (Monk et al., 1987, 1988; Monk and Handyside, 1988). When this method was applied to individual human embryo cells, two problems were encountered. Firstly, there was a considerable dosage variation among individual embryos recovered at the same developmental stage or age. Secondly and more fundamentally, the HPRT activity measured in the embryos studied appeared to be maternally inherited, since HPRT activity was uninfluenced by the addition of alpha –amanitin which blocks transcription. The use of HPRT activity for human preimplantation diagnosis is thus considered to be premature (Braude et al., 1989).

The range of gene expression in the early embryo is limited and whether any inherited disease can be identified by such early markers is unknown, however the potential is present to develop relevant methods in the future. Unfortunately to date most measurements associated with gene expression have utilized destructive methods but with the increasing availability of new fluorescent molecular probes, less destructive methods may be available in future for preimplantation diagnosis of genetic diseases.

1.4.2   Chromosomal techniques (Karyotyping)

It has been calculated that the chance of a woman producing a viable offspring each menstrual cycle during which intercourse occurs is only about 25% (Edmonds et al., 1982). Exactly why this is, is unclear, but there is indirect evidence for a high frequency of embryo death in the normal reproductive processes in humans (Roberts and Lowe, 1975). The main reason for embryo death is believed to be chromosome abnormalities which arise during oogenesis, spermatogenesis, fertilization or during the early cleavage divisions. Most of the errors which occur are believed due to non-dysjunction in the oocyte, especially during meiosis I (Hassold and Matsuyama, 1979; Mattei et al., 1979) and embryo death from chromosome abnormalities is presumably part of natural selection. In addition, spontaneous abortion is common and a high percentage of abortion material is associated with chromosome abnormalities (Boue et al., 1975; Hassold et al., 1980; Kajii et al., 1980). About 0.5%–0.6% (Lubs and Ruddle, 1970; Evans, 1977; Nielsen, 1975) of live-born babies carry a recognisable chromosome abnormality. The incidence of chromosome abnormalities in newborn babies derived from IVF is about 0. 3% (Edwards, 1985).

The chromosome complement of individual human sperm can be visualized following penetration of zona-free hamster eggs (Yanagimachi et al., 1976; Rudak et al., 1978; Martin et al., 1983; Brandriff et al., 1985; Kamiguchi and Mikamo, 1986). The rate of recognisable chromosome abnormalities in sperms was found to be about 9% (range 1.9 to 15.8%). However these results only reflect the karyotyping of those sperms which have penetrated hamster oocytes, and may not be representative of the whole sperm population.

The results of several studies of human oocytes which have remained unfertilized after IVF have indicated a range of normality from 68%-77% normal (Plachot et al., 1987a, Veiga et al., 1987, Martin et al., 1986). These results were similar to multicentric data (74% normal) (Plachot et al., 1988).

The karyotype results from several studies of human embryos also demonstrated a range of normality, from 60%-100%, (Angell et al., 1983, Edwards, 1983, Plachot et al., 1987b, Angell et al., 1988, Papadopoulos et al., 1989). A multicentre study showed 71% of embryos had normal chromosomes (Plachot et al., 1989). It must be recognised however that the majority of embryos analysed were those considered less optimal for transfer and may therefore be subject to bias on morphological grounds.

Cytogenetic techniques of human and mouse embryos have been performed for preimplantation diagnosis (Watt et al., 1987; Roberts and O'Neill, 1988). After single cell mouse embryo biopsy, the rate of successful karyotyping varied between 71%-80% (Bacchus and Buselmaier, 1988, Roberts et al., 1990, Kola and Wilton, 1991). Due to the time required for preparing karyotypes, the low rate of metaphases, the loss of chromosomes through scatter and the practical difficulties associated with chromosome overlapping, research now is more directed towards in situ hybridization or chromosome painting to detect chromosomal status rather than karyotyping, except in those chromosomal diseases.

1.4.3   In situ hybridization

In the field of preimplantation diagnosis, most methods of in situ hybridization have related to determining the sex of embryos. The procedure may involve radioactive sources or non-radioactive sources.

Most experiments with in situ hybridization have been directed to the preimplantation diagnosis of sex in cattle. Three different repetitive male-specific bovine chromosomal DNA probes have been used to sex the cell mass from the 6 or 7 day embryos. When radioactive isotopes were used for in situ hybridization, insufficient data was available for the consistency and accuracy of the experiments to be assessed (Bondioli et al., 1989). Biotinylated probes have also been used to sex bovine embryo cells with Y chromosome-specific sequences. The technique presented difficulties due to the numerous steps required but was used successfully to sex bovine embryos

at different stages of development (Kirszenbaum et al., 1990). Interestingly, it was found that the faster developing embryos were significantly different from the 1/1 ratio with more than 75% being male (Marquant et al., 1992). The mouse has also been used as a model for single cell embryo biopsy and for in situ hybridization (Grifo et al., 1990, 1992a).

For the sex determination of human embryos, the most popular probe has been pHY 2.1 (named Ambrobe RPN 1305X, Amersham) which is a 1.5 Kb sequence in a pSP 65 Vector which recognizes a Y-specific repeat of which 2000 copies are present on the Y chromosome, and less than 200 on an autosome (Cooke et al., 1982). Radioactive tritium and biotin methods have been compared with in situ hybridization using the human male-specific repetitive sequence probe 102d[2]. The radioactive method was shown to be more reliable but required 1-3 weeks to achieve the results (Jones et al., 1987). The radioactive method showed a 5% false negative rate and a 1.3% false positive rate with interphase 'male' lymphocytes (West et al., 1987). These results were better than the 88% efficiency using dual fluorescent in situ hybridization of similar lymphocytes (Griffin et al., 1992). Discrimination between male and female early embryos using in situ hybridization is more difficult than between individual samples of male and female derived lymphocytes. Early embryo cells have only a few large interphase nuclei and Y-bodies and tend to be diffuse and less obvious than those cells in later stage embryos or lymphocytes. It has also been shown that the biotin method is a faster (48hr), cheaper and more convenient method (West et al., 1988; West, 1989) when compared with the radioactive method.

The fluorescent method is the most rapid method available requiring between 6 and 24h to achieve the results (Griffin et al., 1992; Penketh et al., 1989). The diagnostic ability has been reported as 66% (interphase) (Penketh et al., 1989), 82-85% (human embryos) (Griffin et al., 1991), 72% (interphase) and 89% (metaphase) (Griffin et al., 1992). Misdiagnosis of male embryo cells as female was possible either because of failure of hybridization with the Y-specific probe or as a result of the presence of a tetraploid cell with multiple X when the X-specific probe (pBam X7, Willard, 1985) was used. Also, XY cells have been classified as XO cells due to a failure of the Y signal (Griffin et al., 1992).

To date, we must conclude that in situ hybridization has not yet been demonstrated to be reliable enough to perform human preimplantation diagnosis with confidence.

1.4.4 PCR Amplification

The development of PCR, principles and application

It is fascinating to recall the history of this development which has had profound benefits to molecular biology. On a Friday night in April, 1983, Kary Mullis was driving along Highway 1, heading north from San Francisco to Mendocino. He pulled over to one side of the road, and started doing mental arithmetic. 'Ten cycles would get me a thousand and 20 would

get me a million', mused Mullis. He had just invented the miraculous polymerase chain reaction (PCR) (Cherfas, 1990; Mullis, 1990).

In his laboratory at the Cetus Corporation near San Francisco, Mullis repeated the time-consuming and laborious cyclic amplification. The high temperature which denatured double stranded DNA also destroyed the polymerase enzyme, so that fresh polymerase was required to be added each cycle. The answer was to find a thermostable polymerase. Mullis was aware that certain bacteria (Thermophilus aquatics) which lived in hot springs, had been investigated, but no one had purified this Taq's polymerase nor did anyone in Mullis' department at Cetus want to do this work (Cherfas, 1990). Later, however, the thermostable DNA polymerase was isolated, and significantly improved the specificity, yield, sensitivity and length of products. Single-copy genomic sequences were able to be amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified (Saiki et al., 1988a).

In the spring of 1984, Mullis presented a poster (Mullis et al., 1984) describing the PCR at the annual Cetus Scientific Meeting, a meeting which attracted some first-rate scientific advisers. However, nobody seemed to be interested in his poster. At last, he snared Joshua Lederberg, President of the Rockefeller University, into looking at his results, and had 'an enjoyable talk' (Mullis, 1990).

The primers for PCR amplification were oligonucleotides, first made available by synthesis in 1981 (Alvarado-Urbina et al., 1981;

Hunkapiller et al., 1984; Itakura et al., 1984; Caruthers, 1985). The first applications of PCR technique to genetic medicine (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987) rapidly popularised the technique and more than 1,000 reports of its use have since been published (Mullis, 1990).

The polymerase chain reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA; after denaturation of the DNA, each primer hybridizes to one of the two separated strands such that extension from each 3' hydroxyl end is directed toward the other. The annealed primers are then extended on the template strand with a DNA polymerase. These three steps (denaturation, primer binding, and DNA synthesis) represent a single PCR cycle. Consequently, repeated cycles of denaturation, primer annealing, and primer extension results in the exponential accumulation of a discrete fragment whose termini are defined by the 5' ends of the primers (Erlich et al., 1991).

The technique of PCR amplification has been thoroughly described (Vosberg, 1989; Rapley et al., 1992; Eisenstein, 1990; Lynch and Brown, 1990). The simplicity of the technique (after setting the conditions) allows for wide application in many fields associated with DNA and RNA, including archaeology, species evolution (Erlich et al., 1991), basic molecular biology (Vosberg, 1989), genetic medicine, prenatal diagnosis, preimplantation diagnosis, virology, bacteriology, cancer diagnosis and therapy, and forensic medicine (Lynch and Brown, 1990).

PCR amplification and preimplantation diagnosis (animal)

Transgenic animals have proved useful for the perfection of PCR techniques because the transgenic sequences are known and the transgenic parent animals are well characterised. When transgenic hemizygous male mice were bred to normal female mice, the new gene was detected in approximately half of the blastocyst embryos by PCR amplification (King and Wall, 1988). Other workers have reported the identification of foreign genes in transgenic mouse embryos (Ninomiya et al., 1989) and the sheep (Walton et al., 1991) by PCR amplification. The transgenic mouse containing the directed mutation associated with cystic fibrosis has also been used as a model for PCR amplification in preimplantation diagnosis (Koller et al., 1991).

Transgenic mouse models with beta-thalassaemia have been used for preimplantation diagnosis. These models which have the whole beta-major haemoglobin gene deleted (Skow et al., 1983; Goldberg et al., 1986) have shown a 89% correct diagnosis rate, 9% false negative and 2% false positive by PCR amplification of single blastomeres (Holding and Monk, 1989). Another report showed a 69% correct diagnosis rate from single blastomeres with this kind of model (Lindeman et al., 1990). The sparse fur mouse model has been used for X-linked diseases (ornithine transcarbamylase deficiency) to detect mutations after biopsy of a single blastomere (Morsy et al., 1992). Rapid preimplantation detection of mutant (Shiverer) and normal alleles of the mouse myelin basic protein gene has allowed selective implantation and birth of live young, with a 64% correct diagnosis rate following assay of biopsied trophectoderm cells (Gomez et al., 1990).

1.5   Adjuvant techniques

1.5.1   Cryopreservation

Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo was reported in 1983 (Trounson and Mohr, 1983). Following embryo biopsy and preimplantation diagnosis, it is usually desirable to transfer the embryo which has been subjected to preimplantation diagnosis in the same menstrual cycle. However in some conditions, embryo cryopreservation may be either obligatory or be beneficial by choice (Kaufmann et al., 1992). Some kinds of mutation or chromosomal disorders will need more time to be analysed or to be repeated following the initial failure of the diagnostic result.

At other times embryos following trophectoderm biopsy may miss the interval of endometrial receptivity due to the shortened luteal phases of the 'stimulated cycle'.

Embryo transfer in subsequent natural cycles may achieve improved results when compared with stimulated cycles and may reduce the risk of multiple pregnancy. Excess embryos having undergone preimplantation diagnosis may be cryopreserved for a subsequent child without the additional need for repeated examination. Couples may wish to transfer only homozygous normal embryos first, and save the heterozygous carrier embryos for subsequent attempts.

While the cryopreservation of mouse embryos following single cell embryo biopsy or zona drilling has shown successful results (Wilton, et al., 1989; Depypere et al., 1988) and thawed embryos with one or more blastomeres damaged during freezing shown to have the same capacity to produce pregnancies as did those with all blastomeres intact (Hartshorne et al., 1990), the potential for deleterious effects due to cryopreservation/thawing itself must be considered.

1.5.2   Co-culture Systems

A high percentage of mouse blastocyts developed and hatched when co-cultured in vitro on a feeder layer of irradiated HeLa cells (Cole and Paul, 1965).   Co-culture techniques also maintained the viability of mammalian embryos once they had been removed from the female's uterus (Thibodeaux and Godke, 1992).   The development of embryo co-culture systems have been dependant on either the use of trophoblastic vesicles, cellular monolayers, or the chicken embryo.   In virtually all instances, co-culture systems have been reported to improve embryo development compared with incubating similar embryos in culture medium alone (Thibodeaux and Godke, 1992).

Embryonic blocks are well recognized in various species of animals and co-culture system appears to overcome some blocks (Rexroad, 1989). These embryonic blocks appear to be due therefore to artefacts in the culture environment (Bavister, 1988). Even with strictest quality control measures and the best laboratory conditions, only 25-30% of 'excess' human

embryos cleave normally to the expanded blastocyst stage in the usual culture conditions (Fehilly et al., 1985). This is the reason why human embryos are replaced into the uterus at the 4- to 8-cell stage to avoid in-vitro fragmentation and degeneration of embryos at later stages (Bongso et al., 1990). One report that 70% of co-cultured embryos (v.s. 33% control) reached the early blastocyst stage has been presented as evidence that the critical 8-cell and morula stages have been overcome in the human (Bongso et al., 1990).

Human embryos, co-cultured with the cattle cell monolayers, have been transferred to human subjects with a pregnancy rate significantly greater than those embryos cultured in conventional Earle's medium alone (35%, n=40 versus 17%, n=36; P<0.05) (Wiemer et al., 1989). Human day one embryos co-cultured with human oviductal cells have achieved significantly higher hatching rates than the control (Yeung et al., 1992). Co-culture with the human material may therefore help human IVF and in turn the preimplantation diagnosis programme (Critser, 1992). Agreed scoring methods for human embryos would be helpful for the better evaluation of the current co-culture system (Cummins et al., 1986; Bolton et al., 1989; Hardy et al., 1989b).

Since single cell embryo biopsy for preimplantation diagnosis is most ideally performed at the 8- to 16-cell embryo stage, the embryo should be between the 8-cell and morula stage when the diagnostic results become available. However, after biopsy most human embryos remain stationary at the 8 or 16 cell stage during this interval (personal observation). If development could continue normally during the diagnostic time, using co-

culture techniques, the expectation of pregnancy should be higher, an aspect which may be very critical given the fewer normal embryos available for transfer after preimplantation diagnosis.

1.6 Critical Analysis of the Current State of Human Preimplantation Diagnosis

1.6.1 Sexing human embryo by PCR amplification

Over 300 recessive X-linked diseases have been reported, including Duchenne muscular dystrophy (DMD), Lesch-Nyhan syndrome, adrenoleukodystrophy, X-linked mental retardation, X-linked anaemia and hemophilia. Only the heterozygous male will be clinically affected by these kinds of diseases since the female carries a normal allele. The determination of the sex of the preimplantation embryos would therefore allow only female embryos to be transferred to the uterus thus the fetus, while still a carrier (50% possibility) of the genetic disease, would be clinically normal.

Sex selection has important implications for agriculture. In the mouse, southern blot analysis of pY353/B following PCR amplification from single blastomeres identified the sex of 72% of the embryos with the birth of 8 mice pups correctly predicted (Bradbury et al., 1990). A reliable method to determine the sex of the bovine blastomeres using PCR amplification showed a 100% correct diagnosis rate (Peura et al., 1991). Another bovine experiment showed 98.5% diagnosis rate, and found that male bovine preimplantation

embryos developed faster than female embryos (Avery et al., 1992). The use of Y chromosome–specific sequences for PCR amplification also allowed 100% correct diagnosis rate in mouse (Kunieda et al., 1992). These animal results have greatly enhanced the credibility of PCR amplification for human preimplantation diagnosis.

By contrast in human experiments, the reported results of sexing embryos for preimplantation diagnosis have been very unstable and unreliable. The 3.4kb Y repeated sequences of DYZ$_1$ family (Nakahori et al., 1986; Kogan et al., 1987) has been used for PCR amplification by several research centres with similar results. The first report showed a 60% PCR amplification rate (Handyside et al., 1989). The report of pregnancies from biopsied human preimplantation embryos sexed by Y–specific DNA amplification showed a 92% amplification rate but with faint non–specific, non-identical bands. In a few cases, a regular ladder of amplified fragments prevented analysis (Handyside et al., 1990) and unfortunately a misdiagnosis occurred of one of the six pregnancies (Winston et al., 1991; Soussis et al., 1991; Hardy and Handyside, 1992).

To predict the gender of the human embryo reliably, two criteria must be met: 1) there must be no contamination with even minute quantities of foreign DNA, and 2) the PCR must be 100% successful (Strom et al., 1991a). For example, an incorrect diagnosis of a female fetus would be made by the presence of either a faint Y–specific band or by the failure of amplification of the Y–specific band (Handyside et al., 1989, 1990). Therefore, the absence of a Y–specific band can indicate either the presence of a female fetus or the failure of the amplification process itself (Strom et al., 1991a).

In order to reduce the risk of misdiagnosis, several modifications have been published (using the same Y repeated sequences) including the inclusion of a 'control' band to ensure proof of the PCR amplification. Unfortunately the Y-band does not always show in male blastomeres (3 out of 11 blastomeres) after PCR amplification (Kontogianni et al., 1991) and sometimes the female cells have shown stronger Y-bands than the male cells (Strom et al., 1991b). Moreover, when the amplification products were elongated from 149 base pairs to 500 base pairs, or shortened to 130 base pairs, the results provided only a 77% diagnosis rate in the two IVF cycles of a patient with hemophilia (Grifo et al., 1992b). The results repeatedly showed non-confident targeted bands with 6% error rate (Grifo et al., 1992b). This problem of misdiagnosis in sexing human embryos for preimplantation diagnosis must be solved before routine clinical practice. To this end particular attention is now directed toward the 'Y-specific repeated sequences' used to date.

Y-repeated sequences and their relative chomosomal and male specificity

The Y-repeated sequence which has been used for PCR amplification in human preimplantation diagnosis (Handyside et al., 1989) is a 149 base pair of a 3.4kb repeated sequence (DYZ$_1$ family) on the Y chromosome (Kogan et al., 1987; Nakahori et al., 1986). Given the preimplantation diagnosis misdiagnosis utilising this sequence, it is necessary to analyse the Y-repeated sequence with respect to its chromosomal and male specificity.

More than 50% of the human Y chromosome is composed of a variety of repeated sequences which include:

1.   The alphoid repeats and the major human SINE (Alu repeat family) (Schmid and Jelinek, 1982). The alphoid sequences (about 170 bp) are tandemly clustered near the centrometre on the Y chromosome and can be distinguished from those on other chromosomes by both the nature of the sequence and repeat organization. The majority of Y-chromosome Alu repeats have little homology with genomic consensus Alu sequences (Smith et al., 1987).

2.   The Y-chromosome LINE repeats (Adams et al., 1980), also designated the Kpn family (Shafit-Zagardo et al., 1982) or L1 family (Voliva et al., 1983), cannot be distinguished from LINEs found on other chromosomes (Smith et al., 1987).

3.   The 3.4kb Y repeats includes about 39 families including the DYZ_1 family (Handyside et al., 1990). The 3.4kb Y repeats contain at least three distinct sequences with autosomal homologies interspersed in various ways with a collection of several different Y-specific repeat sequences (Burk et al., 1985; Smith et al., 1987). Individual recombinant clones derived from isolated 3.4kb Hae III Y fragments have been shown to contain some cross-reaction in both men and women (Cooke, 1976).

4.   The 2.1kb Hae III Y fragment cross-reacts with a 1.9kb Hae II autosomal fragment (Smith et al., 1987).

5.   The repeated sequences on the short arm of the Y chromosome, are present in XX males and in reduced copy number in an XY female (Muller et al., 1986).

To identify the absolute chromosomal specificity in the Y-repeated sequences is rather difficult. The absence of an homologous band in the restriction pattern is not sufficient to rule out the possibility that female DNA contains a similar sequence, since, the detection method of restriction analysis would not detect less than 1,000 copies which might be present (Cooke, 1976).

Additionally, what is known about the evolution of repeated sequences suggest that the sequence could be represented on the X chromosome (Cooke, 1976). It has been suggested that the Y chromosome has acquired genetic material from both X chromosome and autosomes at various times during primate evolution (Koenig et al., 1985) and certainly X-Y crossing over has been recognized in the chimpanzee (Weber et al., 1988). In the human, a probe from q13-q22 region of the X chromosome can recognize a 98% homologous sequence on the Y chromosome (Koenig et al., 1985) and the human 'Y-specific' probe pDP35 is highly homologous to the human X chromosome (Page et al., 1987). Definitive evidence for the short arm of the Y chromosome associating with the X chromosome during meiosis in the human male has also been reported (Pearson and Bobrow, 1970). Twenty-six

human Y-chromosome-derived DNA sequences, free of repetitive material, were used to probe male and female genomic blots. Extensive sequence-homologies between Y and other human chromosomes were found, and these data were consistent with the hypothesis of a common evolutionary origin for the mammalian sex chromosomes and revealed structural similarities between Y-located and autosomal non-repetitive sequences (Bishop et al., 1984). The above reports suggest that the Y-repeated sequences only reflect relative rather than absolute Y chromosome specificity.

A series of twelve XX males and their relatives have been examined by southern blot analysis with fourteen different Y recombinants. The pattern of Y sequences present showed considerable variation between XX males. Moreover, the so-called heterogeneous 3.4kb Y-repeated sequences did not show any results from these XX males (Affara et al., 1986). Such results indicate that the Y-repeated sequences are not closely related to male specificity.

It has become clear that the human misdiagnosis (Handyside et al., 1990, Winston et al., 1991) was indeed predictable. A number of issues were of concern. *No controls* (to confirm PCR amplification) were included thus the lack of amplification of the Y band would have been interpreted as a 'female' (Handyside et al., 1989; Strom et al., 1991a; Trounson, 1992; Grifo et al., 1992a). The repeat sequence used (149bp) appeared to be *amplified incorrectly (10%)* when *female blood* DNA was analysed (Lo et al., 1989) and when *blastomeres* were amplified (Handyside et al., 1989). Some women showed a positive signal when the 149bp fragment was amplified (Handyside et al., 1990; Nakagome et al., 1990). Of concern was the limited (60%)

amplification rate (Handyside et al., 1989) rather than 100% (Strom et al., 1991a) with some 'male' blastomeres showing false negative results (Kontogianni and Handyside, et al., 1991). Other groups have found the *sequence to be amplified (149bp) was unsuitable for sexing diagnosis in human* (reviewed by Nakagome et al., 1990 – the authors of DYZ_1 (pHY10) DNA sequence), with *some female bands being stronger than the male bands* and the existence of a number of errors (Strom et al., 1991b; Grifo et al., 1992b). The choice of the DYZ_1 family for sexing human embryo must be considered inappropriate.

Thus the start of human clinical preimplantation diagnosis was characterised by the use of an unreliable choice of target sequence and methodology far from optimal (Lo et al., 1989; Nakagome et al., 1990). If the PCR diagnostic method had been initially proven by basic experiments (Handyside et al., 1989), the predictably high percentage of fetal misdiagnosis would have been avoidable (Winston et al., 1991). Many authors emphasised this risk prior to (Jarmulowicz, 1989; Nakagome et al., 1990) and after the report of the human birth (Strom et al., 1991a; Pickering et al., 1992; Trounson, 1992; Simpson and Carson, 1992).

1.6.2 The detection of mutant gene for cystic fibrosis

Cystic fibrosis was first described in 1905 (Landsteiner, 1905; Fanconi et al., 1936; Anderson, 1938) and its autosomal recessive nature clarified later (Carter, 1952). Except for the X-linked diseases, cystic fibrosis is the most common genetic disease in the white population, affecting about 1 in 2500 births, with the frequency of the carriers ranging from 1 in 20 to 1 in 25 (Boat et al., 1989). The disorder is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7q31 (Riordan, et al., 1989; Rommens et al., 1989; Kerem et al., 1989). To date more than 200 mutations have been described, but the most common mutation, occurring in 70% of CF genes from Northern Europe and Northern America, is the deletion of amino acid phenylalanine at position 508 in the first nucleotide binding fold (ΔF508) (Harris, 1992). It is possible to diagnose reliably the ΔF508 mutation in blood DNA with the southern blot method (Kerem et al., 1989).

The genetic analysis of DNA from single human oocytes for preimplantation diagnosis of cystic fibrosis had been reported with primers identifying the locus CS.7, which maps only close to, rather than directly at the mutation responsible for cystic fibrosis (Coutelle et al., 1989). The reliability of this indirect method of diagnosis requires further confirmation. Both direct electrophoresis of the 95/98 bp bands and heteroduplex formation have shown only an 84% amplification rate with unclear bands in single human polar bodies and blastomeres (Strom et al., 1990; Verlinsky et al., 1992). Handyside et al (1992) showed a 75% amplification rate using the method of heteroduplex formation. The basic science underlying the formation of heteroduplex to diagnose the point mutation is not understood (Myers and Maniatis, 1986).

Only 50-70% of all mutations could be detected with this method (Myers and Maniatis, 1986), and the fidelity of the method was markedly influenced by the poor separation of the normal and mutant homoduplexes (Rommens et al., 1990; Kerem et al., 1990). Thus misdiagnosis with this method when used for human preimplantation diagnosis might be predicted.

Furthermore it is of continuing concern that a further report, detailing the birth of an unaffected child following preimplantation diagnosis for cystic fibrosis, has relied on a 75%-93% single cell amplification rate using the heteroduplex method (Handyside et al., 1992). It appears likely that more episodes of misdiagnosis from this suspect method of preimplantation diagnosis will follow.

1.7   Aims of Thesis

Preimplantation diagnosis is a new branch of prenatal diagnosis. At present, over 5,000 different gene defects are known, most of them are diagnosable (Brock, 1990). In U.S.A., the Human Genome Project has begun. The objectives are to create high-resolution genetic and physical maps. The results will localize the estimated 100,000-200,000 human genes, and acquire a better understanding of the relationship between genome structure and function (Rose, 1991). At the same time, the DNA basis of more genetic diseases will become known.

At present preimplantation diagnosis is in the earliest stages. Although some babies have been born from preimplantation diagnosis, reliable methods to precisely diagnose the preimplantation embryos have not yet been established and the safety of embryo biopsy still needs to be confirmed.

The aims of the thesis are to develop reliable procedures to (1) determine the presence of individual genes within single human cells including the male gamete and individual blastomeres of the human embryo in order to confidently identify sex and (2) identify the 3 point genetic mutation characteristic of cystic fibrosis within a single biopsied cell of the early embryo.

CHAPTER TWO

EXPERIMENTS TO ADDRESS THE SAFETY OF EMBRYO BIOPSY AND TO DETERMINE A MARKER OF EMBRYO HEALTH FOLLOWING BIOPSY OF THE MOUSE EMBRYO

2.1   Introduction

Given the limited numbers of human embryos available, it is important that the technique of single blastomere biopsy be performed successfully without injury to the embryo.   Since the procedure itself is technically demanding, an accurate method of monitoring the safety of the procedure would be valuable.   The rates of blastocyst formation, implantation and fetal development (Wilton and Trounson, 1989; Krzyminska et al., 1990) have all been proposed as useful indicators of the safety of blastomere biopsy, the results of the following experiments suggest that the blastocyst hatching rate is a particularly sensitive indicator.

2.2   Materials and Methods

Oocyte collection, in vitro fertilization and culture

Female (CBA X C57) F1 mice (6 weeks old) were injected (i.p.) with 10 IU PMSG (pregnant mare's serum gonadotrophin, Folligon, Intervet Pty. Ltd., Sydney, N.S.W. Australia) for superovulation followed by 10 IU hCG (human chorionic gonadotrophin, Profasi, Laboratories Serono, S.A. Aubonne, Switzerland) 51h later.   Females were killed about 14h after hCG administration and oocytes were collected from the oviducts into hyaluronidase medium (295u/ml) for a 5 minute treatment.   After washing twice in the alpha-modification of Minimum Essential Medium, (MEM, Sigma Chemical Co. St.

Louis, Mo, U.S.A.) containing 5mg/ml BSA (bovine serum albumin), oocytes were transferred to MEM and fertilized using the capacitated epididymal sperms. After insemination, oocytes were transferred to culture medium (MEM) for further development.

Micromanipulation

Embryos at the late 4-cell and early 8-cell stage after culture were incubated in Ca++ and Mg++ – free Human Tubal Fluid Medium (HTFM) (Quinn et al., 1985) supplemented with 3mg BSA/ml for 1-3 hours before micromanipulation. All micromanipulations were performed with an inverted microscope (Nikon, Diaphot). Embryos were transferred to Ca++ and Mg++ – free Hepes HTFM containing 3mg BSA/ml in 36mm Petri dish. Two kinds of micropipette were used: (1) holding pipette, with an outer and inner diameters of approximately 100um and 40um respectively, flame polished to a flat blunt opening; (2) biopsy pipette, bevelled to 40-45º with an outer diameter of approximately 20um. The embryos were biopsied by puncturing the zona pellucida directly and a single blastomere was aspirated and expelled into the medium (Fig. 1A and B).

Control embryos without micromanipulation were also pre-incubated in Ca++ and Mg++ – free MEM and placed in Ca++ and Mg++ – free Hepes HTFM similar to biopsied embryos.

Fig. 1 A. Single blastomere biopsy from an 8 cell mouse embryo. The biopsied blastomere in the needle is intact. **B.** Embryos and single cells in the medium immediately after single blastomere biopsy. All single cells had intact membranes. No cell lysis was apparent in the majority of the biopsied embryos.

A
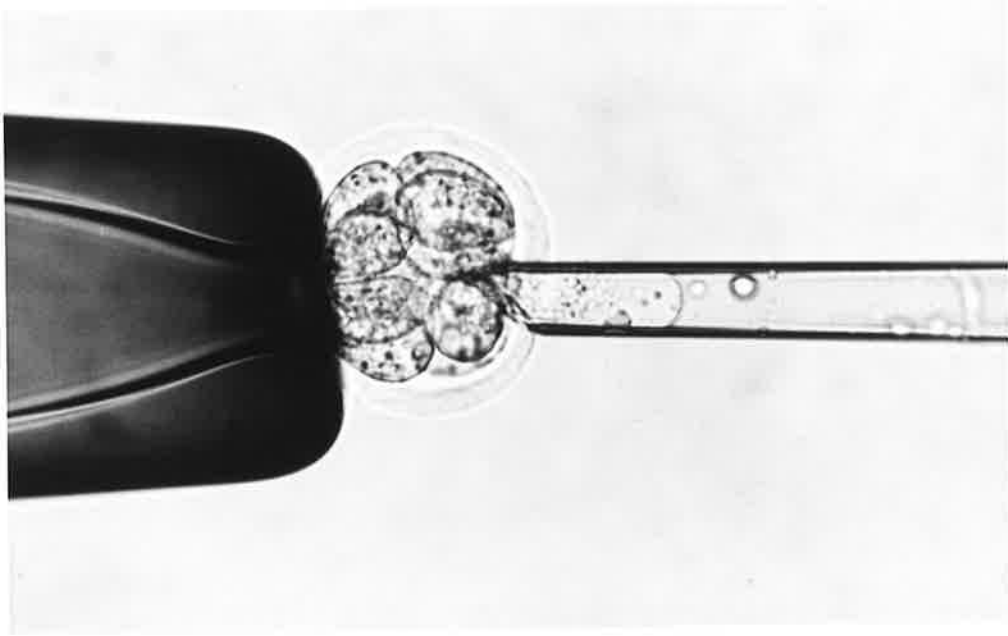
B
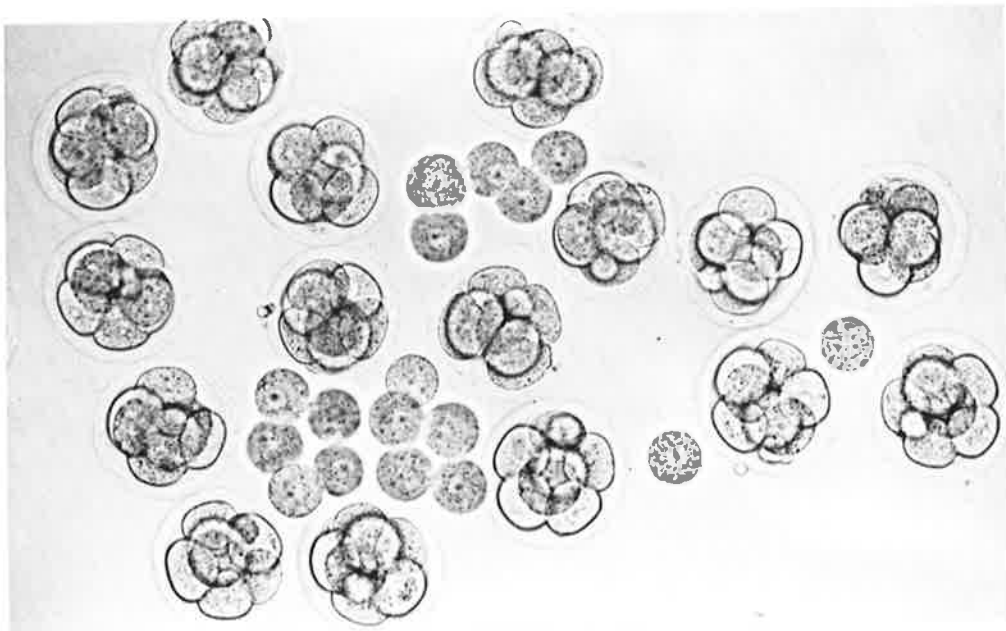
Post-biopsy embryo culture, assessment and embryo transfer

After biopsy, embryos were washed twice and cultured in MEM (BSA 5mg/ml) to the hatching stage. Two experiments were undertaken:

Experiment 1.

The development in vitro of the biopsied and control embryos (4–8 cell stage) was investigated, and the rate of blastocyst formation (42h after embryo biopsy) and the hatching rate (54h after embryo biopsy) were compared (Fig. 2A and B).

Experiment 2.

The implantation rate and the fetal viability rate were investigated. Two days after embryo biopsy (at the 8 cell stage), the biopsied and control embryos at the hatching stage were transferred to the uteri of day 3 pseudopregnant recipient (BALB X C57) mice. Four biopsied embryos were transferred into the right uterus, and 4 control embryos into the left uterus of each recipient. On day 14 after embryo biopsy, the implantation rate and fetus rate were detailed at autopsy.

Statistical analyses

Data were analysed by student T-test and Chi-square test.

2.3 Results

Considerable practice was required to perfect the biopsy of a single blastomere to avoid embryo damage which included aspirating more

Fig. 2.   **A.** The hatching biopsied embryos. **B.** One hatched embryo and another mostly hatched embryo after embryo biopsy.
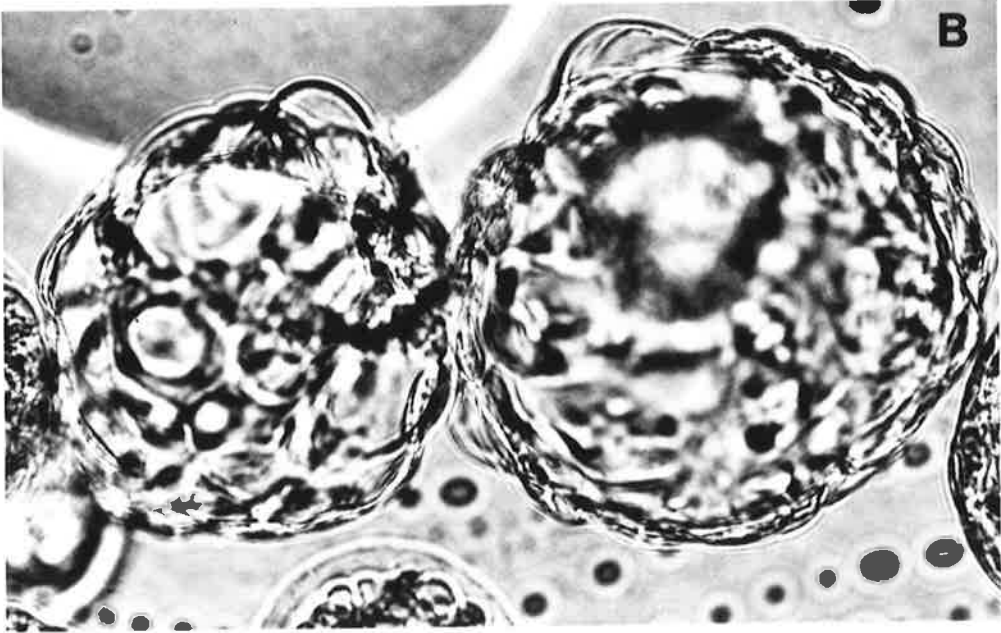
than one cell, breakage of the aspirated cell or lysis of the remaining cells of the embryo. With experience a single intact blastomere could be smoothly aspirated from almost all embryos except in a few instances when the membrane of the aspirated cell was damaged. Biopsied embryos were cultured to hatching stage without any microscopic differences when compared with control embryos except for the biopsy site on the zona pellucida being visible in some embryos.

Experiment 1 consisted of 13 series of single blastomere biopsy. Each series contained about 20 embryos in each of the biopsied and control groups. Although the rate of blastocyst formation of biopsied embryos was slightly reduced to about 80% in first two series (Fig. 3), the rate of blastocyst formation of both biopsied and control groups in the 3-13 series remained over 90% (Fig. 3). The rate of hatching of biopsied embryos gradually increased in the 1-8 series of biopsy, from 60% to 85% (Fig. 4). The hatching rate of control embryos was always in excess of 90% even from the initial series. The 13 series were divided into 3 groups (1-4, 5-8 and 9-13 series) for statistical analysis (Table 2). The rate of blastocyst formation in all three groups showed no significant differences between the biopsied and the control groups. However, the hatching rate of biopsied embryos in the 1-4 series was lower than control embryos (P<0.001) with a less significant difference (P<0.01) being apparent in the second group (5-8 series) and differences being eliminated in the third group (9-13 series) (Table 2).

In Experiment 2, there were 8 series of single blastomere biopsy and embryo transfer. A total of 100 hatching biopsied embryos and 100 hatching control embryos were transferred to 25 recipient mice. The implantation rate of the biopsied embryos (96%) was not significantly different from that of the control embryos (92%) (Table 3). The rate of fetal

Fig. 3   The development of blastocysts after embryo biopsy compared with non–biopsied controls. Each series consisted of about 20 embryos in the biopsied and control groups. Note the reduced rate of blastocyst formation following biopsy in series 1 and 2 otherwise blastocyst formation was >90% in all series.

Fig. 4.   The rate of hatching after embryo biopsy compared with non–biopsied controls. Note the gradual improvement in the hatching rate of biopsied embryos.
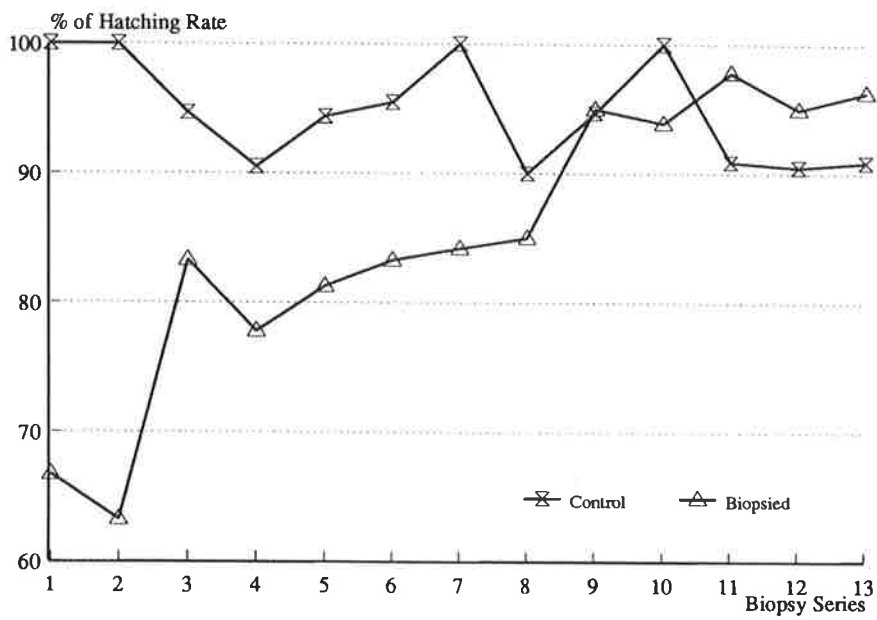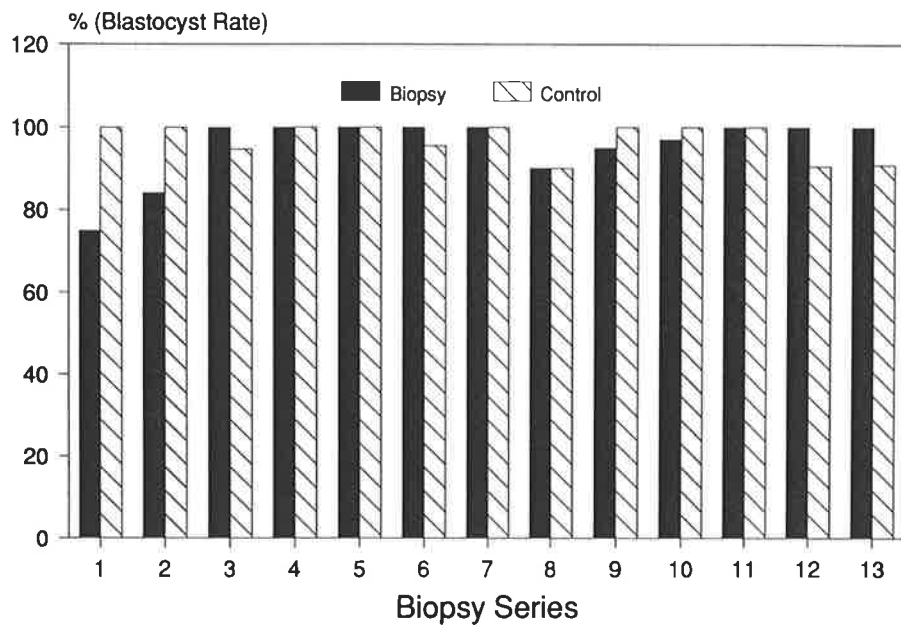
Table 2   **Blastocyst formation and hatching rate following embryo biopsy**

Biopsy Series		Blastocyst Formation (%)	Hatching (%)
1–4 series	Control	64/65 (98.5)	62/65 (95.4)[a]
	Biopsied	61/67 (91.0)	49/67 (73.1)[a.]
5–8 series	Control	77/80 (96.3)	76/80 (95.0)[b]
	Biopsied	71/73 (97.3)	61/73 (83.6)[b.]
9–13 series	Control	129/133 (97.0)	125/133 (94.0)[c]
	Biopsied	143/145 (98.6)	139/145 (95.9)[c]

a: P<0.001;   b: P<0.01;   c:n.s.

Table 3   **The implantation rate and fetus rate on day 14 of pregnancy**

	No. of embryos transfer	Implantation (%)	Reabsorption (%)	Fetus (%)
Biopsied	100	96(96)	26(26)	70(70)
Control	100	92(92)	24(24)	68(68)

development from the biopsied embryos (70%) was also not significantly different from that of the control embryos (68%) (Table 3).

2.4   Discussion

The safety of embryo biopsy is an important issue and the use of mouse embryos to perfect the technique of embryo biopsy for human application is widely utilised.   Clearly, optimal operator skill is required for single blastomere biopsy in the human given the limited number of embryos available from in vitro fertilization procedures and a good training model with the ability to discriminate harmful effects is an important requisite.

Stable embryo quality is a basic requirement for the evaluation of the single blastomere biopsy technique.   Embryo loss can vary markedly between experiments apparently unrelated to the culture regimens and may reflect inherent embryo quality and/or operator skill on the day of biopsy (Roberts et al., 1990), the latter being particularly variable with new operators (Wilton and Trounson, 1989; Roberts et al., 1990).   Although embryos from in vivo fertilization have been shown to cleave and develop better than those derived from in vitro fertilization (Bowman and McLaren, 1970; Streffer et al., 1980), embryos from in vitro fertilization was the basic source of embryos for the study.

Several workers have reported the rate of blastocyst formation as the observed end point of in vitro embryo development after single blastomere biopsy (Krzyminska et al., 1990; Wilton et al., 1989; Takeuchi et al., 1992).   In this study, the rate of blastocyst formation was not significantly different between the biopsied group and the control group, even in the early biopsy series but significant differences were evident in the hatching rate and thus the

hatching rate was considered the more sensitive discriminator. Should the less sensitive rate of blastocyst formation be found to be significantly different between the biopsied and control groups at 4 to 8 cell stage, clearly more training is required to improve operator skills.

Following biopsy, embryos tended to delay development for several hours compared to the control group. While this delay did not appear to influence the eventual potential of the biopsied embryos, a small time delay remained by the time of intended implantation between the biopsied and control embryos, and therefore between embryos and the recipients. It was therefore considered inappropriate to transfer asynchronous embryos to evaluate the rate of implantation and fetal development (Wilton and Trounson, 1989). The delay had been eliminated by the very earliest stage of hatching (42 hours after embryo biopsy) and therefore all embryos were transferred to the recipient uteri at the hatching stage. No significant differences between groups were evident in the rate of implantation or fetal development. The current results and that of Wilton et al. 1989 confirm that the implantation rate and fetus rate are not necessarily the most sensitive indicators of the biopsy technique. Additionally the determination of the implantation rate and fetus rate is time consuming, costly and may itself be subject to inherent variation. Should for example, the hatching blastocoel cavity be ruptured by the transfer pipette during embryo transfer, implantation and fetal development could be expected to be reduced, which may explain the reduced rates found following trophectoderm biopsy (Monk et al., 1988; Gomez et al., 1990).

One possibility to be considered is that the failure of hatching per se prevented optimal embryo development. No objective evidence for or against this view is offered as control embryos were not micromanipulated but microscopic observations suggested that hatching often took place away from

to the site of the zona puncture and it seemed likely therefore that failure of hatching reflected basic embryo health rather than adverse zona changes due to perhaps in vitro culture conditions.

In summary caution is advisable in accepting blastocyst formation as a reliable end point for the assessment of micromanipulative procedures. The rate of hatching appeared to be a simple, sensitive and reliable method to reflect operator skills following blastomere biopsy and the avoidance of embryo transfer further simplified the testing procedure.

CHAPTER THREE

SEX SELECTED BIRTHS OF MICE FOLLOWING SINGLE CELL EMBRYO BIOPSY AND Y-LINKED TESTIS SPECIFIC GENE ANALYSIS

3.1 Introduction

Reliable and safe procedures for the precise diagnosis of a single gene by PCR using a single cell obtained at the preimplantation stage have yet to be demonstrated (see Chapter 1). In this study, the precise amplification and analysis of DNA sequences from two individual genes present in a single cell from the 4–8 cell mouse embryo is determined. Mouse testis and ovary specific genes have been amplified by PCR to correctly predict the sex of all 39 pups born following embryo biopsy. Some indices of the safety of embryo biopsy including histopathological and blood examinations of the pups have been analysed.

3.2 Materials and Methods

Selection of gene primers

The testis specific gene selected is present on the mouse Y chromosome encoding pYMT2/B which is about 1.3kb in length. Its RNA transcripts are only present in testis mRNA and cannot be detected in the mRNA from liver, spleen, kidney, brain, heart or lung tissues of male mice. pYMT2/B is not located within the critically important 'Sxr' region of the Y chromosome (Bishop and Hatat, 1987) and was detected by a Y specific probe (pY353/B), which has been used to test several different male mice strains

(Bishop et al., 1985). The testis specific gene primers used in this experiment were located at mid–sequence of pYMT2/B.

The ovary specific gene used as the control gene encodes a 83,000–85,000 Da glycoprotein (1317 nucleotide–long mRNA) of the murine oocyte zona pellucida (ZP3), which has sperm–binding activity via its 0–linked oligosaccharide side chains (Ringuette et al., 1988). ZP3 is located on chromosome 5 (Lunsford et al., 1990), and its RNA transcripts are only present in ovarian mRNA and are undetectable in liver mRNA (Ringuette et al., 1988). Sequences homologous to ZP3 are conserved among mammals, such as the rat and rabbit. The control gene primers used in this experiment were located at the beginning of 5' region of ZP3.

Blood Testing of selected primers

Mouse blood was used to test the correctness of the testis and ovary specific gene primers. Twenty male and 21 female adult mice (CBA/C57) were decapitated and heart blood was carefully individually collected into separate heparin tubes. DNA from the whole blood was extracted and prepared for PCR amplification (Kawasaki, 1990) prior to storage. All PCR mixtures were freshly combined. Reagents were transferred to 0.5ml PCR specific Eppendorf tubes in order, which included 28ul sterile distilled water, 5ul of 10X PCR buffer (500mM KCl, 100mM Tris–HCl, pH8.3), 1ul of each dNTP (10mM), 1ul of each testis specific gene primers (5'–TTTCTCACAGTTGGAAGGAAGGTA–3'; 3'–CCTCGAGATGTCACTACTGTC CTA–5'), 1ul of each ovary specific gene primers (5'– TGTGGCTTTTGCCG GGTGGAACT–3'; 3'–CTATGGCTGCACCAGTCCAAGTTG–5'), 0.25ul of Ampli Taq DNA polymerase (5u/ul), 4ul of 25mM $MgCl_2$ and 5ul (1.5ug) of individual mouse blood DNA template in a final volume of 50ul. Before overlaying with 50ul of mineral oil, the mixture was gently vibrated. DNA was

denatured for 4 min at 94^0C, followed by 30 cycles 94^0C (1 min), 65^0C (1 min) and 72^0C (2 min), and a final extension at 72^0C (10 min) with Corbett FTS-1 fast thermal sequencer. Amplification DNA (20ul) and the marker was run on a 2% agarose gel stained with ethidium bromide, and examined on an ultraviolet transilluminator with photography.

Embryo, embryo biopsy and sexing embryo

Oocytes were collected from superovulated female (CBA X C57, F_1) mice (6 weeks old) and fertilized using capacitated epididymal spermatozoa. After 5 hours of insemination, embryos were washed and transferred into MEM (Minimum Essential Medium–Alpha modification) for culture in microdrops under paraffin oil in a 5% CO_2 incubator at 37^0C.

Eight cell stage (day 3), embryos were biopsied. Before embryo biopsy, all the embryos were incubated in Ca^{++} – and Mg^{++} – free HTFM (Human tubal fluid medium) (Quinn et al., 1985) for 1–3 hours. Embryos were then transferred to Ca^{++} – and Mg^{++} – free Hepes HTFM for micromanipulation using an inverted microscope (Nikon) with two micromanipulators. Two types of micropipette were used: (1) holding pipette, approximately 100um O.D. and 40um I.D., and (2) biopsy pipette, with approximately 20um O.D. The embryos were secured by the holding pipette, prior to the biopsy pipette puncturing the zona pellucida. One single embryo cell was carefully aspirated out using the biopsy pipette (Fig. 5).

Fig. 5   Single blastomere biopsy from 8 cell mouse embryo. One single blastomere was aspirated and expelled into the medium.

Fig. 6   Amplification of fragments of testis specific gene and ovary specific gene from blood derived from mice of known sex by PCR. Twenty male and 21 female adult mice were tested, but only 10 male (m) and 10 female (f) results are shown. The fragments from the testis specific gene were 151 base pairs amplified by testis specific oligonucleotide primers. The control fragments from the ovary specific gene were 203 base pairs, amplified by ovary specific oligonucleotide primers. All male bloods showed two bands while all female bloods showed one band. M was marker pUC 19. B was the blank.

Fig. 7   PCR amplification of fragments of testis specific gene and ovary specific gene derived from single cells after embryo biopsy. In total, 164 single embryo cells were analysed with ten shown above (1-10). The fragments from testis specific gene were 151 base pairs amplified by testis specific oligonucleotide primers. The fragments from ovary specific gene were 203 base pairs as the control, amplified by ovary specific oligonucleotide primers. The results from embryo 1, 3, 6, 7 and 10 demonstrate both testis specific and ovary specific gene positive products, and they were considered 'male' embryos. The results from embryos 2, 4, 5, 8 and 9 showed testis specific gene negative and ovary specific gene positive products, and they were designated 'female' embryos. Blood controls are also shown. Lane 11 was derived from male mouse blood DNA and lane 12 from female mouse blood DNA. Lane 13 was the blank. M. was marker pUC19.
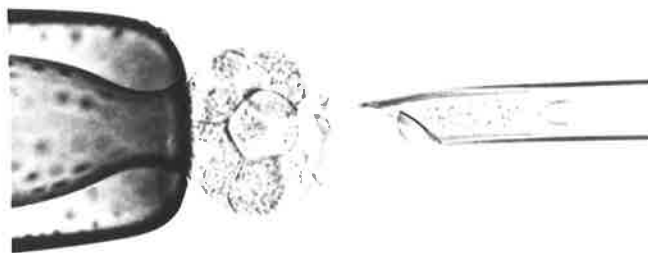
M m f m f m f m f m f m f m f m f m f m f m f m f B

203
151

Embryo   Blood

M   1   2   3   4   5   6   7   8   9 10 11 12 13

203
151
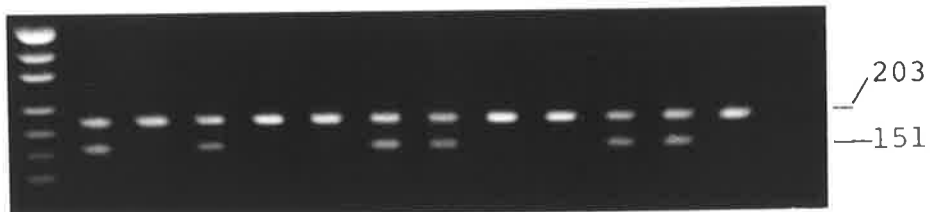
All aspirated single embryo cells and nuclei were separately transferred to a PCR specific tube which contained 10ul PCR buffer (10mM Tris–Cl, 50mM KCl, 2.5mM MgCl2, pH8.0), using a fine hand drawn Pasteur glass pipette. PCR tubes were placed in the 94^0C heating block for 30 minutes in order to denature DNA. Freshly made PCR mixture (50ul), containing 10xPCR buffer, dNTP, MgCl2, Taq polymerase, testis specific gene and ovary specific gene primers, was transferred to the heat treated PCR tubes containing the biopsied embryo cells. The DNA was denatured for 4 min at 94^0C, followed by 30 cycles (as per blood testing) for PCR amplification accompanied by the mouse blood DNA as control. Another batch of PCR tubes were prepared with 50ul PCR mixture containing the nested testis specific gene primers (5'–TTTCTCACAGTTGGAAGGAAGGTA–3'; 3'–CCTCGAGATGTCACTACTGTCCTA–5') and ovary specific gene primers (5'–TGTGGCTTTTGCCGGGTGGAACT–3'; 3'–CTATGGCTGCACC AGTCCAAGTTG–5'), and placed in 1ul first PCR amplication product, (the control blood DNA production in 1:100 dilution first), for reamplification with 30 cycles. Agarose gel electrophoresis was performed to determine the sex of the biopsied embryos. The time taken from biopsy to obtaining an embryo sex result was about 8 hours.

All the embryos following single cell embryo biopsy were separately transferred to individually labelled MEM culture droplets under paraffin oil for continuing culture. When the biopsied embryos began to hatch, all the 'male' embryos determined by PCR amplification were collected into one MEM culture medium disc, and all the 'female' embryos into another disc. A total of 43 'male' embryos and 32 'female' embryos were transferred to day 3 pseudopregnant recipient (BALB X C57) mice using separate recipients for each sex (Table 4).

To determine the effect of the biopsy procedures on subsequent embryo development, control embryos originating from the same batch as the biopsied embryos were treated and cultured similarly to the biopsied embryos but without being subjected to embryo biopsy and gene analysis. Eighty such control embryos were similarly transferred to day 3 pseudopregnant recipient mice housed separately from the sex selected embryo recipients.

General studies after birth

Nineteen or twenty days postcoitum, all recipient mice separately delivered offspring developed from the 'male', 'female' and control embryos. Sex identification was carried on the day of birth and again at 6–10 weeks of age using phenotypic criteria.

The mice pups from the biopsied and control embryos were examined for macroscopic abnormality and the birthweights recorded.

Three pairs of adult mice from biopsied and control embryos were examined by x–ray. Reproductive competence was examined by mating 6 pairs of adult male and female mice derived from the sex selected embryos.

Histopathological studies after birth

Six adult mice (aged 10 weeks male and female) – three in biopsy and three in control group were sacrificed. After studying the macroscopic anatomy of the internal organs in both groups, the organs were immediately fixed in 10% formalin.

Fifteen major organs including the brain, heart, lungs, liver, kidneys, stomach, intestine, voluntary muscle, spleen, pancreas, adrenal, thymus, skin, testis (male) and ovary (female) from these mice had paraffin sections formed. Representative blocks from the organs were stained with heamotoxylin and eosin. Microphotographs at differing magnification were taken from both biopsied and control groups during the microscopic examination.

Haematological/Biochemical studies

Twenty eight and 32 adult mice (aged 20–30 weeks) derived from the biopsied and control embryos respectively were anaesthetized with 2% avertin (0.015–0.017ml/g body weight) by intraperitoneal injection before sacrifice. Blood was collected by heart puncture in EDTA tubes (for haematology) or heparin tubes (for biochemistry).

Blood samples from mice derived from the biopsied and control embryos were analysed for general haematological (n=54) and biochemical indices (n=60).

3.3   Results

Blood testing of selected primers

All 20 male blood DNA samples after PCR amplification were both testis specific gene and ovary specific gene positive, and all 21 female blood DNA samples were ovary specific gene positive but testis specific gene negative (Fig. 6). All backgrounds were clear without obvious 'primer–dimer' effects. These results indicated the selected testis specific gene and ovary

specific gene primers to be satisfactory to determine the sex of the biopsied embryos.

Embryo biopsy and sexing embryo

The procedure of embryo biopsy was generally smooth. Most single embryo cells after aspirating had one or two recognizable nuclei, but some appeared anuclear, (perhaps at the metaphase stage). In total, 165 embryos were biopsied, in which 143 (87%) single cells were aspirated in an intact condition, 17 cells had some cell membrane damage and 5 'cells' had a naked nucleus.

Single embryo cell material was placed in 159 PCR tubes (96.4%), and 2 to 3 embryo cells were placed in 6 tubes for experimental reasons. After PCR amplification, (except one PCR tube which accidentally dried during heating), all PCR tubes (164) which represented 164 embryos showed evidence of PCR amplification without any failure (Fig. 7). Embryos which contained the fragments amplified from both testis specific gene and ovary specific genes were designated as 'male', and all embryos which only contained the fragment amplified from the ovary specific gene were designated as 'female' embryos. Of the 164 biopsied embryos, 94 (57.3%) embryos were 'male' embryos and 70 (42.7%) 'female' embryos. The brightness of the bands from the fragments amplified from the two specific genes was not influenced by the number of embryo cells contained in the PCR tubes, nor influenced by whether the embryo cell membrane was intact or damaged, nor whether the embryo cell contained an obvious nuclear membrane or not.

General studies after birth

In total, 39 mice were born following sex prediction without any misdiagnosis. 'Male' embryos only produced the male pups, and the 'female' embryos only produced the female pups (Table 4 and Fig. 8).

No phenotypic abnormalities were evident in the 39 mice born following biopsy. The birthweights of the pups from the biopsied and the control embryos were not significantly different, nor were the birthweights between the male and female pups (Table 5). X-ray examination showed no differences in the skeletal structures and soft tissues between the biopsied and control animals (Fig. 9A). Adults derived from biopsied embryos showed normal reproductive capacity. Within one month, 4 pairs of mice delivered 35 healthy second generation pups (Fig. 9B). This pregnancy rate is similar to the general breeding data from our animal facility.

Histopathological studies after birth

No macroscopic abnormalities were noted between the organs examined from control and biopsied groups.

In the heart, three distinct layers (endocardium, myocardium and pericardium) were evident with the myocardium displaying interconnecting muscle fibres (Fig. 10.1A.B). The structure of the great vessels were similarly and distinctly identified. The lungs consisted of bronchi, bronchioles and alveolar ducts opening normally into the lobules of alveoli (Fig. 10.2A.B). The pleural membrane was also normal. No structural abnormalities were noted in the kidneys (Fig. 10.3A.B), urethras and urinary bladder. The liver was also

Table 4   Births of sex selected mice pups following embryo biopsy

	Number of embryos	Number of recipients	Number of male pups	Number of female pups
'Male' embryos	43	6	18	0
'Female' embryos	32	5	0	21
TOTAL	75	11	18	21

Table 5   Birthweights of pups from the biopsied and non-biopsied embryos

	No. of embryos transferred	No. of pups	Birthweight $(X \pm SD)$
Biopsied	75	39	1.63 ± 0.21*g
Male pups	43	18	1.63 ± 0.24**g
Female pups	32	21	1.63 ± 0.19**g
Control (Non-biopsied)	80	35	1.56 ± 0.16*g

* P= N.S.   ** P=N.S.

Fig 8.	**A** and **B** are the phenotypic male and female adult mice resulting from the sex selected 'male' and 'female' embryos following single cell embryo biopsy and testis specific gene analysis.

Fig. 9   **A**. No X–ray differences were found in the skeletal and soft tissues of the adult mice from biopsied group (L) and control group (R). **B**. The second generation (10 pups) with parents originally derived from the sex selected embryos.
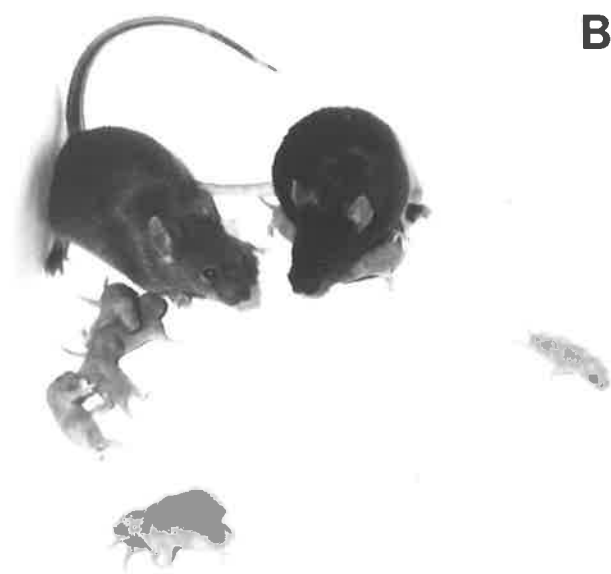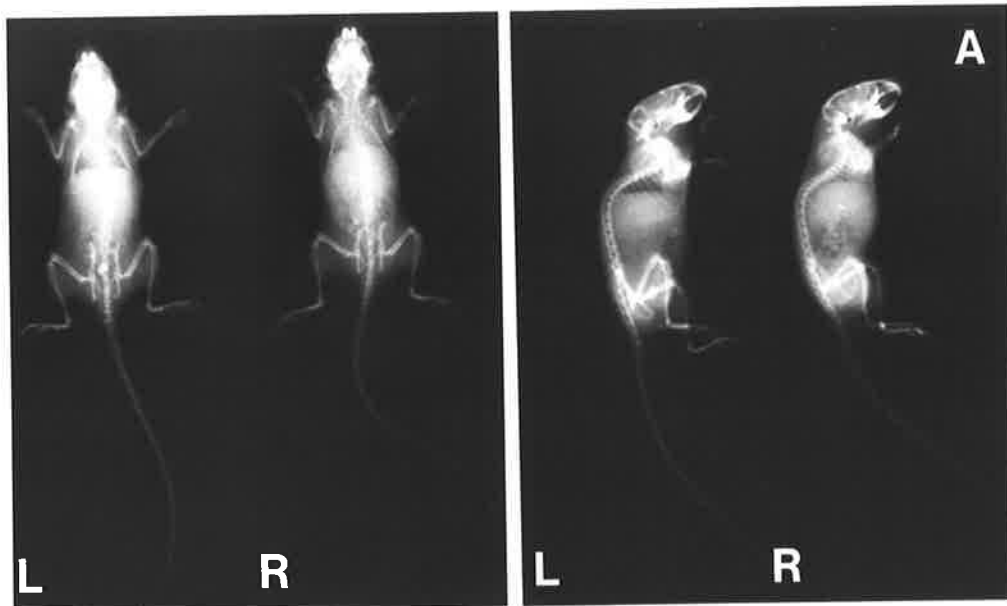
Fig. 10.   1.   A. (Control group) and B. (biopsied group) show normal layers of pericardium, endocardium and myocardium.

2.   A. (Control group) and B. (biopsied group) show normal terminal brochioles and alveoli of the lungs.

3.   A. (Control group) and B. (biopsied group) show normal tubules and glomeruli of the kidneys.

4.   A. (Control group) and B. (biopsied group) show normal liver lobule with hepatocytes, sinusoids and central veins.
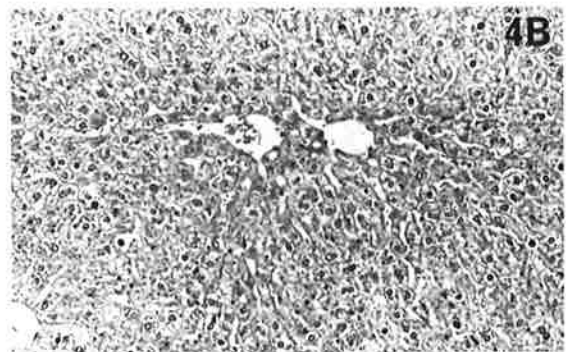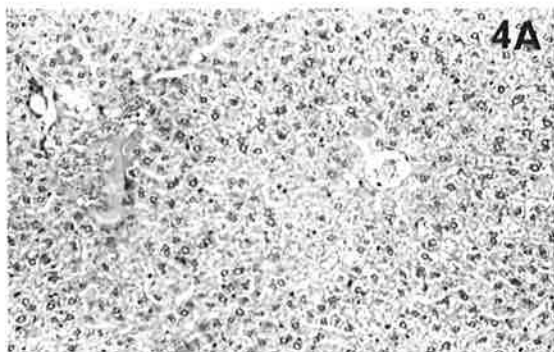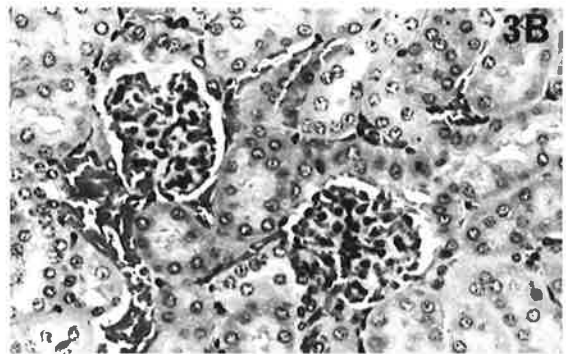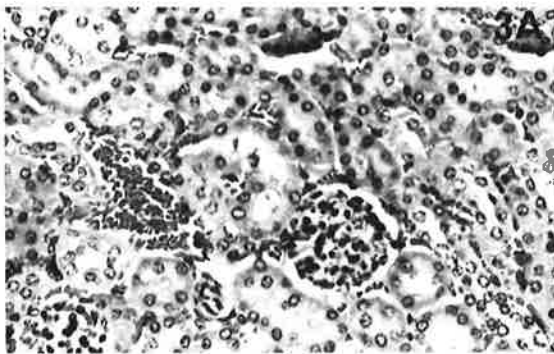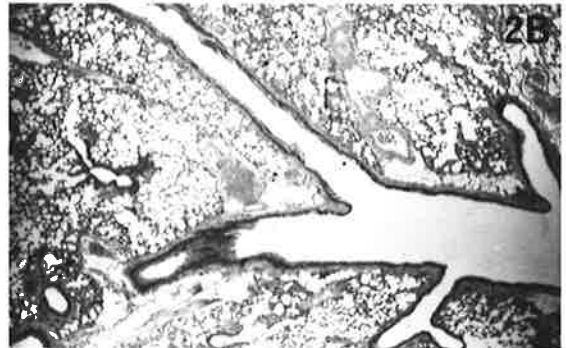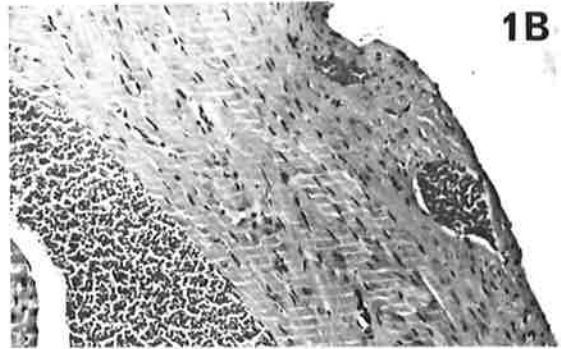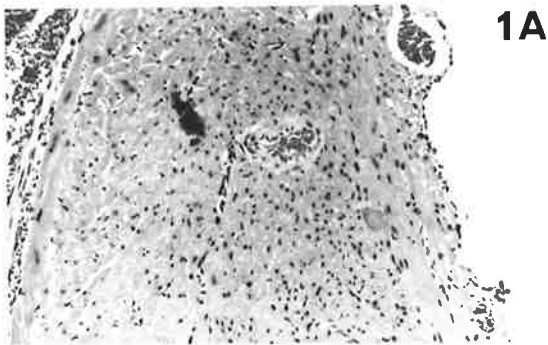
Fig. 11   The normal histological features of the testis (**A**) and ovary (**B**) from the sex selected adult male and female mice following single cell embryo biopsy and gene analysis.
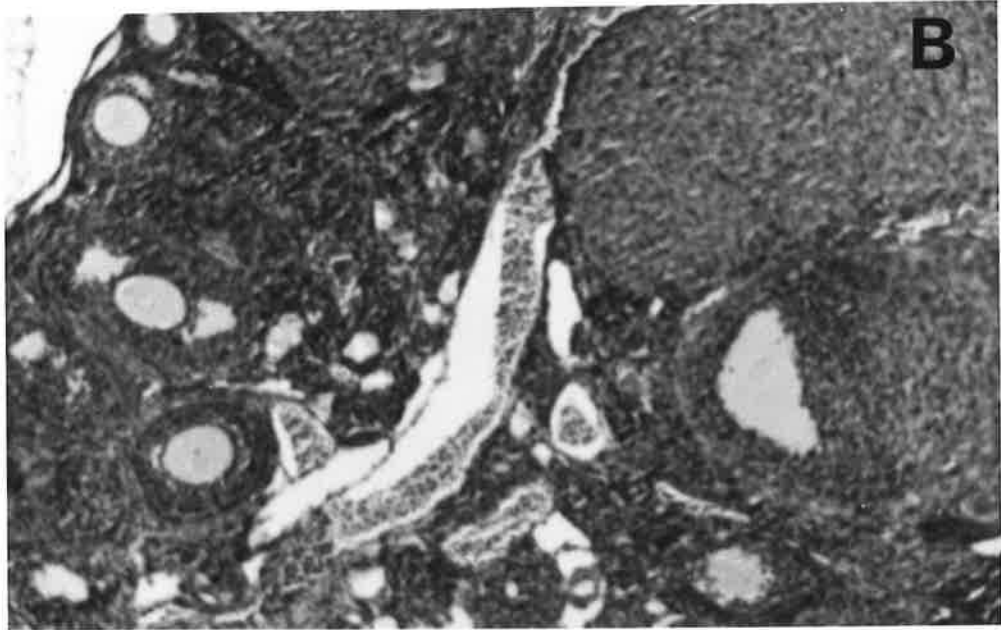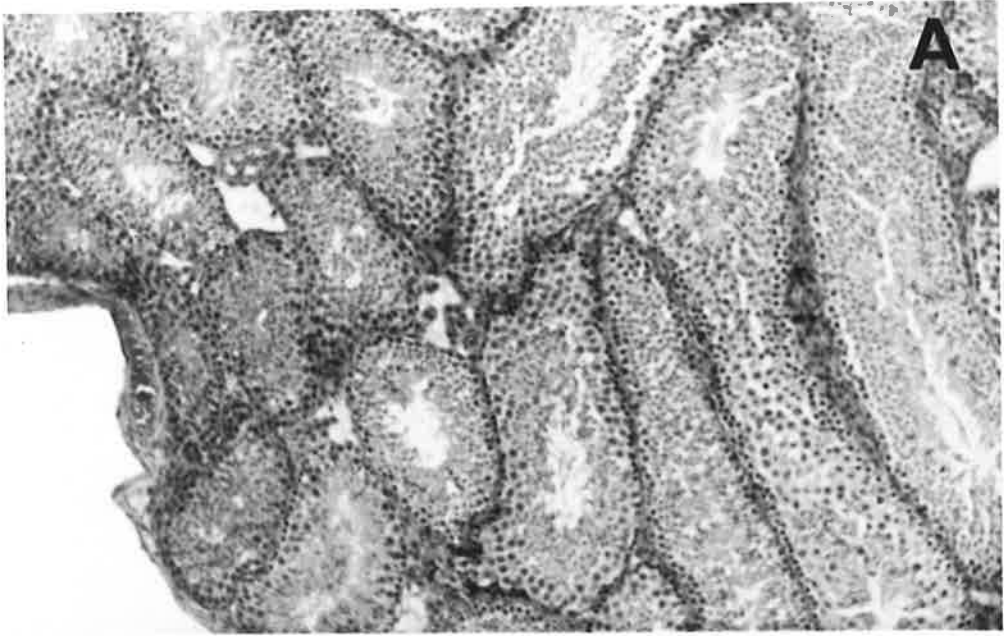
normal (Fig. 10.4A.B). The cerebral hemispheres showed a normal distinct layered composition of a rather narrow grey and wide white matters. The cerebellum also had three layers – molecular, Purkinje's cell layer and granula cell layer. The spinal cord was also normal. The lymphoid cells and Hassall's corpuscles were normal in the thymus. The layers of adrenals both cortex and medulla were normally arranged.

Both male and female reproductive organs were normal – the male testis showing normal seminiferous tubules with spermatogenesis and ovaries showing ova in different stages of development (Fig. 11A and B). In summary, no differences between animals derived from biopsied and non biopsied embryos were evident.

Haematological studies after birth

Haematological values of the mice derived from the biopsied and control embryo, which included white cell count, red cell count, haemoglobin level and platelet count, were not significantly different (Table 6).

Similarly biochemical values including plasma sodium, potassium, chloride, bicarbonate, calcium, glucose, albumin, protein, creatinine, urea, bilirubin, aspirate transaminase, creatine kinase and lactate dehydrogenase of the blood samples from the mice derived from the biopsied and control embryo groups were not significantly different (Table 7).

Table 6   **Blood Analysis of Mice derived from Biopsied (n=26)**

and Non–biopsied (n=28) Embryos

	Control (mean±S.D.)	Biopsied (Mean±S.D.)	P
White Cell Count ($X10^9$/L)	1.7±0.82	1.83±1.23	NS
Red Cell Count ($X10^{12}$/L)	8.70+0.37	8.82+0.36	NS
Haemoglobin (g/dL)	14.3±0.5	14.2±0.6	NS
Platelet Count ($X10^9$/L)	790.6±202.2	862.7±196.6	NS

NS: No significant difference

Table 7   Biochemistry Analysis of the Mice derived from the Biopsied (n=28) and Non–biopsied (n=32) Embryos

	Control (Mean±S.D.)	Biopsied (Mean±S.D.)	P
Na (mmol/L)	146.8±2.7	146.3±2.5	NS
K (mmol/L)	4.51±0.96	4.91±0.81	NS
Cl (mmol/L)	110.5±2.7	110.3±2.7	NS
HCO^3 (mmol/L)	16.6±3.6	16.5±3.6	NS
Glucose (mmol/L)	17.0±6.3	18.5±4.3	NS
Creatinine (μmol/L)	39.2±8.0	43.1±8.1	NS
Urea (mmol/L)	8.68±1.68	8.98±1.80	NS
Calcium (mmol/L)	2.28±0.09	2.34±0.12	NS
Albumin (g/L)	27.0±1.9	27.2±1.5	NS
Protein (g/L)	51.1±4.4	51.1±3.4	NS
Bilirubin (μmol/L)	7.96±6.66	7.67±4.74	NS
*AST (U/L)	129.0±171.0	145.9±163.7	NS
*CK (U/L)	508.0±626.6	567.0±691.4	NS
*LD (U/L)	467.7±431.5	759.8±661.4	NS

NS – No significant difference

*AST – aspartate transaminase

*CK – creatine kinase

*LD – lactate dehydrogenase

3.4   Discussion

The precision of diagnosis and the safety of embryo biopsy remain crucial scientific and ethical questions for the advancement of preimplantation diagnosis in the human (Trounson, 1992).

The precision of preimplantation diagnosis is dependent on knowledge of the known specific DNA sequences for specific hereditary diseases in question and the availability of optimal conditions for the amplification of the different specific DNA sequences. Since, many hereditary diseases in the human are known to be sex linked, the determination of sex is an important basic procedure for preimplantation diagnosis and several approaches have been used.

The most frequent method which has been employed is the use of Y specific repeated sequences. Several different kinds of mouse Y repeated sequences, such as YB10 (Eicher et al., 1989), AC11 (Nishioka and Lamothe, 1986), 145SC5 (Nishioka, 1988), M34 (Singh et al., 1987), pBC15-1.1 (Nallaseth and Dewey, 1986), are known to have variable specificity to the Y chromosome (Nallaseth and Dewey, 1986) and the selection of an highly specific sequence to the Y chromosome is thus critical to the precision of diagnosis. The human 3.4 kb Y-repeated sequence DYZ_1 which has been utilised for the initial reports of human preimplantation diagnosis (Handyside et al., 1989, 1990; Kogan et al., 1987) contains many (>484) TTCCA and other sequence nucleotide substitutions and is very similar to satellite III which originated from chromosome 1 and has been recovered from both male and female DNA (Nakahori et al., 1986). Thus within its 800–5000 'copies', non-specific copies would be expected to be many times more common than the

identical targeted sequence. The high frequency of non-specific templates and a large amount of non-specific products may have been responsible for the misdiagnosis of one of five human pregnancies examined and for the regular ladder of non-specific amplified bands (Handyside et al., 1990; Winston et al., 1991; Soussis et al., 1991). The more non-specific (non-identical) 'copies' of this kind which are present, the more variability and potential for misdiagnosis can be expected. Due to the high percentage rate of misdiagnosis (15%) in the reported human work, Trounson (1992) has proposed that the emphasis should remain 'on basic research rather than continue with clinical cases until amplification failure and any other problems have been solved'.

Measurement of the expression of a precise sex gene dosage has been demonstrated, for example hypoxanthine phosphoribosyl transferase: adenine phosphoribosyl transferase ratios have been shown to be different between the male and female mouse embryos (Monk and Handyside, 1988), however the practical application of this method for sex determination for the human would require additional validation and experience (Braude et al., 1989). One object of the current study was to determine whether the chosen testis specific gene on the Y chromosome and the ovary specific gene on chromosome 5 were present in single embryo cells, and thus avoid the need to determine gene expression, such as testis specific mRNA and/or gonadal proteins which are unlikely to be present at the early embryo stages. Thus Y-linked testis specific gene analysis rather than testis specific tissue analysis was deemed appropriate and optimal.

The use of a testis specific gene on the Y chromosome to sex embryos by PCR amplification would seem to be the optimal choice to provide the required accuracy of diagnosis for preimplantation diagnosis. The

inclusion of a second gene (control) for amplification which is common to both male and female embryos is also critical for confirmation of primer function during PCR amplification. The absence of control gene primers (Handyside et al., 1989, 1990) poses an additional risk for clinical work (Trounson, 1992).

The final proof of accuracy of the preimplantation diagnostic procedure was dependent on the correct prediction of births. The use of the mouse testis specific gene sequence (pYMT2/B) allowed the correct prediction of all births of both sex reported here, and confirmed the usefulness of the sequences selected. Interestingly the male:female embryo sex ratio was higher than 50:50, but similar to other reports (Lehtinen and Pelliniemi, 1984; Bradbury et al., 1990). It remains to be proven whether male sperms are more efficient at fertilizing the oocyte than female sperms or whether there are other explanations for this finding.

The present study confirmed the sensitivity of PCR to allow the precise detection of specific gene sequences in single embryo cells with sufficient amplification of the targeted DNA sequences to enable further molecular analysis. The PCR result was not influenced whether the embryo cell was intact or damaged, or whether a nuclear membrane was obviously present or not. Nor did the presence of more than one nucleus influence the result. During metaphase, the cell membrane remains intact but the nuclear membrane disappears, nevertheless, the genes on the condensing chromosomes appeared susceptible to PCR amplification. The '100%' PCR amplification rate indicated the genes selected were readily susceptible to the PCR procedure. These excellent results are not unique, as a similar success rate of single embryo cells has been reported previously (Kunieda et al., 1992) but are in some contrast to those derived from single sperm typing in which it was difficult to achieve perfect results (Li et al., 1990). It should be noted that

the DNA complement of sperm is highly condensed, full of protamines and haploid, and these aspects may explain the reported '90–95%' results. The genes in the single embryo cell and lymphocyte have been shown to be easily amplified by PCR, and their bands to be identical under UV light (Kunieda et al., 1992; Morsy et al., 1992) in some contrast to those results using a Y–repeated sequence (with a large number of non–specific copies) (Handyside et al., 1990). Thus whether the sequence selected for PCR amplification is a repeated sequence or not is not important to the issue of PCR sensitivity. However, our experiments utilised CBAxC57 mouse embryos, and may not be simply extended to other species of mice, sheep and human embryos which contain cell fragments in the early embryo stage, akin to viable embryo cells, but without amplifiable DNA (Handyside et al., 1990).

The safety of the embryo biopsy is a crucial technical and ethical question. Current results have shown that in the mouse the 8–cell embryo is the most suitable for single cell embryo biopsy (Wilton et al., 1989; Krzyminska et al., 1990). The rate of implantation and fetal development after embryo biopsy (at 8 cell stage) has been shown to be similar to the non–biopsied control embryos (Wilton et al., 1989; Chapter Two). Both total cell numbers and the numbers of trophectoderm and inner cell mass cells of biopsied embryos were not obviously different to controls on day 5 of gestation (Hardy et al., 1990), nor was there any effect of biopsy (at the 8–cell stage) on the fetal weight on day 17 of gestation (Krzyminska et al., 1990).

In the experiments described, no differences were evident in the birthweights. No phenotypic abnormalities were noted in the offspring, and the x–ray results of the adult mice from the biopsied and control animals showed no differences confirming the lack of the influence of embryo biopsy.

Histopathological analyses of 15 major organs from the mice derived from the biopsied and control embryos showed no significant differences.

The normal white cell count, red cell count, haemoglobin and platelet count showed the function of bone marrow and immunity system were not harmed by the single cell embryo biopsy (Ganong, 1985).

The constitution of the extracellular fluid is maintained by the normal kidney function. The normal levels of the electrolytes (sodium, potassium, chloride and bicarbonate) and of substances normally excreted by the kidney (urea and creatinine) is good evidence for the integrity of function (Zilva and Pannall, 1979; Ganong, 1985).

Two aspects of liver function were assessed, excretion of bilirubin and the synthesis of albumin. While minor degrees of hepatic impairment may not have been detected, the normal results exclude major liver dysfunction. Indirectly the albumin and protein levels are markers of adequate absorption of nutrients. Glucose levels reflect both this and a complex interplay of hormones (Zilva and Pannall, 1979); again no differences were noted. Finally, the levels of the enzymes measured. In general, elevated plasma enzyme activities are the most sensitive markers of tissue damage. Of those measured, aspirate transaminase and lactate dehydrogenase are derived from liver and muscle and creatine kinase from muscle only. The wide range of values found may reflect trauma during blood collection, but there were no differences between the biopsied and control populations.

The demonstration of a healthy second generation from the parent mice derived from biopsied embryos is important. Since reproductive

function is normal following embryo biopsy, there is the potential to eliminate some hereditary diseases from some families following embryo biopsy and specific gene analysis.

Given the precision of diagnosis achieved and the proven safety of single cell embryo biopsy of this study and other reports (Kunieda et al., 1992), the removal of more than one cell as a routine for human preimplantation diagnosis (Handyside et al., 1990) may not be necessary.

These basic experiments indicate that blastomere diagnosis in mouse is a useful model for the human. Moreover, the demonstration that one or more individual genes can be identified with confidence allows the extension of these techniques to the conditions where known gene mutations may be present in the embryo.

CHAPTER FOUR

DETERMINATION OF THE SEX STATUS OF PREIMPLANTATION HUMAN EMBRYOS

4.1 Introduction

The experiments in Chapter Three demonstrated satisfactorily the precision of diagnosis and the birth of sex selected mouse pups. In this Chapter the methodology has been extended to human preimplantation embryos.

4.2 Materials and Methods

Selection of gene primers

The testis–determining gene (SRY) is present on the short arm of human Y chromosome encoding a testis–specific transcript which provides an important control of sex developmental differential in mammals. The motif of this gene covers a stretch of 80 amino acids which is encoded by pY35.3 (human), and is 90% homologous with the rabbit Y–specific sequence (Sinclair et al., 1990). The SRY gene primers used in this experiment cover all this motif sequence.

The control gene utilised encodes a 57,000–73,000 molecular weight glycoprotein (1289 nt long mRNA) present in the human oocyte zona pellucida (ZP3), which was isolated with the homologous mouse sperm receptor gene (ZP3) located on chromosome 5 in mouse (Chamberlin and Dean, 1990). The control gene primers used in the experiment were selected from the beginning of 5' region of human ZP3.

Whole blood DNA testing

Human blood was used to test the correctness of the SRY and ZP$_3$ gene primers. DNA from the whole blood of 55 men and 65 women was extracted and prepared for PCR amplification (Kawasaki, 1990) prior to storage. All PCR mixtures were freshly combined. Reagents were transferred to 0.5ml PCR specific tubes in order, which included 28μl sterile distiled water, 5μl of 10 X PCR buffer (500 mM KCl, 100mM Tris–HCl, pH 8.3), 1μl of each dNTP (10mM), 2μl of each testis – determining gene primers (5'–CATGAACGCATTCATCGTGTGGTC–3'; 5'–CTGCGGGAAGCAAACTGCAAT TCTT–3'), 2μl of each ZP$_3$ gene primers (5'–AGCCATCCTGAGACGTCCGTA CA–3'; 5'–CCTGACCACATCTTCTGTGTCCAT–3'), 0.25μl of ampli Taq DNA polymerase (5U/μl), 4μl of 25mM MgCl$_2$ and 5μl (1.5μg) of individual human blood DNA template in a final volume of 50μl. DNA was denatured for 4 min at 94°C, followed by 30 cycles 94°C (1 min), 65°C (1 min)) and 72°C (2 min), and a final extension at 72°C (10 min) with Corbett FTS–1 fast thermal sequencer. Amplification DNA (20μl) and the marker was run on a 2% agarose gel stained with ethidium bromide, and examined on an ultraviolet transilluminator with photography.

DNA extracted from the blood of male and female marmosets, horses, cattle, alpacas, rabbits and mice was used to test for amplification by PCR with the human testis–determining gene primers. Two pairs of SRY gene primers were used: the first pair was above SRY gene primers for the longer SRY DNA sequence amplification; the second pair (5'–CATGAACGCATTCATCGTGTGGTC–3'; 5'–GCCTCCTGGAAGAATGGCCAT TTT–3') was for the shorter SRY DNA sequence amplification. PCR amplification of 30 cycles was performed as above.

Single lymphocyte testing

Single lymphocytes were used to test the precision, sensitivity and stability of PCR amplification with the designed primers. Nineteen single male derived lymphocytes and 19 single female derived lymphocytes were individually aspirated (following dilution) into a fine hand drawn Pasteur glass pipette using an inverted microscope (Nikon), and transferred to the PCR tubes which contained $10\mu l$ PCR buffer (10mM Tris–Cl, 50mM KCl, 2.5mM MgCl2,). PCR tubes were placed in the 94ºC heating block for 30 minutes in order to denature the single lymphocyte DNA. Freshly made PCR mixture ($50\mu l$), containing 10 X PCR buffer, dNTP, Taq polymerase, MgCl2, testis-determining gene and ZP3 gene primers, was transferred to the heat treated PCR tubes containing the male or female lymphocytes. The DNA was denatured for 4 min at 94ºC, followed by 30 cycles (as per while blood testing) for PCR amplification. Another batch of PCR tubes were prepared with $50\mu l$ PCR mixture containing the nested testis–determining gene primers (5'-CATGAACGCATTCATCGTGTGGTC–3'; 5'–CTGCGGGAAGCAAA CTGCA ATTCTT–3') and ZP3 gene primers (5'–AGCCATCCTGAGACGTCCGTACA–3'; 5'–CCTGACCACATCTTCTGTGTCCAT–3'), and placed in $1\mu l$ first PCR amplification product for reamplification with 30 cycles. The results were determined after the agarose gel electrophoresis.

Human embryo biopsy and embryo sexing

Four to 8 cell human polyspermic frozen/thawed embryos were biopsied. Before embryo biopsy, all the embryos were incubated at Ca^{++}- and Mg^{++}- free Human tubal fluid medium (HTFM) (Quinn et al., 1985) for 1 hour. Embryos were then transferred to Ca^{++}- and Mg^{++}- free Hepes HTFM for micromanipulation using an inverted microscope (Nikon) with 3

micromanipulators. Three types of micropipette were used: (1) holding pipette, approximately 100μm O.D and 40μm I.D., (2) drilling pipette, 20μm O.D., and (3) biopsy pipette, with approximately 20μm O.D. The embryos were secured by the holding pipette. The zona was drilled using Ca++– and Mg++– free Tyrode's solution (0.8g NaCl, 0.02g KCl, 0.1g Glucose, 0.4g Polyvinyl pyrrolidone/100ml H_2O, pH 2.3). The biopsy pipette was passed through the zona hole and a single embryo cell for gene PCR amplification was aspirated from each embryo. All aspirated single embryo cells were transferred to separate PCR tubes for 2 steps of 30 cycles of PCR amplification as per single lymphocyte testing.

4.3 Results

Whole blood DNA testing

All 55 male blood DNA samples after PCR amplification were both testis–determining (SRY) gene and ZP3 gene positive, while all 65 female blood DNA samples were ZP3 gene positive and testis–determining gene negative (Fig. 12). These results indicated the selected testis–determining gene and ZP3 gene primers to be satisfactory for human sex determination.

The DNA extracted from male and female marmoset, horse, cattle, alpaca, rabbit and mouse blood failed to show PCR amplification with the human testis–determining gene primers for the longer SRY DNA sequence fragment (254 base pairs) (Fig. 13A). However the DNA from the male marmoset blood showed PCR amplification with the human testis–determining gene primers for the shorter SRY DNA sequence fragment (150 base pairs) (Fig. 13B), with a similar location band as human DNA.

Fig. 12 PCR amplification of fragments of testis–determining gene and ZP3 gene from blood derived from humans of known sex. Fifty five men and 65 women were tested, but only 10 male (m) and 10 female (f) results are shown. The fragments from the testis determining gene were 254 base pairs and from the ZP3 gene 177 base pairs. All male derived blood showed two bands while all female derived blood showed one band. M was marker pUC 19. B was the blank.

Fig. 13 PCR amplification of fragments of human testis–determining gene from the blood derived from human (Hu), marmoset monkey (Mo), horse (Ho), cattle (Ca), alpaca (Al), rabbit (Ra) and mice (Mi) of known male (m) and female (f) sex. M. was marker pUC 19. B was the blank. A. PCR amplification of the 254 base pair fragment of human testis–determining gene from the blood. Only human male blood showed the human testis–determining gene positive. **B.** PCR amplification of the 150 base pair fragment of human testis–determining gene from the blood. Only the human male and the male marmoset blood (Mo) showed the human testis–determining gene positive.

Fig. 14 Amplification of single human lymphocytes of known sex by PCR with routine 'lymphocyte' (or blastomere) method. All male lymphocytes (m) showed two bands –254bp and 177bp (SRY and ZP3 bands) positive while female (f) only one band –177bp (ZP3 gene band) positive.
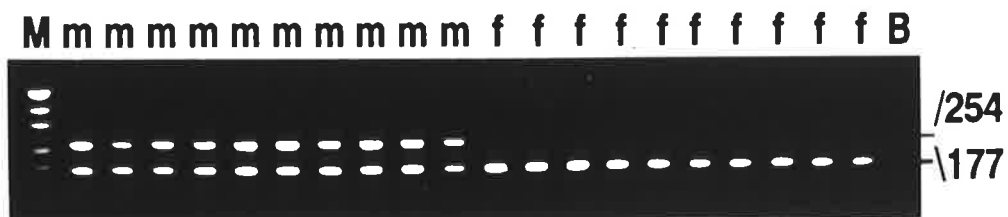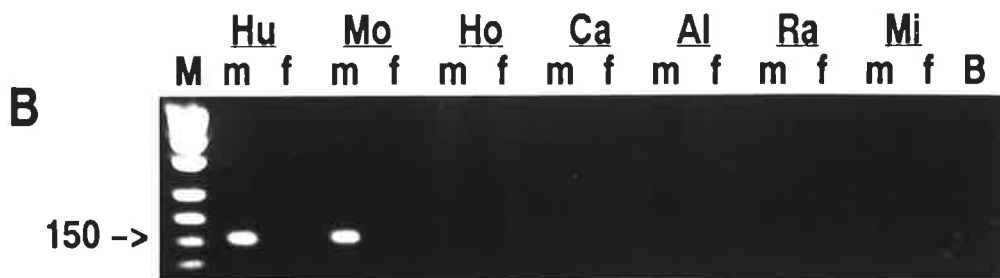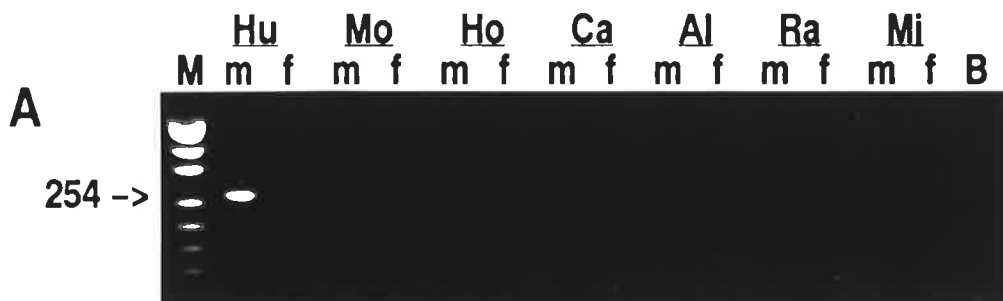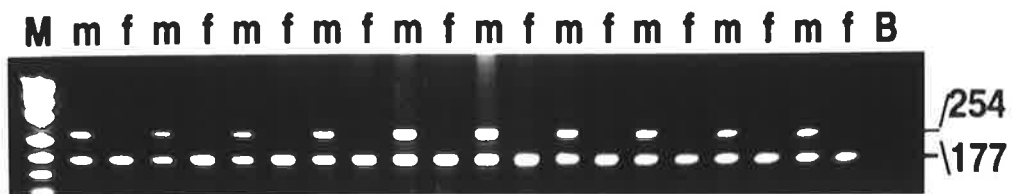
Fig. 15   Single cell embryo biopsy from 7 cell human polyspermic embryo (after thawing). One single embryo cell was aspirated after the biopsy pipette was passed through the drilled zona hole. This single embryo cell was degenerate with a disappearing nuclear membrane and with condensed, shrunken and irregular chromatin (arrow).

Fig. 16   PCR amplification of fragments of testis–determining gene and ZP3 gene derived from human single embryo cells after embryo biopsy. In total, 21 single embryo cells were analysed with 16 demonstrated (1–16). The 254 base pair bands were amplified by testis–determining gene primers. The 177 base pair bands were amplified by ZP3 gene primers. The results from embryos 1, 5, 7, 10, 11, 12 and 15 demonstrate both testis–determining gene and ZP3 gene positive products, and therefore considered "male" embryos. The results from embryos 2, 3, 4, 6, 8, 13 and 14 showed testis–determining gene negative and ZP3 gene positive products, and therefore designated 'female' embryos. Embryo 9 had a degenerated cell (Fig. 15) for this PCR amplification, and the degenerated chromatin (DNA) failed to amplify. Embryo 16 was another degenerate cell without any PCR amplification. B was the blank. M was marker pUC 19.
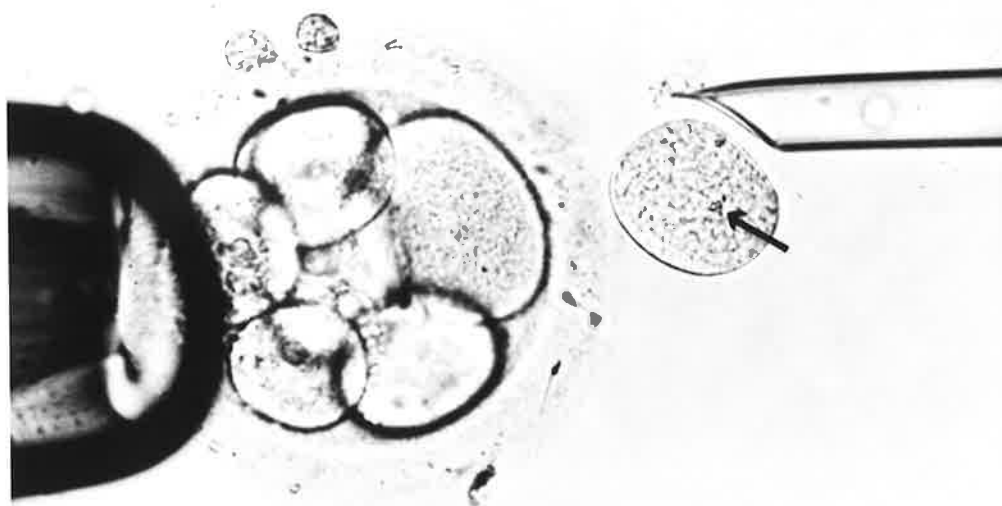
Human Embryos

M 1 2 3 4 5 6 7 8 9 10 1112 13 14 1516 B

/254

\177

Single lymphocyte testing

All 19 single male lymphocytes after PCR amplification were both testis–determining gene and ZP3 gene positive, and all 19 single female lymphocytes were ZP3 gene positive and testis–determining gene negative (Fig. 14). These results confirmed the sensitivity of PCR amplification to amplify both gene fragments from the single cell DNA without any misdiagnosis. All the individual amplification results were bright, 'identical' and clear.

Sex determination of human embryo

In total, 21 human polyspermic embryos were biopsied. Except for 2 single biopsied embryo cells (two biopsied embryos) which were recognised at biopsy to be degenerate and failed to show PCR amplification (Fig. 15), all 19 single biopsied embryo cells (19 biopsied embryos) showed satisfactory PCR amplification results (Fig. 16). Embryos which were both testis–determining gene and ZP3 gene positive were designated as 'male' embryos, and embryos which were ZP3 gene positive but testis–determining gene negative were designated as 'female' embryos. Of the 19 biopsied embryos with PCR amplification, 11 (57.9%) were 'male' embryos and 8 (42.1%) were 'female' embryos.

4.4 Discussion

The primers utilised appear to be very satisfactory to enable the precise diagnosis of the sex of the human preimplantation embryo. One hundred and twenty blood DNA preparations together with 38 single lymphocytes all confirmed correctly the sex of origin (100%) following PCR

amplification without any contamination.   Results of a similar technical standard were achieved following PCR amplification of 21 single blastomeres.

Specificity of testis–determining gene

The target DNA sequence was the motif of the human Y–linked testis determining gene (SRY). The transcripts of this gene are testis specific and the gene is believed to be importantly involved in testicular determination in the human. Primers designed to amplify the longer DNA sequence (254 base pairs) were specific to the human when tested against blood DNA preparations from the marmoset, horse, cattle, alpaca, rabbit and the mouse. When the shorter DNA sequence (150 base pairs) was targeted, only the male marmoset DNA showed amplification confirming the highly specific nature in the human of the longer sequence chosen. Interestingly, even with an 88% base pair homology of the 150 base sequence present in the rabbit and a 98% identical primer sequence (Sinclair et al., 1990), PCR amplification failed in rabbit blood DNA.   This exemplified the different specificity of PCR amplification of the same testis–determining gene in different species.

Y–repeated sequences

A method to determine the sex of human embryos has been described, however the potential for misdiagnosis has also been recognised (Handyside et al., 1989, 1990; Winston et al., 1991). These workers utilised a 3.4kb Y specific repeat target sequence (DYZ1) (Nakahori et al., 1986). This choice is vulnerable to misdiagnosis given the >484 TTCCA and other sequence nucleotide substitutions.   The DYZ1 sequences are similar to satellite III which originated from chromosome 1 and has been recovered from both male and female DNA (Nakahori et al., 1986). In rare situations the DYZ1

sequence is also deleted in males and can be translocated to autosomes or chromosome X and thus carried by females (Lo et al., 1989; Handyside et al., 1990).

Within the 800–5000 copies of the DYZ1 repeat sequences, non-specific copies are more common than copies of the identical targeted sequence. While such sequences are ideal for in situ hybridization studies, they are harmful for PCR amplification and may result in non-specific regular ladder effects, non-identical bands or amplification failure.

It is noteworthy that similar problems were encountered when a mouse Y repeat sequence (pY353/B) was utilized to sex mouse embryos with failure of diagnosis occurring in 28% of mouse embryos due to a variety of causes including unclear bands, non-amplification of PCR, non-specific ladder effects, male and female co-amplified sequences and non-identical bands (Bradbury et al., 1990). However when the same Y repeat sequence was utilized to target the mouse Y-linked testis specific gene (pYMT2/B), amplification with this Y-linked testis specific gene was perfect and the bands obtained were specific and identical with perfect separation of the sexes (Chapter 3).

It is imperative when sex determination is required to include a control gene sequence in order to recognise failure of cell transfer or DNA amplification which failure would allow misdiagnosis. Either an autosomal sequence or an X located gene is suitable for the control. In this study, the ZP3 gene was utilized and provided excellent results in both male and female substrates. The inclusion of a control gene may not be critical for the recognition of some autosomal gene mutations as the loss of DNA material may be apparent, but it is crucial for the diagnosis of sex-linked diseases.

Changes to the PCR conditions were required when single cells were the subject of amplification compared to general DNA (blood) preparations. This is likely to be due to the minimal number of copies present in the starting material but could also be dependant on changes in the DNA structure as profound changes to conditions are required when sperm DNA is to be amplified which contains large amounts of protamines within its condensed and haploid structure. It is essential therefore to have appropriate single cell model systems in place before clinical diagnosis is attempted.

Avoidance of contamination

The avoidance of contamination is crucial. The possibility of the incorporation of spermatozoa (bound to the zona), granulosa or cumulus cells into the biopsied cell material can be avoided by careful and precise aspiration techniques under microscopic observation together with thorough washing procedures of the embryo prior to biopsy. Following transfer of the biopsied cell, the transfer pipette should be washed and checked microscopically to avoid the adherence of any cell previously aspirated.

Two possibilities of contamination exist. Firstly, the contamination of a 'female' embryo by a male cell (or DNA). Should amplification occur, the embryo would be misdiagnosed as a male but would not be transferred, as only female embryos would be considered safe for transfer in X–linked disease. If female cell (or DNA) contamination of a female embryo were to occur, then there would be no consequences to the correct diagnosis (Fig. 17) as the female embryo would be transferred without risk. If female contamination of a male embryo occurred, then the PCR product would be diagnosed as male (unless there was a concomitant failure of amplification of the male cell) and the embryo not transferred with no clinical consequences.

Theoretical Results of Contamination in Embryo Sexing
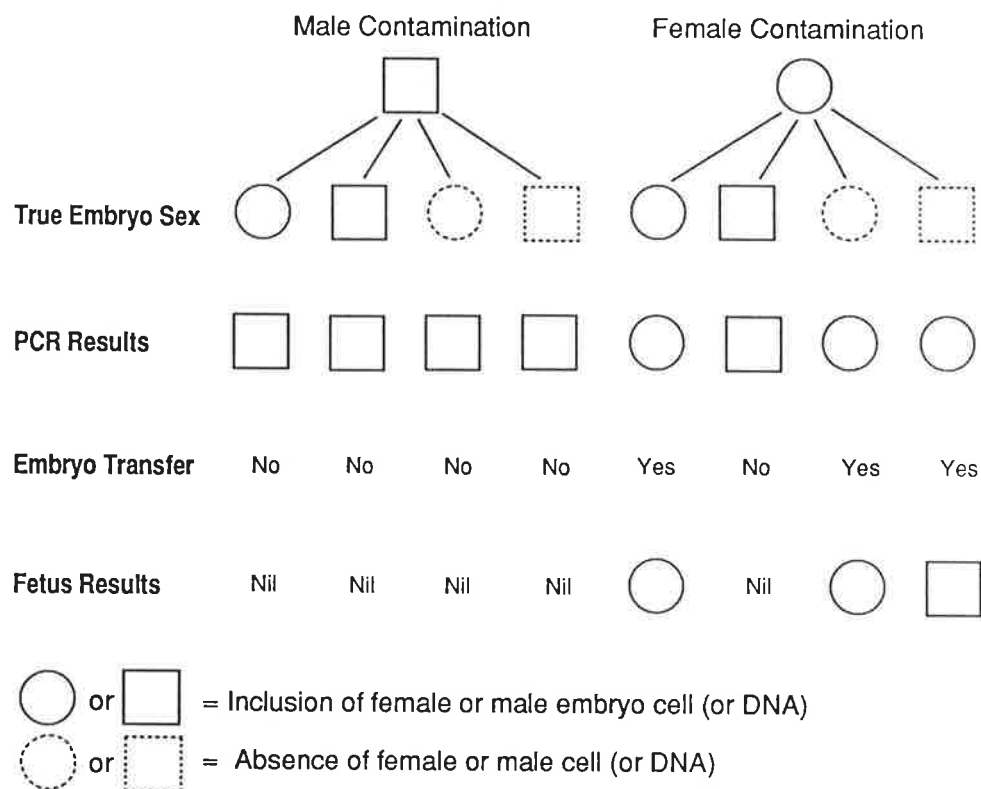
Note the single risk situation (last column) where a male fetus would be the result of contamination with female cell (or DNA) and the biopsied cell (or DNA) from a male embryo was excluded.

Fig. 17   Theoretical analysis of the consequences of contamination in PCR amplification for preimplantation diagnosis of sex-linked disease. 1). If a foreign male cell (or DNA) is included, no male child would be born. 2). If a female cell (or DNA) is included and a male embryo cell was not included in the PCR reaction then the male embryo would be diagnosed as a 'female' and perhaps transferred.

The single theoretical risk is where contamination with a female cell (or DNA) occurs to which no blastomere cell or DNA has been transferred. In this case a biopsied affected male embryo could be misdiagnosed as female and transferred.   Selections of only nucleated blastomeres for biopsy would reduce this risk as might the inclusion of multiple negative DNA (control) tubes, however if such rare possibilities combined then misdiagnosis would be a possibility.

In the case of genetic mutation disease, only if homologous normal embryos were chosen for transfer would all risks be eliminated when no biopsied cell (DNA) is missed (see Chapter 5).

Contamination from reagents requires extreme caution.   Fresh batches of reagents should be frequently used and stored separately. Scrupulous care and cleanliness is required for all reagent racks and multiple DNA negative control tubes included to detect contamination.

Environmental contamination should be addressed with similar precision.   Gloves are changed frequently, hair and face masks are essential and all laboratory work performed within UV hoods.   Separation of the PCR preparation facility from the electrophoresis and DNA extraction facility is desirable.

Embryo biopsy

Biopsy of the individual blastomeres from the embryo requires adaptation to the circumstances. In this study, polyspermic embryos which had been cryopreserved were utilized. The human zona pellucida was found to be very soft and minimal zona drilling was found to be the most optimal technique since direct puncture of the embryo allowed the escape of additional cells in different directions through the soft zona. Previous report with zona drilling of the mouse oocyte indicated the potential for oocyte damage (Payne et al., 1991), a phenomenon which does not appear so apparent for the human embryo (Hardy et al., 1990). Nevertheless, this study utilized minimal zona drilling in order to thin or to breach the zona prior to the aspiration pipette being introduced. Whether fresh embryos require a different methodology remains untested in this series.

Human 4-8 cell embryos appear to contain a proportion of degenerate cells which contrast with findings in the mouse and may reflect less optimal culture conditions or may be inherent. In this series, two of the 21 cells biopsied failed to be amplified. In the mouse, occasional cells which did not display a nuclear membrane amplified satisfactorily. However, in the human every care should be taken to select cells with the best morphological nuclear outline in the hope of avoiding amplification failures. Again, the inclusion of the control gene was critically important to recognise this risk.

CHAPTER FIVE

IDENTIFICATION OF HUMAN Y BEARING SPERMATOZOA BY Y-LINKED TESTIS-DETERMINING GENE AMPLIFICATION.

5.1   Introduction

The theoretical basis of X and Y sperm was proposed in 1923 (Painter, 1923). Since then, several methods have attempted to recognise X and/or Y spermatozoa including fluorescence (Barlow and Vosa, 1970; Sumner et al., 1971) and karotypic analysis of pronuclear formation following hamster oocyte fusion (Rudak et al., 1978). With the advent of the polymerase chain reaction, the ability to recognise the characteristics of X and Y individual spermatozoa has become feasible.

5.2   Materials and Methods

The selection of the SRY and ZP3 gene primers were identical to those described in Chapter 4.

Testing whole blood DNA

Initial testing of the primers utilised blood DNA extracted from 21 men and 20 women (Kawasaki, 1990). Individual blood DNA were transferred to PCR tubes containing PCR buffer, which included deoxyribonucleotides, $MgCl_2$, Taq DNA polymerase, SRY and control gene primers which amplify the

selected regions of the motif of SRY and ZP3 genes. Following DNA denaturation, 30 cycles of amplification were performed.

Modified Li-Cui's method and testing single lymphocytes

Ten single male lymphocytes and 10 single female lymphocytes were individually aspirated under microscopic observation, individually transferred to a PCR tube which contained 5μl sperm lysis medium (4mg DTT, 1.7μM SDS, 1mg proteinase K in 1ml PCR buffer (50mM KCl, 10 mM Tris-HCl, 2.5mM MgCl$_2$ at pH8.3)). After one hour at 56°C, samples were heated in 92°C for 12 min (Li et al., 1988). After cooling, 2μl of an alkaline solution (5mg DTT/1ml 0.2M KOH) was added to each sample and heated at 65°C for 10 min. After addition of 2μl of 0.3M KCl/0.9M Tris, pH8.3 (Cui, X.F. et al., 1989), the samples were brought to 20μl with PCR reagents together with the SRY and ZP3 gene primers (but without the addition of 10 x PCR buffer) for 30 cycle amplification. One μl of first PCR product was added to the prepared 20μl PCR mixture containing the nested SRY and ZP3 gene primers (sequences in Fig. 18) for another 30 cycle amplification. Agarose gel electrophoresis was performed to show the results.

Sexing motile sperm

Freshly ejaculated human semen were collected from 6 different donors. One drop of semen was mixed with 2ml of MEM (Minimum Essential Medium – Alpha modification) and cultured in 5% CO$_2$ at 37°C before aspiration for PCR amplification. One to 5μl of this diluted medium containing spermatozoa was further mixed and diluted one or more times with 250μl medium on the slide to ensure that only one or two motile sperms were present in a 100x microscopic field. A single motile sperm was then aspirated under microscopic observation, and transferred to an individual PCR tube and

lysed. One hundred and eleven (one sample), 112 (5 samples) single motile spermatozoa were randomly aspirated from each donor sample and placed in the individual PCR tubes and subjected to the modified Li–Cui's method for sperm DNA decondensation prior to amplification.

After 60 cycles of PCR amplification with the SRY and ZP3 gene primers, some sperms demonstrated both SRY and ZP3 gene bands to be positive, and were designated as 'Y' sperms, while other sperms showed only the presence of the ZP3 gene band and were designated as 'X' sperms (Fig. 20).

Sexing immobilised sperms following photography

For photography, sperm aspiration procedures were as for motile sperm, following which 1–2μl giemsa stock solution (25 g Giemsa / 1600 ml glycerol+1600 ml methanol) was added to 250μl diluted sperm medium to inhibit totally sperm movement. Individual ovoid shaped and horizontal lying spermatozoa were selected for photography (400X), and then were individually aspirated into individual PCR tubes. Nineteen to 25 single spermatozoa from each of 11 donors' were analysed. Satisfactory PCR amplification was performed for 217 (93.1%) of the 233 photographed spermatozoa and showed 106 (48.8%) were 'Y' and 111 (51.2%) were 'X' sperms. Under 'blinded' conditions, photographs of individual spermatozoa were further magnified (x30) by image projection and measurements were made of the length, width, perimeter and area of the single sperm heads together with the length of the sperm neck and tail. In addition, digitized measurements (using a software program, Flinders Imaging) was performed for individual spermatozoa. After measurement, the individual PCR (X or Y) results were correlated with the corresponding measured indices of each spermatozoa.

Fig. 18   PCR amplification of fragments of SRY (254bp) and ZP3 (177bp) genes from human blood DNA. M was marker pUC19. B = blank. m = male. f = female. All male blood DNA showed both SRY and ZP3 gene positive. All female blood DNA showed only ZP3 gene positive. PCR amplification with SRY gene primers (5'-CATGAACGCATTCATCGTGTGGTC-3'; 5'-CTGCGGGAAG CAAACTGCAATTCTT-3')   and   ZP3   gene   primers   (5'- AGCCATCCTGAGACGTCCGTACA-3'; 5'- CCTGACCACATC TTCTGTGTCCAT-3') was performed by 30 cycles.

Fig. 19   Amplification of single human lymphocytes of known sex by PCR with modified Li-Cui's method. Except one male and one female lymphocytes were not amplified, all male lymphocytes showed both SRY (254bp) and ZP3 (177bp) gene bands to be present and all female lymphocytes only the ZP3 gene band positive.

Fig. 20   Amplification of 112 motile single sperms of donor 5 with modified Li-Cui's method. The sperms with both SRY (254bp) and ZP3 (177bp) gene positive were designated 'Y' sperms, and the sperms with only ZP3 gene positive were designated 'X' sperms.

Table 8.   Distribution of X and Y bearing Spermatazoa

Subject	Number of Sperm	Number Amplified (%)	Number "Y" Sperm (%)	Number 'X' Sperm (%)
1	111	109 (98.2)	53 (48.6)	56 (51.4)
2	112	109 (97.3)	56 (51.4)	53 (48.6)
3	112	110 (98.2)	62 (56.4)	48 (43.6)
4	112	109 (97.3)	55 (50.5)	54 (49.5)
5	112	107 (95.5)	64 (59.8)	43 (40.2)
6	112	109 (97.3)	65 (59.6)	44 (40.4)
TOTAL	671	653 (97.3)	355 (54.4)*	298 (45.6)*

*P=n.s.

Table 9   Parameters of Measurement of X and Y bearing

Spermatozoa   (Mean ± S.D.)

	'Y'	'X'	P
Sperm Head (Length)	$5.23 \pm 0.40 (\mu m)$	$5.38 \pm 0.43 (\mu m)$	<0.01
Sperm Head (Width)	$3.53 \pm 0.30 (\mu m)$	$3.53 \pm 0.33 (\mu m)$	>0.05
Sperm Neck & Tail (Length)	$41.18 \pm 3.47 (\mu m)$	$42.22 \pm 4.37 (\mu m)$	<0.05
Sperm Head (Perimeter)	$14.73 \pm 1.07 (\mu m)$	$15.26 \pm 1.17 (\mu m)$	<0.001
Sperm Head (Area)	$13.93 \pm 1.79 (\mu m)^2$	$14.74 \pm 2.09 (\mu m)^2$	<0.001

5.3 Results

All 21 men's blood DNA samples were both SRY and ZP3 genes positive, and all 20 women's blood DNA samples were only ZP3 gene positive but SRY gene negative (Fig. 18).

Nine 'male' and 9 'female' single lymphocytes were amplified and all satisfactorily confirmed the correct sex origin of the single lymphocytes (Fig. 19). The male derived single lymphocyte confirmed both SRY and ZP_3 genes to be positive while the female derived single lymphocyte contained only the ZP_3 gene. These results confirmed the potential of the selected SRY and ZP_3 gene primers to determine the Y and the likely X constitution of individual spermatozoa.

Of a total of 671 single motile sperms examined (Fig. 20), 653 (97.3%) sperms showed satisfactory PCR amplification, 355 (54.4%) were 'Y' sperms, and 298 (45.6%) were 'X' sperms (Table 8). No significant difference was found in the ratio of 'Y' and 'X' sperms and the expected 1:1 ratio.

Statistical analysis showed the length, perimeter and area of the 'X' sperm heads and the length of the 'X' sperm neck and tail were significantly larger and longer than those of the 'Y' spermatozoa (Table 9).

5.4 Discussion

The reliable identification of human X or Y bearing spermatozoa will not only allow precise investigation of the ratio across a wide spectrum of conditions of human fertility but will be invaluable to examine purported existing techniques to sort spermatozoa or to assess new methods. The

modified Li-Cui's methodology provided satisfactory rates (>97%) of PCR amplification and confident 'Y' identification using a Y-linked testis determining gene in the presence of a control gene.

Although the primers from the X chromosome were not available in these experiments, the PCR conditions were refined to ensure that the SRY gene was amplified efficiently, so that the X status of individual spermatozoa could be confidently inferred by the lack of SRY gene amplification. The error free results following the use of single lymphocytes from known sex sources together with the confirmation of the anticipated 1:1 ratio of X and Y spermatozoa also provided confidence that the methodology utilized was satisfactory.

A previous attempt at sperm DNA amplification using a general lymphocyte method was limited to 30% amplification possibly due to the failure of decondensation of the protamine rich haploid sperm DNA (Gledhill, 1988). Experience suggests that differences in PCR conditions are required for single spermatozoa compared with single lymphocytes or single blastomeres. The necessary specificity of the conditions (for spermatozoa) was illustrated by the less optimal amplification (90%) of the modified Li-Cui's method when applied to single lymphocytes. Giemsa showed little inhibitory effect on the PCR amplification of single spermatozoa, and was found to be preferable to the use of formalin or alcohol.

The X chromosome bearing spermatozoa have been postulated to be likely to be larger than the Y bearing spermatozoa because the X chromosome is itself larger (Roberts, 1972). Shettles using direct microscopic observation claimed an absolute bimodel distribution (X sperms are all larger than Y sperms) (Shettles, 1960) but was subject to immediate criticism

(Rothschild, 1960). Later Sumner et al. (1971) using fluorescent optical density considered there to be no size differences between X and Y spermatozoa. The current findings based on individual spermatozoa measurements are likely to be more precise than the more indirect fluorescent optical methods.

The DNA content of individual spermatozoa can be measured with sufficient precision to detect differences between the X and the Y spermatozoa in a number of mammals. The separation of X and Y bovine sperm based on a 7% difference in DNA content utilizing flow cell cytometry is now commercial. It has been calculated that the DNA content of human X and Y sperm would differ by 1% in total cellular dry mass (Sumner and Robinson, 1976; Pogeny et al., 1981). The results presented here suggest statistical differences on morphological measurements but not all X sperms were larger. Size differences are unlikely to be necessarily solely related to DNA content but perhaps to the cytoplasm content and structure or as a consequence of the stage of maturity or senescence of spermatozoa.

A variety of procedures have been proposed to obtain enriched populations of human X and Y spermatozoa but the results of all methods have remained questionable to date due to the lack of a reliable discriminating assay of the product. The application of PCR techniques to this area may assist the development of useful programs of sperm selection for insemination in preimplantation diagnosis, especially in X-linked diseases.

CHAPTER SIX

PREIMPLANTATION DIAGNOSIS OF CYSTIC FIBROSIS: PCR AMPLIFICATION OF THE ΔF508 MUTATION

6.1   Introduction

Cystic fibrosis (CF) is the most prevalent in the recognised inherited diseases within the Caucasian population with an incidence of about 1 in 2500 births and a carrier frequency of approximately 1 in 25 (Boat et al., 1989).   The disorder is caused by mutations of the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7q31 (Rommens et al., 1989). While more than 200 mutations have been described, the most common mutation is the deletion of amino acid phenylalanine at position 508 in the first nucleotide (ATP)–binding fold (i.e. ΔF508) (Harris, 1992) and is responsible for 70% of CF in Northern Europe and Northern America. Due to the most important sequences in the CF gene being analysed, it has been possible to reliably diagnose the ΔF508 mutation in blood DNA by southern blotting (Kerem et al., 1989).

The direct PCR amplification and identification of the gene sequences of the ΔF508 mutation is now demonstrated to allow precise and reliable diagnosis of the CF status of the early embryo.

6.2   Materials and Methods

Blood Testing

Human blood DNA was used to determine the ability of the designed primers to detect the ΔF508 mutations in cystic fibrosis. DNA from the whole blood of 8 homozygous normal, 7 heterozygous abnormal, and 7 homozygous abnormal individuals was extracted and prepared for PCR amplification (Kawasaki, 1990) prior to storage. All PCR mixtures were freshly combined. Reagents were transferred to 0.5ml PCR tubes in order, which included 25µl sterile distiled water, 4µl of 10 X PCR buffer (500mM KCl, 100mM Tris-HCl, pH8.3), 1µl of each dNTP (10mM), with either 1.6µl of each normal CF gene primers (5'-GGCACCATTAAAGAAAATATCATCTTTG-3'; 5'-AGCTTCTTAAAGCATAGGTCATGTG-3') (designated as N tubes with 1µl of phenol red 1mg/10ml H_2O), or 1.6µl of each ΔF508 mutation gene primers (5'-CTGGCACCATTAAAGAAAATATCATTG-3'; 5'- AGCTTCTTAAAGCATAGG TCATGTG-3') (designated as F tubes), 0.2µl of ampli Taq DNA polymerase (5U/µl), 4µl of 25mM $MgCl_2$ and 4µl (or 1.5µg) of individual human blood DNA template in a final volume of 40µl. DNA was denatured for 6 min at 94ºC, followed by 30 cycles 94ºC (1 min), 65ºC (1 min) and 72ºC (2 min), and a final extension at 72ºC (10 min) with Corbett FTS-I fast thermal sequencer. Amplified DNA (20µl) and the marker were run on a 2% agarose gel stained with ethidium bromide, and examined on an ultraviolet transilluminator with photography.

Single lymphocyte testing

Single lymphocytes were used to test the precision, sensitivity and stability of PCR amplification with the designed normal and mutation

primers. Sixty two homozygous normal single lymphocytes (from a homozygous normal male), 59 heterozygous abnormal single lymphocytes (from a heterozygous abnormal female) and 53 homozygous abnormal single lymphocytes (from a homozygous abnormal young male) were diluted and individually aspirated into a fine hand drawn Pasteur glass pipette under the inverted microscope (Nikon), and transferred to the PCR tubes which contained $5\mu l$ PCR buffer (10mM Tris-Cl, 50mM KCl, 2.5mM $MgCl_2$, pH8.0). After DNA denaturing (Chapter Three), freshly made PCR mixture ($15\mu l$), containing 10 x PCR buffer, dNTP, Taq polymerase, $MgCl_2$, cystic fibrosis gene primers were transferred to the heat treated PCR tubes which contained the single homozygous normal, heterozygous abnormal and homozygous abnormal lymphocytes. The DNA was denatured for 6 min at 94oC, followed by 30 cycle amplification and similar processing as blood DNA for PCR amplification of the common sequences covering the mutation points CTT. Another batch of PCR N and F tubes were prepared with $20\mu l$ PCR mixture containing the nested normal and mutation gene primers identical to those used in the blood testing, and placed in $1\mu l$ first PCR amplification product for reamplification with 30 cycles. The results were determined after the agarose gel electrophoresis.

Human embryo biopsy, blastomere and embryo diagnosis

Four to 8 cell human polyspermic frozen/thawed embryos from 7 couples were used in these experiments (with the appropriate consent of the couple). Before embryo biopsy, all the embryos were incubated at Ca++- and Mg++- free HTFM (Human tubal fluid medium) for 1 hour. Embryos were then transferred to Ca++- and Mg++- free Hepes HTFM for micromanipulation using an inverted microscope (Nikon) with 3 micromanipulators. The embryos were secured by the holding pipette. The targeted nucleated blastomere was

determined and located in the biopsy position. The drilling pipette was approximated to the zona, and Ca++– and Mg++– free Tyrode's solution (0.8g NaCl, 0.02g KCl, 0.1g Glucose, 0.4g Polyvinyl pyrrolidone/100ml H_2O, pH2.3) was gently expelled to dissolve the zona. The biopsy pipette was passed through the zona hole and a single nucleated embryo cell was aspirated from each embryo. All aspirated single embryo cells were separately transferred to PCR tubes for 2 stages of 30 cycles of PCR amplification (as per single lymphocyte testing) to detect the ΔF508 mutation. Eleven nucleated single blastomeres were biopsied from 11 embryos and a further 10 whole embryos were subjected to PCR amplification.

6.3   Results

Blood testing

All N tube solutions used to detect the normal CF gene fragments were red coloured and easily differentiated from the colourless F tube solutions which were directed toward the detection of the ΔF508 mutation. The blood DNA from the 8 homozygous normal individuals showed normal gene fragments only (N tubes or N bands positive), and mutation gene fragments (F tubes or F bands) negative (Fig. 21). The blood DNA from 7 heterozygous individuals were all both normal and mutation gene fragments (N and F bands) positive. The blood DNA from 7 homozygous abnormal subjects were all mutation gene fragments (F bands) positive, but normal gene fragments (N bands) negative. All the bands were bright and clear.

Fig. 21   Amplification of fragments of normal CF gene and mutant CF gene (ΔF508) derived from blood DNA of people of known CF conditions. Twenty two people were tested, but only 12 individual results are shown. NN_1 to NN_4 denotes the first to the fourth homozygous normal subjects, NF_1 to NF_4 the first to the fourth heterozygous abnormal carriers and FF_1 to FF_4 the first to the fourth homozygous abnormal patients. M was the marker pUC 19. B was the blank. N was the PCR reagent tube with normal CF gene primers (with red colour). F was the PCR reagent tube with mutant CF gene primers (without colour). The blood DNA from homozygous normal people only produced N bands, while heterozygous abnormal carriers both N and F bands, and homozygous abnormal patients only F bands.

Fig. 22   PCR amplification of fragments of normal CF gene and mutant CF gene (ΔF508) derived from single lymphocytes from the people of known CF conditions. Denotation is as in Fig. 21. One hundred and seventy four single lymphocytes were tested, but only the results of 12 single lymphocytes are shown. NN denotes homozygous normal, NF heterozygous abnormal and FF homozygous abnormal lymphocytes. The single lymphocytes from homozygous normal blood only produced N bands, while those from heterozygous abnormal blood both N and F bands, and those from homozygous abnormal blood only F bands. The self–control bands allowed the confidence of DNA presence in the N or F tubes for the second run PCR amplification. Some weak cross–amplification bands from the products of first and second sets of primers can be seen faintly between the aimed (targetted) and self–control bands (such as in NN_3) without influence on the precise diagnosis of the aimed bands.
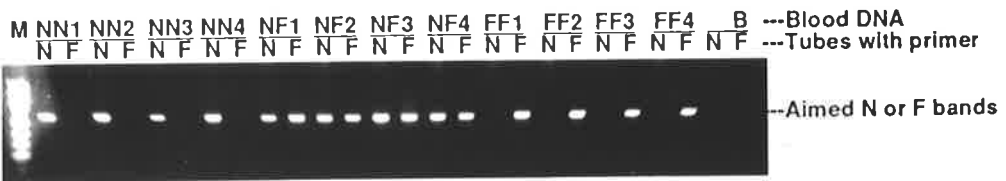
Single lymphocyte testing

All 174 single lymphocytes tested showed 100% amplification and were diagnostically correctly identified. The 62 single lymphocytes from homozygous normal sources all showed normal gene fragments (N bands) positive, and mutation gene fragments (F bands) negative (Fig. 22). All 59 heterozygous abnormal single lymphocytes showed both normal and mutation gene fragments (N and F bands) positive. The 53 homozygous abnormal single lymphocytes were all mutation gene fragments (F bands) positive, but normal fragments (N Bands) negative. Weak common DNA bands (self–control bands) derived from the initial set of primers were present which served as proof of DNA inclusion from the single lymphocytes. Although occasional other very weak bands occurred from cross amplification of the products of the first and second sets of primers, the chosen conditions precluded these bands influencing the results of the targeted bands. The presence of self–control bands was considered essential not only for single lymphocyte testing, but also in the diagnosis of human embryos and single blastomeres. Another gene (ZP3) was investigated as the control gene, however it failed due to the presence of numerous non–specific bands produced by the cross–reactions of the primers from CF and control genes. The self–control bands provided confident results with simpler procedures.

Diagnosis of human embryos and blastomeres

Ten human embryos (Fig. 23) and 11 selected nucleated blastomeres (Fig. 24) biopsied from another 11 human embryos showed 100% PCR amplification. They were all normal gene fragments (N bands) positive but mutation gene fragment (F bands) negative, i.e. all homozygous normal. The targeted bands were bright and clear and the presence of weak

Fig. 23   PCR amplification of fragments of normal CF gene and mutant CF gene (ΔF508) derived from single polyspermic human embryos. Denotation is as in Fig. 21. Ten embryos were tested, but only results of 7 embryos are shown, and all of them have been identified as CF (ΔF508) homozygous normal embryos.

Fig. 24   PCR amplification to diagnose CF conditions of single biopsied blastomeres. Eleven nuclear selected blastomeres biopsied from 11 human polyspermic embryos were tested, but only results of 8 single blastomeres are shown, and all of them have been identified as CF (ΔF508) homozygous normal embryos.
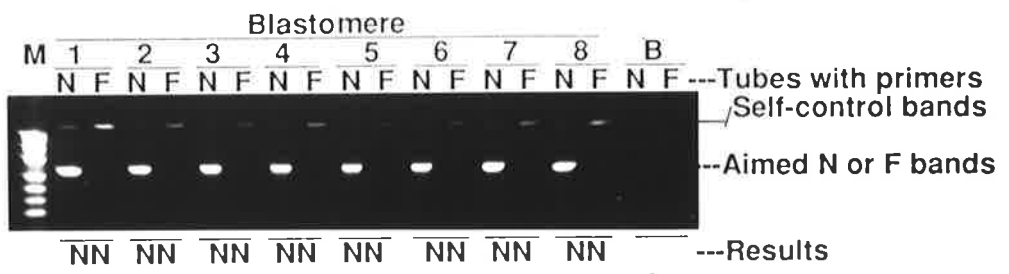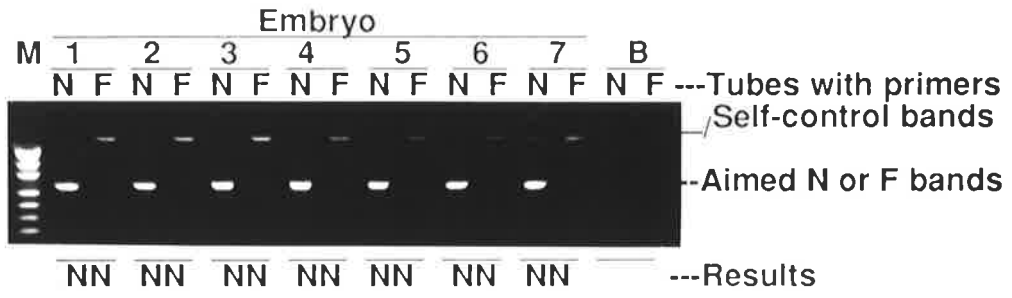
self-control bands ensured the correct diagnosis. The homozygous normal condition of these 21 embryos (derived from 7 couples) was not unexpected given a ΔF508 mutation carrier incidence of about 1 in 25.

6.4 Discussion

For preimplantation diagnosis, the most favoured material to be used is the single human embryo cell (Chapter 1.3.3). Gene analysis of the single embryo cells should be simple, fast, sensitive, stable and precise. Simplicity can assist in avoiding technical misdiagnosis and fast diagnosis can allow embryo transfer to be performed in the same IVF cycle. Sensitivity is especially important for gene amplification from the DNA of the single embryo cell, and PCR amplification is the most suitable. The stability of the technique allows the visualisation of bright, identical and clear PCR amplification products thus avoiding the need for frequent changes of primers or PCR conditions. Precision of the diagnosis is the end point and the key to preimplantation diagnosis. Without precision of the diagnosis, further confirmatory procedures for prenatal diagnosis and unnecessary termination of pregnancy may be required. The rate of misdiagnosis is an inverse ratio of the precision of diagnosis. For example, when sexing the human embryo, a 60%-90% PCR amplification rate (Handyside et al., 1989, 1990) produced a 15% to 18% misdiagnosis rate. Roughly the rate of PCR amplification (%) + the rate of misdiagnosis (%) ≈ 100%. However, even though the rate of PCR amplification may be perfect, the possibility of misdiagnosis due to technical mistakes, contamination, chromosomal translocation and gene mutation could still occur but the risk can be almost eliminated by setting up strict procedures.

The possibilities of misdiagnosis due to contamination in diagnosis of X-linked diseases has been shown to be very limited (Chapter 4). In gene mutation diseases, cystic fibrosis disease can be examined as an example (Fig. 25). **If only the *homozygous normal* embryos are to be transferred (excluding the possibility of a missing blastomere (DNA)), then no fetal misdiagnosis would occur, because:** (1) When contamination with the normal gene occurs to an *homozygous abnormal* embryo, it would produce PCR misdiagnosis of an *heterozygous abnormal* embryo. This embryo would not be transferred. (2) When contamination with a normal gene occurs to an *homozygous normal* or *heterozygous abnormal* embryo, there would be no alteration of the PCR diagnosis. (3) When contamination with an *homozygous abnormal* gene occurs to an *homozygous* or *heterozygous abnormal* embryo, there would also be no alteration of the PCR diagnosis. (4) When an *homozygous abnormal* gene contaminates an *homozygous normal* embryo, misdiagnosis of the PCR result would occur but the embryo would not be transferred but wasted.

If only the homozygous normal embryos would be transferred with missing blastomere (or DNA) following contamination, the possibility of healthy, heterozygous and homozygous abnormal babies would be about 25%, 50% and 25%.

However, if heterozygous embryos are to be transferred, contamination would raise the possibility of healthy, heterozygous and homozygous abnormal children in the ratio of about 25%, 50% and 25%.

A number of different methods have been examined for the diagnosis of cystic fibrosis. Some primers were directed towards the locus

Fig. 25   Theoretical analysis of the consequences of contamination in PCR amplification for preimplantation diagnosis of cystic fibrosis (ΔF508). (Note: The natural frequency of *homozygous normal, heterozygous* and *homozygous abnormal* babies is about 25%, 50% and 25% in cystic fibrosis when parents are *heterozygous abnormal*).

If only *homozygous normal* embryos are to be transferred then the only risk situation is where the PCR result of *homozygous abnormal* embryos shows the embryo to be *homozygous normal* because the biopsied cell (or DNA) has not been included in the PCR tube but a contamination with a *homozygous normal* gene has occurred (marked *).

If *heterozygous abnormal* embryos are to be transferred, two risks exists: (1) when an *homozygous normal* gene contaminates a *homozygous abnormal* embryo (marked**) and (2) when the PCR results of a *homozygous abnormal* embryo shows the embryo to be *heterozygous abnormal* because the biopsied cell has been contaminated with a *heterozygous abnormal* gene (marked ***).

NB: Contamination is likely to be "operator contamination" and the gene status of the operator should be established.

Theoretical Results of Contamination in the Preimplantation Diagnosis of Cystic Fibrosis

Normal Gene Contamination   Heterozygous Mutant Gene Contamination   Homozygous Mutant Gene Contamination

True Embryo Condition

PCR Results

Embryo Transfer   Yes   Yes   No   Yes   No   Yes   Yes   Yes   Yes   No   Yes   No   Yes   No   Yes   No   Yes   No   Yes   No   Yes   No   Yes   No   No   No   No   No

Fetal Result   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil   Nil

**   *   ***   ***

○ : Homozygous normal embryo cell (or DNA)   ◌ : Homozygous normal biopsied cell (DNA) excluded

◐ : Heterozygous mutant embryo cell (or DNA)   ◑ : Heterozygous mutant biopsied cell (DNA) excluded

◍ : Homozygous mutant embryo cell (or DNA)   ◍ : Homozygous mutant biopsied cell (DNA) excluded

NB: The "Yes or No" relating to whether embryo to be transferred or not after PCR amplification

*,**,*** : See the detailed explaination of the figure

CS.7 which maps only close to, rather than directly covering the mutation points which cause cystic fibrosis (Coutelle et al., 1989). This indirect map linkage diagnosis method is helpful for the location and isolation of some genes but was not suitable for the detection of the point mutations for preimplantation diagnosis and later was abandoned by the same research group.

Direct electrophoresis of 95/98 bp bands and heteroduplex formation showed a 84% amplification rate in single human polar body and blastomere experiments but with the unclear and unstable bands (Strom et al., 1990; Verlinsky et al., 1992). Until recently the reason for heteroduplex formation rather than the homoduplex formation has not been thoroughly understood. This method was originally published several years ago to detect point mutations, however only about 50–75% of all mutations could be detected in the β-globin gene with the separation problems remaining unsolved (Myers and Maniatis, 1986). The fidelity of the method was adversely influenced by the poor separation of the normal and mutant homoduplexes of the cystic fibrosis gene even from blood (Rommens et al., 1990; Kerem et al., 1990). However the heteroduplex method has been used for preimplantation diagnosis in the human (Handyside et al., 1992) with poor outcomes, namely the PCR diagnosis rate was only 75% using nucleated blastomeres for amplification. In addition the routine use of the *two* cell embryo biopsy (Handyside et al., 1992), for the embryos at the 8–cell to underpin the shortcomings of the diagnostic method is also inappropriate. The use of human pregnancies rather than the basic scientific experiments to 'prove' this method raises many important ethical concerns. The validity of this experimental approach is questionable and has been subject to severe and increasing criticism (Jarmulowicz, 1989; Strom et al., 1991a; Pickering et al., 1992; Trounson, 1992; Simpson and Carson, 1992).

In preimplantation diagnosis for cystic fibrosis, polar body biopsy and double biopsy (polar body and embryo) have been reported (Verlinsky et al., 1992). It was recognized that polar body biopsy 'will not make it possible to establish genetic diagnosis of the embryo' because: (1) paternal alleles cannot be analysed; (2) the test might not be efficient due to loci crossing-over; and (3) gender determination is not possible (Verlinsky et al., 1992). Possibly due to the acidic influence on the oocytes or the harmful effect of double biopsy, no pregnancy occurred in five couples (Verlinsky et al., 1992).

In the basic experiments, described here the results of 22 blood samples, 174 single lymphocytes, 10 total embryos and 11 nucleated blastomeres representing 11 additional embryos showed 100% PCR amplification and a correct diagnosis. This direct PCR amplification method could substitute for the current radioactive southern blot and heteroduplex methods producing quicker, cheaper and reliable results. These satisfactory primers make routine clinical preimplantation diagnosis of cystic fibrosis ΔF508 possible.

CHAPTER SEVEN

THE FUTURE OF PREIMPLANTATION DIAGNOSIS

About 5,000 single gene defects are now recognized and about 1 in 100 births are affected by severe genetic disease including congenital abnormalities. The prevention of genetic disease must remain an important target of medical research. The current methods of prenatal diagnosis are invasive to the established pregnancy, not without risk and can have severe emotional consequences. Each of these problems is relevant to each pregnancy conceived. Preimplantation diagnosis is a different option, able to provide widening choice which is clearly acceptable and desired by some couples. The procedures are in their embryonic stages themselves and are characterized to date by a real risk that the precision of diagnosis is vulnerable. A major principle objective of this thesis has been to impose rigour and precision in all the areas investigated.

Currently the main disadvantage of preimplantation diagnosis is the necessity for couples to undertake in vitro fertilization procedures which are not required for conception of fertile couples. IVF is currently demanding, invasive, expensive and is not guaranteed of success. Nevertheless, IVF is an accepted clinical procedure with predictable results in the infertile. The results of IVF in the fertile couple are not known but can be expected to be better since a significant proportion (40%) of the current indications for IVF is male infertility. More invasive techniques such as zygote intrafallopian transfer (ZIFT) may be associated with better rates of embryo implantation and is a further additional option to consider. In addition, the results of

cryopreservation of embryos are successful enough to provide an added important dimension to preimplantation diagnosis and raises the real possibility that a single preimplantation diagnostic procedure could result in the achievement of more than one birth (Chapter 1.5.1).

One currently difficult approach to preimplantation diagnosis of sex linked disease is the creation of embryos with single gender spermatozoa. Methods to identify the X or Y bearing capacity of sperm are only now beginning to be developed (Chapter 5) and it is likely that sperm separation techniques will follow. Clearly until separation techniques or recognition patterns (supported by sperm injection techniques) are perfect then this area will remain undeveloped. Alternatively pre-conception screening of the oocyte (by polar body biopsy) for maternal based mutations would seem unlikely to gain popularity given the difficulties of genetic recombination (see Chapter 1.3.2). Similarly, non-invasive testing of the embryo requires considerable more development before clinical usage (Chapter 1.4.1). At this stage of development, single blastomere biopsy of the early embryo is the favoured technique (Chapter 1.3.3) but it may be that biopsy of the later embryo will be further explored in the future and a simple co-culture system may be helpful to the better pregnancy rate and precision of diagnosis.

The focus of clinical programmes is likely to remain directed to genetically inherited disease using informative DNA sequences and PCR amplification (Chapter 1.4.1). The detection of chromosomal abnormalities by in situ hybridization and karyotypic techniques will require further research (Chapter 1.4.2).

This thesis has addressed the methodology associated with the commonest inherited disease within the Caucasian population and has described for the

first time confident technology able to be applied to the clinical programme. Preimplantation diagnosis of cystic fibrosis illustrates the power of the technology namely the ability to eliminate genetic disease from the family in one generation without interruption of the pregnancy.

The future for preimplantation diagnosis appears bright but great care and high ethics are required for clinical practice to avoid disastrous consequences for couples.

CHAPTER EIGHT

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