CHAPTER I

Introduction

A. General Background

During the past two decades, an increasing concern with respect to human genetic and congenital abnormalities has arisen from the considerations that, first, genetic abnormalities are among the major causes of perinatal and infant mortality and are responsible for a high proportion of infant and adult morbidity. Secondly, couples who are at high genetic risk may protect their quality of life by preventing the birth of an affected child. Thirdly, recent advances in Assisted Reproductive Technology (ART), such as in vitro fertilization; donation and cryopreservation of oocytes/embryos; and micromanipulation assisted fertilization have increased public awareness towards genetic regulation of early embryonic growth and development. Plachot et al. (1987a) estimated the frequency of the chromosome abnormalities in oocytes at 32 %, in sperm cells at 8 %, in fertilized oocytes at 37%, in preimplantation embryos at 20.6 %, in 1st trimester fetuses at 8-10% and in newborns at 0.6 % (figure 1). Indeed, if 0.6 % of living newborns carry a chromosomal disorder (Nielsen, 1975), this incidence is ten times higher in stillbirths (6%) (Machin and Crolla, 1974) and one hundred times (60%) in spontaneous abortions (Boue and Boue, 1976). In spite of the strong pressure

of natural selection, about five million infants (approximately 1-2%) with at least one serious congenital disorder are born in the world annually (Kuliev, 1985). Some are lethal at birth or severely affect the quality of life, while others become apparent in middle age only. Lastly, they may interfere with human reproduction, either by acting directly on gonadal development or indirectly on gametogenesis. Modern medical advances have significantly improved the chances of survival in some diseases, which in turn has led to increasing chronic disability due to lack of prevention and hence to heavier financial and social burdens. To date, prevention has been achieved by genetic counseling and prenatal genetic diagnosis which provide information to help people work out for themselves the best course of action to prevent genetic disorders. Prenatal diagnosis of genetic disorders traditionally involved the screening of fetal diseases after a pregnancy is in progress. Routinely, prenatal diagnosis can be performed in combination with molecular methods for mutation detection employing three approaches: direct imaging of the fetus, such as ultrasonography and fetoscopy; invasive tests to obtain fetal materials such as amniocentesis (Verp and Gerbie, 1981), chorionic villi sampling (Hoggs et al., 1985), cordocentesis (Hobbins et al., 1985): and other methods, such as isolation of fetal cells from the maternal circulation (Lo et al., 1989; Briggs et al., 1995) and coelocentesis (Jurkovic et al., 1993). If the fetus is diagnosed as affected at this stage, the couples have only one of two choices: termination of pregnancy or having a diseased child. However, selective termination of pregnancy in the first or second trimesters is a difficult and

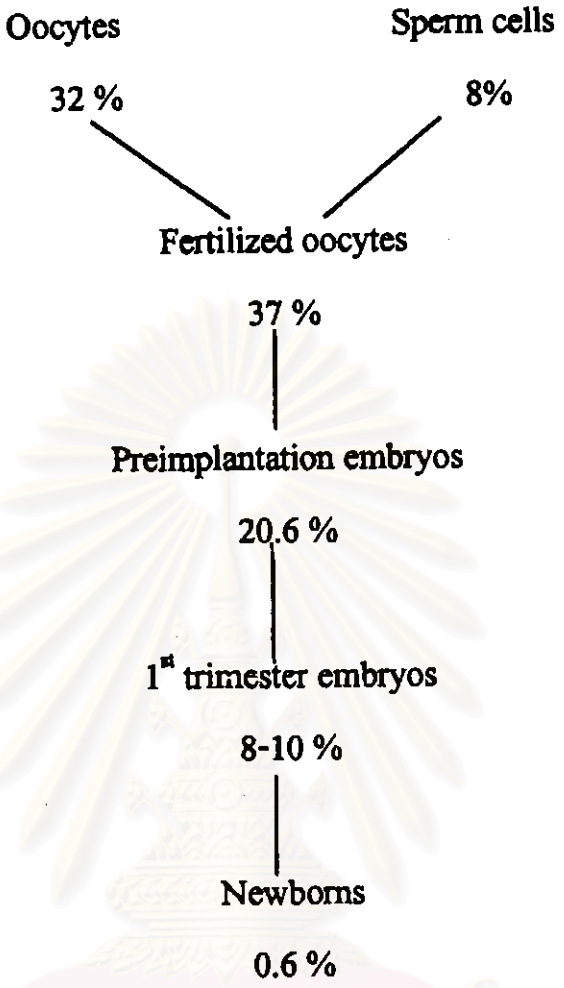


Figure 1 Estimated frequency of chromosome abnormalities in unfertilized oocytes, sperm cells, fertilized oocytes, and various stages of prenatal development. During embryonic development, the rate of chromosome abnormalities decreases during embryonic development, from 37% in the fertilized oocytes to 0.6% in the newborns (from Plachot et al., 1987a)

painful decision faced by the couples and might be unacceptable for moral and some religious reasons.

The recent development in the area of gamete and embryo micromanipulation concurrent with advances in molecular biology techniques have brought forth a new procedure for diagnosing genetic diseases before implantation ,namely, preimplantation genetic diagnosis. The availability of a technique for the diagnosis of genetic diseases in the earliest stage of development of a human embryo will enable the carriers of the mutant genes to avoid a pregnancy with an affected fetus (McLaren, 1985; Whittingham and Penketh, 1987).

B. Development of preimplantation diagnosis

Preimplantation diagnosis refers to a technique whereby the genetic diseases or chromosome abnormalities of an oocyte or early developmental stage embryo are recognized before implantation (Edwards aand Holland, 1988). At first, this approach has been successful with the rabbit blastocyst in which sex chromatin can be identified histologically (Gardner and Edwards, 1968). These techniques have been further developed to determine the gender of cattle blastocysts for application in commercial embryo transfer (Bondioli et al., 1989; Singh and Hare, 1980; Wintenberger and Popesen, 1980). There have been a number of mouse model systems for preimplantation diagnosis. Monk et al. (1987) have diagnosed a deficiency of the X-linked enzyme hypoxanthine phosphoribosyl transferase (HPRT) in mouse preimplantation embryos as a

model for Lesch- Nyhan Syndrome. Morsey et al. (1992) have demonstrated that in mice, preimplantation diagnosis of X-linked recessive mutations could be achieved by blastomere biopsy and followed by DNA-specific sequence amplification. To date, work in preimplantation diagnosis is still largely focused on animal or laboratory studies, with very little direct clinical application. The first successful preimplantation diagnosis in humans has been undertaken for X-linked diseases by Handyside and his collegues in 1989 and for autosomal recessive conditions by Verlinsky et al. in 1990. Since then, after preimplantation diagnosis of some X-linked conditions there have been babies born free from genetic diseases, such as cystic fibrosis (CF)(Handyside et al., 1992; Liu et al., 1994a), haemophillia A (Grifo et al., 1992a) and Tay-Sachs disease (Gibbons et al., 1995). In theory, preimplantation diagnosis could be applied to any genetic disease in which the responsible gene is known. This would provide an alternative for couples wishing to prevent the birth of an affected child, but not willing to consider pregnancy termination.

C. Methods for preimplantation diagnosis of genetic diseases

Preimplantation diagnosis is based on the progress achieved in in vitro fertilization and micromanipulation of gametes and embryos and on methods used to detect specific gene in single cells. This approach can be carried out either with gametes (preconception diagnosis) or embryos. The main reasons for preimplantation diagnosis are to preserve the developmental potential of the micromanipulated oocytes or embryos, to reduce technical losses of the

biopsied specimens and to devise reliable methods for each particular type of analysis.

1. Micromanipulation of gamete and embryo

Micromanipulation technology has provided the means whereby the polar body and/or a single blastomere or trophectoderm cells can be removed and used in genetic analysis (Verlinsky and Kuliev, 1992). These techniques require the use of instruments designed specifically for handling gametes or oocytes under the microscope. Micromanipulation techniques have been used in animal production to multiply embryos and to improve the efficiency of reproductive technology (Baker and Shea, 1985; Barns et al., 1990). For the purpose of preimplantation diagnosis, more than 20 years ago the micromanipulation technique was used for the biopsy of rabbit blastocysts for sex determination by examining the presence of sex chromatin (Gardner and Edwards, 1968). Similar experiments were also carried out in mice (Epstein et al., 1978; Monk et al., 1987), cattle (Betteridge et al., 1981) and humans (Handyside et al., 1990).

2. Preconcepton diagnosis

Preconception diagnosis refers to the genetic analysis of either oocytes or spermatozoa. These approaches provide options for couples at risk of conceiving a genetically abnormal fetus to avoid not only selective abortion of an affected fetus but also fertilization of the oocytes containing a mutant gene.

Preconceptin diagnosis in spermatozoa have been used for sex preselection to prevent X-linked diseases. Several methods exist for identification of X- and Y-bearing spermatozoa which all presuppose the existence of fundamental differences between X- and Y- bearing spermatozoa which can be exploited to enrich one population or the other. These methods include multi-layer discontinuous Percoll density gradient centrifugation (Iizuka et al., 1987), albumin gradient (Ericsson et al., 1973), and flow cytometry (Fantes et al., 1983; Johnson et al., 1993). Of these, flow cytometric separation of X- and Y-bearing spermatozoa is the most promising and the sorting is based on the DNA of X spermatozoa having 2.8% more than the Y spermatozoa (Summer and Robinson, 1976; Edwards and Beard, 1995). Johnson et al. (1993) used flow cytometry cell sorting technology and reported the resulting separation purity, which was evaluated by using fluorescent in situ hybridization (FISH) with both X- and Y-specific probes was 82% for the X-enriched-sorted population and 76% for the Y-enriched-sorted population. They also found that sorted X- and Y-bearing spermatozoa can maintain their viability for several hours after sorting and can be used for in vitro fertilization with oocytes. However, the safety of sorting spermatozoa by flow cytometry for clinical use is of concern because the technique uses two mutagenic treatments, ultraviolet right and a DNA binding agent, Hoechst 33342, chemically know as bisbenzimide. Ultraviolet light has been reported to produce an increase in chromosome structural abnormalities in mouse spermatozoa (Matsuda and Tobari, 1988). Information on the toxicity of bisbenzimide on spermatozoa is lacking. Still, the first clinical pregnancy resulting from applying the flow

cytometric separation procedure for the prevention of X-limited hydrocephalus conjunction with IVF was recently reported (Levison et.al 1995). Although the genetic analysis of a single spermatozoon is technically possible (Li et al., 1988; Arnheim et al., 1990), analyzing the genotype of a single sperm for preimplantation diagnosis is not feasible since spermatozoa are destroyed during analysis. Therefore, preconception diagnosis is currently defined as indirect preimplantation diagnosis which involves the removal and analysis of the first polar body of an oocyte. During normal developmental events, the mammalian oocyte remains in a stage of dictyate arrest for a variable period of time until it is triggered to undergo resumption of meiosis in response to a preovulatory luteinizing hormone surge. The completion of the first meiotic division results in the extrusion of the first polar body, which contains a diploid complement of chromatin. This chromatin is virtually a mirror image of the chromatin of the oocyte. Thus, the assessment of its genetic composition could indirectly determine the chromosome status of the oocytes. The first attempt at visualizing the polar body chromosomes have been undertaken in the mouse model by Modlinski and McLaren (1980). In humans, genetic analysis of the first polar body is technically feasible. The approach is based on removing the first polar body from unfertilized oocytes and performing genetic analysis on its DNA (Coutelle et al., 1989; Verlinsky et al., 1990; 1992; Strom et al., 1990; Munne et al., 1995). The advantages of first polar body analysis are: (1) The first polar body has no known critical function in further development after fertilization of the oocytes; (2) Removal of the first polar body does not cause any damage to the developing embryo as no embryonic materials are

removed. Normal development of the embryo to the blastocyst stage after removing the first polar body was reported (Vrelinsky et al., 1991). Recently, two human pregnancies were reported after in vitro fertilization of unaffected oocytes (International Working Group on Preimplantation Genetics, 1994). However, this method has some limitations for use in genetic diagnosis. A major problem with this scenario is that chromosomal crossing over between homologous chromosomes may occur with high frequency. If crossing over does occur, the primary oocyte would be heterozygous for the two alleles, rather than homozygous for a single allele. Thus, the genotype of the oocyte could not be predicted without further testing either by removal of the second polar body or by blastomere biopsy from the embryos. Another disadvantage of removing of the first polar body is that this procedure only allows the detection of maternal genetic defects, the paternal allele cannot be analyzed. Gender determination is not possible either because the sex of the embryo is determined by the sperm that fertilized the oocyte. The timing is also critical for this approach because of the adverse effects of aging on the ability to successfully fertilize of the oocyte. In these situations preimplantation diagnosis in blastomeres becomes an important adjunct to first polar body analysis.

3. Embryo Biopsy

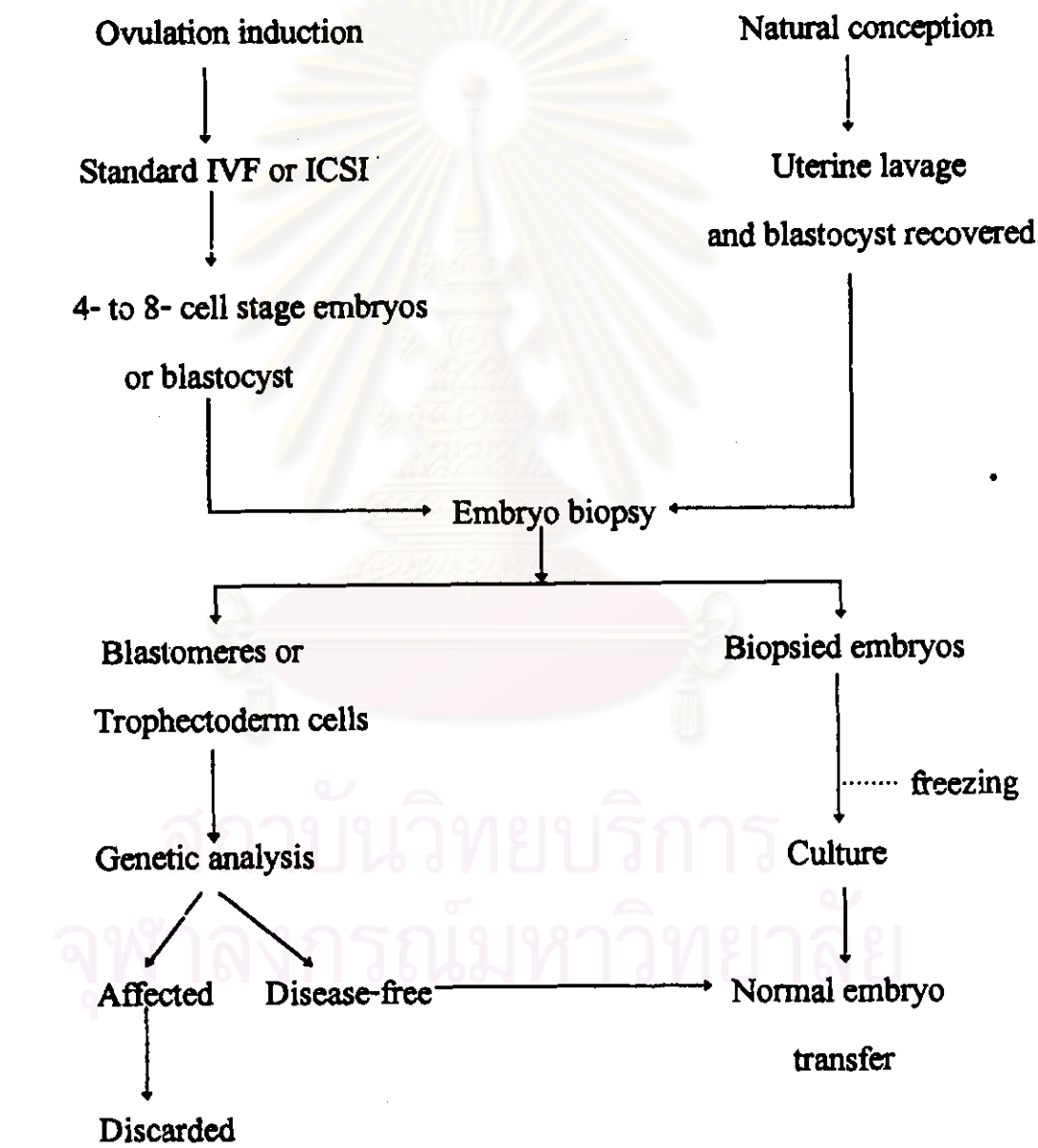
Preimplantation diagnosis of genetic diseases in embryos currently requires the biopsy of one or more cells from embryo. The ability to successfully obtain biopsy samples from embryos depends on the cell stage,

number of cells removed, and biopsy technique. Theoretically, biopsies could be performed at any developmental stages between the two-cell and the expanded blastocyst stage. However, not all these stages have the same prospects of potential success for preimplantation diagnosis. There are two possible sources of embryos for preimplantation diagnosis . First, preovulatory oocytes can be fertilized by standard in vitro fertilization (Edwards, 1985; Tan et al., 1990) or by direct injection of a single sperm into the oocyte' s cytoplasm (Palermo et al., 1992; 1993 ; Van steirteghem et al., 1993a ; 1993b), with the embryos subsequently grown in culture. Secondly, embryos could be collected from donors 5 days after ovulation using uterine lavage (Buster et al., 1985; Brambati and Formigli, 1991). The altogether considerable variety of methods available for preimplantation diagnosis of human genetic diseases are shown in figure 2.

3.1 Cleavage stage biopsy

At the early cleavage stage, each cell of the mammalian embryo remains developmentally totipotent until at least the 8-cell stage and thus can contribute to all the tissues of the conceptus. The experiments on the totipotency of isolated blastomeres initiated by Tarkowski (1959) demonstrated the ability of a partially intact mouse embryo to form a blastocyst. This was achieved by dissociation of embryos at the 4- or 8- cell stage and subsequently culturing to the blastocyst stage. Further experiments have shown that these partially intact embryos can develop into normal offspring following transfer to a recipient female (Papaioannou and Ebert, 1986).

Figure 2 Methods considered for preimplantation genetic diagnosis. (Adapted from Brambati and Formigli, 1991)



These experiments have also demonstrated that the embryo contains more cells than necessary to form an entirely normal term fetus. The observation that dissociated 4-cell embryos give rise to quadruplets indicates that at this stage as many as four times the minimum number of cells required to produce a viable offspring are present. However, this number declines rapidly with cells isolated at progressively later stages because of the commitment of cells to the trophoblast lineage. The ability to safely biopsy embryos thus creates the opportunity to perform genetic diagnosis during cleavage. Epstein et al. (1978) used a similar method to identify the sex of mouse embryos, using one twin embryo to determine the sex of the other, while it is still growing and before it must be replaced in its mother's uterus. However, a reduction of 50% or more of the cell mass frequently results in abnormal differentiation. Many isolated blastomeres developed into trophoblastic vesicles devoid of an inner cell mass (ICM) component (Tarkowski and Wroblewska, 1967; O'Brien et al., 1984). The alternative approach for diagnosis of preimplantation embryos consists of removal of one or two blastomeres from the cleavage stage embryos with subsequent genetic analysis. This method has been applied in mice in order to identify enzyme levels of a genetically defined strain of mice where HPRT was segregating (Monk et al., 1987; Monk and Handyside, 1988). In 2-cell embryos, Nijs et al. (1988) isolated individual blastomeres from 2-cell mouse embryos either by enzymatic or mechanical segregation of the blastomeres after enzymatic removal of the zona pellucida. However, this method had a negative impact on both in vivo and in vitro embryonic growth (Nijs et al., 1988). Wilton and Trounson (1989) developed a technique for removing a

single blastomere from the 4-cell mouse embryos which did not compromise their continued development in vitro or in vivo. They also demonstrated that biopsied mouse embryos could be successfully cryopreserved by ultrarapid freezing even despite a punctured zona pellucida (Wilton et al., 1989). In humans, the first embryo biopsy was reported by Handyside et al. (1989) using Acid Tyrode's solution to drill a hole through the zona pellucida and aspirate single blastomere from a human embryo at the 6- to 10-cell cleavage stage three days after in vitro fertilization. Currently, many successful human embryo biopsied at the 6- to 10-cell stage for preimplantation genetic diagnosis have been reported (Handyside et al., 1990; 1992; Grifo et al., 1992; Harper and Handyside, 1994; Liu et al., 1995). This might be due to the fact that the chance of having 6- to 10-cell stage human embryos is much higher than of having blastocysts after in vitro fertilization. The other advantage of cleavage stage biopsy is that many biopsy techniques can be used including direct aspiration (Wilton and Trounson, 1989; Roudebush et al., 1990), displacement (Roudebush et al., 1990; Krzyminska et al., 1990; Roudebush and Dukelow, 1991; Takeuchi et al., 1992), chemical or mechanical zona drilling and blastomere aspiration (Handyside et al., 1989; Gordon and Gang, 1990; Han et al., 1993). One limitation of cleavage stage biopsy is the small number of cells available for genetic analysis. However, it is possible to increase the number of cells by allowing the biopsied blastomere to proliferate in culture before genetic analysis (Wilton and Trounson, 1989; Geber et al., 1995).

3.2 Blastocyst biopsy

At the blastocyst stage of development, the mammalian blastocyst typically contains 30 (in mice, Smith and McLaren, 1977) to 58-126 (in humans, Hardy et al., 1989) cells which have initiated differentiation into two distinct cell types: an outer epithelial layer of trophectoderm (TE) and an inner cell mass (ICM). Following implantation, the TE gives rise to components of the placenta and extraembryonic membrane while the ICM contributes to all three germ layers of the fetus as well as to the extraembryonic membrane (Gardner and Papaioannou, 1975). On the assumption that the excised cells are not essential for the growth of the embryo, it is possible to selectively excise a small piece of TE from the blastocyst. The excised cells are then used to type the embryo. This constituted the method first used to type mammalian embryos, when the sex of rabbit blastocysts was diagnosed, and the embryos were thereupon replaced in recipient females (Gardner and Edwards, 1968). Its feasibility was confirmed in mice (Gardner, 1985; Monk et al., 1988; Nijs and Van Steirteghem , 1990; Carson et al., 1990), cattle (Betteridge et al., 1981), and small primates (Summers, 1988). Carson et al. (1993) reported that the optimal blastocyst biopsy technique appears to be zona incision at the pole opposite the ICM followed by excision of the herniated trophectoderm cells. One obvious advantage of blastocyst biopsy is that at this stage the embryo attains the maximum number of cells, therefore, more cells can be biopsied for analysis and the accuracy of the preimplantation genetic diagnosis can be increased in comparison with using the first polar body or the blastomeres of the cleavage embryos.

Dokras et al. (1990) first reported successful biopsy of human "spare" IVF blastocysts cultured in vitro from which 10-40 trophectoderm cells had been excised. Although subsequent development of these biopsied blastocysts was neither impaired in terms of morphology nor regarding in vitro production of human chorionic gonadotropin and furthermore showed an increased rate of hatching, no human blastocysts have been transferred after biopsy (Dokras et al., 1991). Blastocysts can be obtained following IVF procedures which requires an extended period of culture. However, a relatively low percentage (maximum 25-50%) of normally in vitro fertilized oocytes develop to the blastocyst stage (Bolton et al., 1989; Lopata and Hat, 1989; Hardy, 1992) and of those embryos which reach the blastocyst stage on day 5, only 45% can be successfully biopsied (Dokras et al., 1990). This substantially reduces the number of embryos available for analysis and ultimately the number of unaffected embryos available for transfer, thereby reducing the chance of establishing a pregnancy. Alternatively, blastocysts can be obtained by means of uterine lavage of either natural cycles or following ovulation induction (Brambati and Tului, 1990; Formigli et al., 1990; Brambati and Formigli, 1991). As a result of natural fertilization the zygote undergoes a rapid succession of mitotic divisions as it moves down the fallopian tube and into the uterine cavity, from where it may be recovered at the blastocyst stage for trophectoderm biopsy. It has been argued that biopsy of such selected embryos may be more efficient, since due to IVF procedures those embryos with limited development potential have already been eliminated.

There are, however, some disadvantages of blastocyst biopsy. For example, there are a lot of cell to cell interactions in the developing blastocyst, the cells become very sticky, making their manipulation technically difficult. Moreover, there may be limited incubation time available for genetic diagnosis. In addition, blastocyst transfer has only recently been successful in humans yielding a low and inconsistent percentage of pregnancy rate (Dawson et al., 1988; Bolton et al., 1991; Menezo et al., 1992a; 1992b).

It appears that embryo biopsies in both cleavage stage embryo and blastocyst are possible for human application, but both embryo biopsy procedures exhibit various advantages as well as disadvantages (table 1).

4. Genetic diagnostic techniques

Preimplantation genetic diagnosis relies on the assumption that the removed cell is representative of the remaining cells of the embryo that will be transferred to the patient after analysis. For this to be true, the molecules assayed must be embryonic and not maternal in origin, and each cell of the embryo should be identical biochemically. However, the most difficult factor in preimplantation genetic diagnosis is precision of analysis. Tests for genetic abnormalities can be performed at one of two levels: (1) direct detection of the underlying defect in the DNA or an associated abnormal region; (2) detection of the abnormal protein product of the gene, which, if in the form of an enzyme, can be recognized by it abnormal level of activity.

Table 1 Advantages and disadvantages of different stages of embryo biopsy for preimplantation genetic diagnosis.

Stage of embryo	Advantage	Disadvantage
Cleavage stage biopsy	* cell totipotency	* limited number of cells to be removed
	* sufficient time for diagnosis	* greater reduction of cellular mass
	* high pregnancy rate	
Blastocyst biopsy	* many biopsy techniques	* exist between TE and ICM
	* cell differentiation	* limited time for diagnosis
		* manipulation technically difficult
	* greater cell number to be removed	* low pregnancy rate
	* obtained by uterine lavage	

The various methods currently provide new tools for genetic analysis of single cells, including karyotyping, microenzyme analysis, sex chromatin body or F body, and DNA hybridization such as polymerase chain reaction (PCR)and in situ hybridization (ISH). Three types of preimplantation genetic diagnosis could be performed: embryo sexing, detection of either specific single gene defects or chromosome abnormalities.

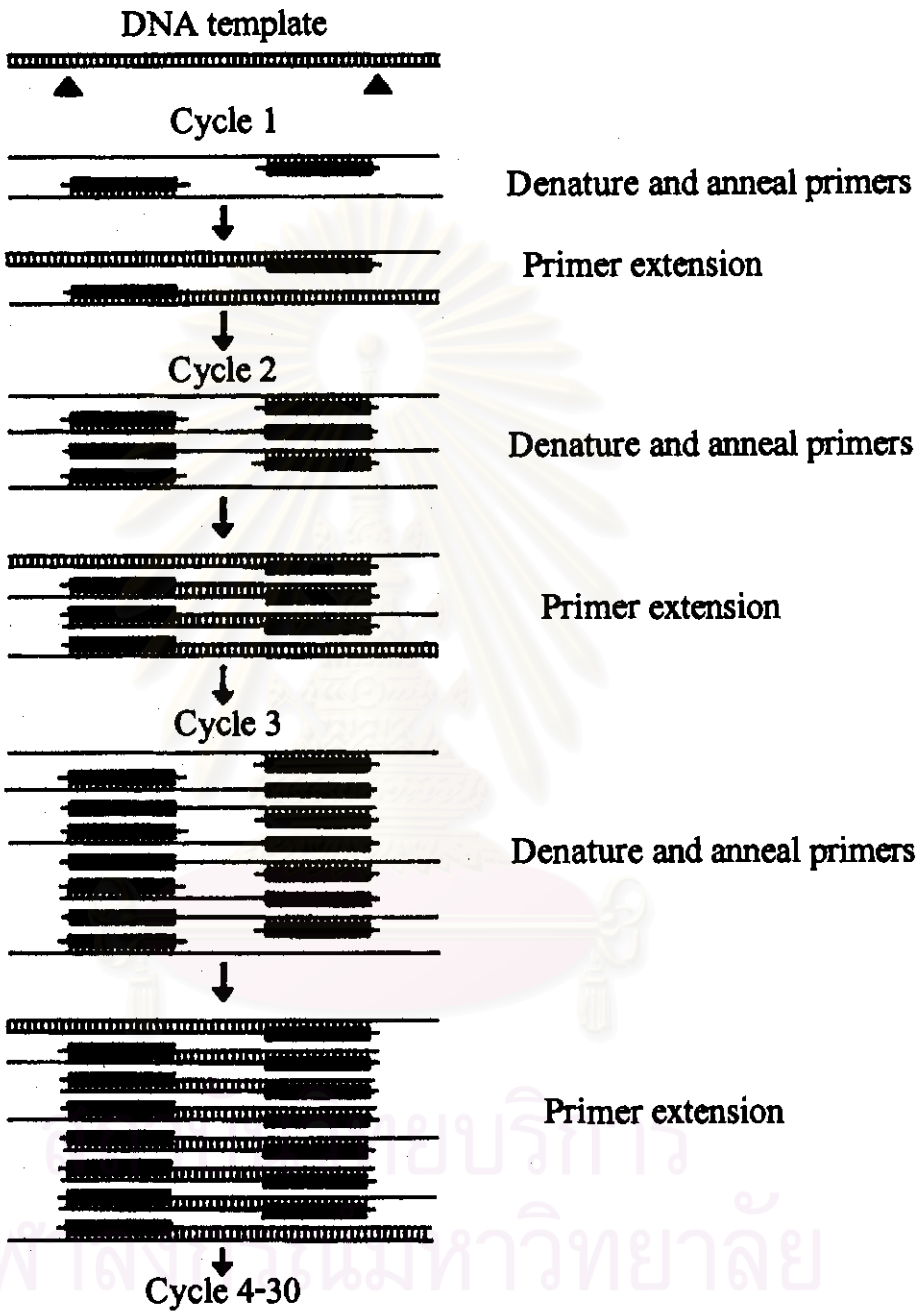
4.1 Embryo sexing to avoid X- linked disease

In humans, more than 100 clinical disorders have been mapped to chromosome X (Frezal and Schinzel, 1991) and approximately 300 X-linked recessive disorders have been characterized by transmission via largely unaffected female carriers to their affected male offspring (McKusick, 1992). Among these are some of the most tragic disabilities such as Lesch-Nyhan syndrome, the muscular dystrophies, Fragile X syndrome and Hunter's syndrome. Female carriers of an X-linked recessive allele have a 1 in 4 chances of having an affected child and a 1 in 2 chances in case of a male child (Zarutskie et al., 1989). However, specific diagnoses are available for only a few of them and the majority of X-linked diseases have to be diagnosed on the basis of sex determination. Since half of the male offspring will be affected, preimplantation gender determination of embryo offers the opportunity to start a normal pregnancy after transfer of unaffected female embryos. The advantage of identifying the sex is that it is equally applicable to any X-linked recessive disease and does not require the identification of the specific genetic defect in each family at risk for any particular disease. There are two basic diagnostic

techniques currently used for sex determination of human preimplantation embryos. The first involves amplification of known DNA sequences by the polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis and Faloona, 1987; Saiki et al., 1988). The second involves detection of one or both sex chromosomes in an intact nucleus by in situ hybridization (ISH).

a) The polymerase chain reaction

The polymerase chain reaction (PCR) is an in vitro method for enzymatical amplification of a specific DNA sequence (Saiki et al., 1985). The reaction uses two oligonucleotide primers that hybridize to opposite strands of the target DNA to allow the PCR to logarithmically amplify the internal sequence (Mullis et al., 1987)(figure 3). The application of PCR for sexing human embryos, as first described by Handyside et al., (1989; 1990) consists of amplifying a repetitive sequence on the long arm of the Y chromosome of single blastomeres. Diagnosis was made on the basis of the presence or absence of the Y-specific fragment as visualized on a polyacrylamide gel and an embryo was diagnosed as female if no Y-specific band was present. This approach led to the successful transfer of female embryos to mothers who were carriers of serious X-linked disorders (Handyside et al., 1990). However, in one case, failed amplification of the Y-specific band led to misdiagnosis. This fetus was diagnosed as male by CVS and subsequently terminated . The failure of the PCR for technical or other reasons will also result in the absence of the Y-specific band. Therefore, the absence of the Y-specific amplification product



At least 10^5-fold increase in DNA

Figure 3 A schematic representation of the polymerase chain reaction. The DNA fragment to be amplified is shown by triangles. In the first PCR cycle the double stranded DNA template is converted to single stranded form (denatured). A pair of oligonucleotide primers then hybridize to a specific nucleotide sequence in each of the separated DNA stranded. The hybridized primers serve as starting point for a DNA polymerase enzyme, which copies both The DNA template strands by adding deoxynucleoside triphosphate to the end of the primer. In the next PCR cycle the new double stranded DNA molecules are denatured, the primers hybridize to the separated DNA strands, and again the DNA polymerase makes a copy of each of them. The number of DNA melecules is thus doubled in each PCR cycle. After 20 to 30 PCR cycles millions of copies of the DNA fragment flanked by the primers have been synthesized. (adapted from Perkin Elmer Cetus, 1990)

may indicate either the presence of a female embryo or failure of the amplification process. To overcome these difficulties, two strategies were developed by several groups. In the first strategy, simultaneous amplification of X- and Y-specific sequences derived from the same single blastomere is performed (Strom et al., 1991; Kontogianni et al., 1991; Grifo et al., 1992a; Liu et al., 1994). Female embryos are characterized by a single X-specific amplification product, whereas male embryos are characterized by the presence of both X- and Y-specific amplified bands. When the assay fails or nuclear material is not present, no band will be detected. Since there has been evidence in other systems that amplification from different primer pairs can fail independently of each other (Li et al., 1988; Boehnke et al., 1989), the second strategy was developed. It relies on single amplification of two homologous X- and Y-linked genes or pseudogenes with the same primers, such as amelogenin (Nakahori et al., 1991a; 1991b; Levinson et al., 1992), ZfY/ZfX (Chong et al., 1993) which yield fragments of different size in males and females.

b) In Situ Hybridization

The development of in situ hybridization (ISH), with nucleic acid probes specific for a given chromosome, allows direct visualization of the DNA sequences of interest in metaphase or interphase nuclei. ISH has been applied in blastomeres removed from bovine embryos in an effort to detect the Y chromosome in the absence of a metaphase chromosome spread (Leonard et al., 1987). The use of ISH to sex human preimplantation embryos was first achieved in the late 1980's using Y-specific probes (Jone et al., 1987; West et

al., 1987; 1988). Initially, ISH was carried out with radiolabelled probes and detected post-hybridization by autoradiography, therefore, it takes several days before the results are known, which is not convenient for preimplantation diagnosis.

However, over the past few years, with the development of non-radioactive methods the results can be obtained within a day (Pinkel et al., 1986; Penketh et al., 1989; Pieters et al., 1990; Grifo et al., 1990). Briefly, non-radioactive in situ hybridization involves modifications of the DNA probe with a reporter molecule by enzymatic or chemical methods (such as biotin, digoxigenin, or dinitrophenol). The labeled probes, once denatured, are hybridized withdenatured cells fixed to a glass slide. After hybridization, the bound probes are detected by fluorescently labelled reporter-binding molecules (such as fluorescein isothiocyanate, FITC; or rhodamine) which give different color signals (Litcher and Cremer, 1992). The fluorescence in situ hybridization (FISH) can also offer analysis of multiple chromosomes which requires the simultaneous use of multiple DNA probes labeled with different fluorochromes. This technique has been successfully applied for determination of embryo sex and chromosome aneuploidies in single blastomeres (Grifo et al., 1992b; Munne et al., 1993a; 1993b; 1994; Griffin et al., 1991; 1992; Delhanty et al., 1993; Schrurs et al., 1993; Harper et al., 1996). The advantages of using FISH are: (1) the procedure can now be completed within two to four hours (Coonen et al., 1994; Harper et al., 1994) thus allowing embryos to be transferred to the patient on the same day of analysis, (2) this approach

includes information on the copy number of chromosomes present and hence detection of aneuploidy and polyploidy since chromosome-specific probes are now available for more than half of the autosomes as well as for the sex chromosomes (Cremer et al., 1986; Pieters et al., 1990) and (3) the fact that it can also be used on both interphase and metaphase nuclei. Although FISH appears to be ideal for determining sex and detecting aneuploidy in preimplantation embryos, significant technical problems exist. Grifo et al.(1992b), Griffin et al. (1992) , and Munne et al. (1993) reported the inability to retain about 10% of intact blastomeres on the slide, apart from difficulties during embryo biopsy.

c) Other procedures

Several non-invasive methods have been applied to sex preimplantation embryos. Initially, Gardner and Edwards (1968) reported the first preimplantation diagnosis by gender determination of rabbit blastocysts. They karyotyped the interphase nuclei for sex chromatin body or the F body, which is detectable in the majority of female cells with an inactive X chromosome at this stage. But the F body identification was unsuitable to sex human embryos (Edwards, 1972; West et al., 1987) because the timing of X chromosome inactivation is unknown in the human and sex chromatin is only visible in a proportion of female cells.

The histocompatability Y (H-Y) antigen, a putative Y chromosome-specific gene product, has been used by White et al. (1987) to sex animal embryos. Various subsequent studies confirmed the use of H-Y antigen for sex

selection of porcine (White et al., 1987a), bovine (White et al., 1987b), and ovine (White et al., 1987c) embryos. However, in none of the previous H-Y assays 100% accuracy was achieved and the results were not reproducible.

Another approach is to sex embryos by measuring differences in X-linked gene expression. The rationale for this approach is based on the phenomenon of X-chromosome inactivation. In mammals, X-inactivation takes place to compensate for the fact that both X chromosomes are active in female embryos for a short period of time in morulae and blastocysts (Lyon, 1974). During this period, female embryos contain twice the dosage of sex-linked genes or gene products compared with males. Epstein et al. (1978) used the sex-linked enzyme HPRT to identify male and female mouse blastocysts. The sexing procedure depended on calculating the ratio between the levels of HPRT and an autosomally-regulated emzyme, adenine phosphoribosyl transferase (APRT). Monk et al. (1987) and Benson and Monk (1988) used the same approach for microassay of these enzymes in single blastomeres or in several cells removed from blastocysts to detect HPRT in mice to be used as a model of the Lesch-Nyhan syndrome in humans. Monk et al. (1988) subsequently reported their success in identifying embryo sex by this means. Microassay of the sex-linked enzyme B- N- acetylhexosaminidase was also feasible in mouse embryos (Sermon et al., 1991). Unfortunately, when the assays for HPRT and B-N- acetylhexosaminidase were evaluated on preimplantation human embryos (Braude et al., 1989; Sermon et al., 1992), throughout development, constant enzyme levels were found in human oocytes or embryos and in spent media.

Therefore, they could not identify the expected transition from maternal to embryonic enzyme activity by this assay, making it unsafe to assume that any activity detected in a biopsied blastomere was embryonic and not maternal. In humans, during the early stage of development, the embryo is under the direction of the maternal genome and does not express embryonic genes until the four- to eight-cell stage of development (Braude et al., 1988; Tesarik et al., 1988). Moreover, not all genes are expressed at this early stage; many are not expressed until the blastocyst stage or later, therefore, this approach cannot be extended to testing for genes not expressed during early development. These evidences indicate that microenzyme assays cannot be applied to human preimplantation genetic diagnosis.

Diagnosis of embryo sex by karyotyping provides the basic assessment of the gross morphology of chromosomes and has been the standard technique for prenatal diagnosis of developmental abnormalities, as well as potential genetic disease conditions (Tarkowski, 1966). This technique has been applied in preimplantation embryos by many working groups (Watt et al., 1987; Roberts et al., 1990; Selva et al., 1991). Despite numerous attempts, such conventional cytogenetic analysis of human embryos has been only partly successful (Plachot et al., 1987a; 1987b; Bongso et al., 1991). The primary limitations of this technique to be applied in human preimplantation diagnosis are: (1) blastomeres have to be arrested at the metaphase stage. It is difficult to prepare adequate metaphase from a limited number of cells, and (2) the chromosomes

are short and often clumped together, resulting in poor quality of chromosome preparations (Angell, 1989; Zenzes and casper, 1992; Jamieson et al., 1994).

4.2 PCR - based diagnosis of single gene defects

Preimplantation diagnosis for detecting single gene defects may be attempted either by the use of linked markers or, if the gene has been cloned, by the use of specific primers. For both approaches, a high efficiency of DNA amplification is required. PCR is so sensitive that a single copy gene can be amplified. Theoretically, PCR can be used for any genetic disease if the mutant gene is known. A mouse model for diagnosis of human beta-thalassemia has been applied using nested PCR to amplify DNA in single excised blastomeres (Holding and Monk, 1989). The first clinical application was in couples at risk of transmitting cystic fibrosis. Accurate detection of the common delta- F508 mutation is possible using nested PCR to amplify the affected exon from single cells followed by heteroduplex formation (Lesko et al., 1991; Liu et al., 1992; 1993) The birth of the first unaffected child following embryo selection using this method was reported in 1992 (Handyside et al., 1992). A similar approach is possible for Tay-Sachs disease (Sermon et al., 1995) and Duchenne Muscular Dystrophy (DMD) and unaffected child have been born free of DMD (Gibbons et al., 1995; Liu et al., 1995).

D. Genes involved in sex determination

In mammals, the sex difference between males and females is controlled by the presence of a Y chromosome, regardless of the number of X chromosomes. Koller and Darlington (1934) observed that X and Y chromosomes are dissimilar but could pair along part of their lengths. They proposed that the Y chromosome is composed of a pseudoautosomal region (PAR) and a Y-specific region. In humans, the PAR comprises the terminal region on the short arms of the X and Y chromosomes and is required for correct pairing during male meiosis (Pearson and Bobrow, 1970; Ellis, 1991). The male determining activity of the Y chromosome has been attributed to a gene or genes termed testis-determining factor (TDF, in humans) or testis-determining Y (Tdy, in mice) gene located in the Y-specific region. During embryogenesis TDF is activated to commit the undifferentiated genital ridge to the testicular pathway and in its absence female characteristics develop. As a consequence of testicular formation, subsequent hormone production induces male sexual differentiation. By using 135 human Y-specific DNA probes, Page et al. (1987) made a chromosome walk and defined the region in which TDF must lie as 140-280 kilobases (kb) from the PAR boundary (figure 4). This gene was later named zinc finger Y (ZFY, Page et al., 1988). The ZFY encodes a putative protein containing several zinc fingers and activating domain proteins of similar structure are known to be transcription factors.

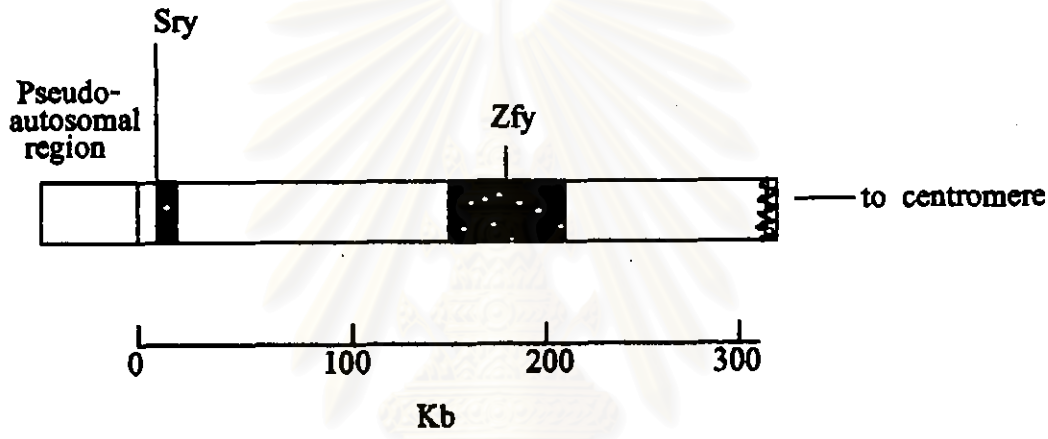


Figure 4. Localization of the sex-determining region, Sry and the zinc finger gene, Zfy on the Y-chromosome (from Harley and Goodfellow, 1994)

Schneider-Gadicke and his colleagues also isolated and cloned the homologous sequences from the X chromosome (ZFX). The base composition of this gene has a high degree of similarity with its Y counterpart. Moreover, the ZFX also showed the same origin and almost the same exon spacing as ZFY. Initially, the ZFY gene was proposed to be a good candidate for TDF. The data on mice provided further support for this assumption. Mardon et al.(1989) and Nagamine et al. (1989) found two Zfy loci in mice, Zfy-1and Zfy-2, known to contain Tdy. There is, however, some further evidence which conflicts with the hypothesis equating TDF and ZFY. Since it has been shown that the gene homologous to ZFY is autosomal in marsupials (Sinclair et al., 1988) and furthermore, the expression pattern of Zfy-1 is inconsistent with its role in sex determination. Although Zfy-1 transcripts are detected in the fetal testis, no Zfy-1 transcript is detected in the gonads of male mouse embryos that develop testes lacking germ cells. Accordingly, Zfy-1 expression was proposed to be necessary for germ cells but not for testis development (Koopman et al., 1989). Simultaneously with the finding in mouse embryos, Palmer et al. (1989) found four XX human males who had a Y segment on an X chromosome but lacked the ZFY sequence in their genomes.

The four patients described by Palmer et al. (1989) had inherited only 35-40 kb of Y-specific sequences. This implies that the TDF must be located within approximately 35 kb adjacent to the boundary with the PAR. A search of this region led to the discovery of a highly conserved sequence that mapped to the Y chromosome of all placental mammals tested. This sequence formed

part of the gene which was named SRY (gene in the sex determining region of the Y) in humans, and Sry in mice (Sinclair et al., 1990; Berta et al., 1990; Gubbay et al., 1990). There is now convincing evidence that this gene is genetically and functionally equivalent to TDF/Tdy. DNA sequencing of the SRY gene in humans, rabbits, and mice revealed the presence of a conserved open reading frame known as HMG box. This indicated that SRY is a member of the HMG box containing DNA-binding proteins which play a role as tissue - specific Pol II transcription factor (Harley and Goodfellow, 1994). The DNA - binding properties of SRY have been investigated in relation to genes involved in gonadal differentiation((Haaq et al., 1993).

E. Aim of the study

1. To compare the embryo biopsy techniques and assess their respective effectiveness as to the developmental potential of biopsied mouse embryos both in vitro and in vivo particularly since embryo biopsy constitutes a crucial step in preimplantation diagnosis.

2. To compare in vitro and in vivo the developmental potential of embryos biopsied at the 4-cell, 8-cell, and the early morula stages.

3. To assess the reliability of sex determination by simultaneously detecting of X- and Y-chromosome specific sequences in a single mouse blastomere using PCR analysis.

F. Experimental design

This thesis study was divided into three sections according to its aim.

1. Section I

This section compared in vitro and in vivo developmental potential of mouse embryos biopsied at the 4-cell, 8-cell, and morula stages by means of two different mechanical biopsy techniques, direct aspiration and PZD-push. Thus it could be ascertained which technique would be safest to perform at what particular stage of embryo and therefore, would be most applicable to human preimplantation genetic diagnosis.

2. Section II

The accuracy and sensitivity of the selected oligonucleotide primers and the optimal conditions for the polymerase chain reaction assay for detection of X- and Y-chromosome specific sequences in a single mouse blastomere were tested using two-step (nested) PCR.

3. Section III

The reliability of sex determination in a single blastomere was assessed by transfering sexed embryos to the uterine horn of pseudopregnant recipients and allowing them to develop to full term. The sex of newborn mice was phenotypically determined and compared with the results obtained by PCR.