CHAPTER IV

Discussion

A. Effects of biopsy procedure on embryo development

Preimplantation genetic diagnosis (PID) refers to a technique whereby the genetic diagnosis of an oocyte or an early cleavage-stage embryo is carried out before implantation. This diagnostic procedure requires the micromanipulation techniques that can be used for removing tiny cells from oocytes or embryos for analysis and molecular genetic techniques, which can be used for analysis of chromosome abnormalities, gender determination and specific single-gene defects. The safety of embryo biopsy and the precision of diagnosis are important issues and remain crucial scientific and ethical questions for the advancement of PID in humans (Trounson, 1992). The use of mouse embryos to standardize the technique of embryo biopsy for human application is now widely utilized. Mouse preimplantation embryos can tolerate a significant reduction in cell number and retain their developmental potency. This has been demonstrated for early stage embryos in which a reduction in up to half the cell number was compatible with postnatal development (Papaioannou et al., 1989). However, the use of two-cell stage embryos for PID in humans has not been utilized. This is probably associated with the high risk of damage to the embryo (there are only two cells) and the

delay in embryonic gene expression, which may be required for some analysis. Two studies (Tsunoda and McLaren, 1983; O'Brien et al., 1984) support the hypothesis that a critical number of cells is necessary for the development of a viable fetus.

A method of embryo biopsy used for PID must not have deleterious effects on embryo development both in vitro and in vivo. Several different methods of embryo biopsy have been reported to date. The most widely reported technique is direct penetration of the zona and aspiration of a blastomere with a beveled micropipette (Wilton et al., 1989; Vertinsky et al., 1990; Roudebush et al., 1990). Aspiration is also possible by opening the ZP by zona drilling with a direct flow of acidified Tyrode's solution (pH 2.3-2.5) or by partial zona dissection (PZD) with a sharpened microneedle. As an alternative to aspiration, the blastomere can be displaced through the ZP opening with a flow of medium from a micropipette inserted through the ZP, so called displacement (Roudebush et al., 1990; Takeuchi et al., 1992; Roudebush and Dukelow, 1991) or by exerting pressure against the ZP with a blunt-ended micropipette (Gordon and Gang, 1990; Han et al., 1993). However, not all have the same potential of success for PID because embryo and/or blastomere viability may be affected after biopsy. Even though the development of mouse embryos is not affected following the use of acidic Tyrode's solution to open the ZP, the application in human embryos appears to be limited, due to the increased size and resilience of the ZP and the limited buffering capacity of the small amount of perivitelline fluid. Microvilli on the

plasma membrane are reduced by the acidic solution, and blastocyst formation is inhibited (Malter and Cohen, 1989).

A few comparative studies of different methods of embryo biopsy have been reported, such as displacement versus aspiration methods (Roudebush et al., 1990; Takeuchi et al., 1992) no comparative study has been reported using the push and aspiration techniques. This study was therefore conducted to compare direct aspiration and PZD-push techniques with respect to efficacy and efficiency of embryo biopsy procedures. In the direct aspiration method, a single blastomere is removed by direct puncturing through the ZP and aspiration with a beveled-micropipette (figure 7 and 8). The PZD-push technique comprises partial dissection of the ZP, squeezing of a single blastomere through the ZP slit by pushing against the ZP at some distance from the ZP opening using three types of micropipettes (figure 9, 10 and 11). The results show that the PZD-push technique appears to be superior to the direct aspiration technique. The PZD push biopsy technique offers several advantages over direct aspiration methods. The preparation of biopsy pipettes is simple and does neither require beveling as is necessary with the direct aspiration method, nor the use of three sets of micromanipulators for simultaneous control of the holding, zona dissecting, and biopsy micropipettes to avoid changing micropipettes during biopsy. The biopsy procedure is relatively rapid compared to other methods that used zona drilling with acidic Tyrode's solution combined with aspiration (Gordon and Gang, 1990) or displacement (Pierce et al., 1997). Gordon and Gang (1990) reported that the

time required for embryo biopsy by zona drilling was between 4 sec and 3 min. They separated the blastomere attached to other blastomeres by tapping the micromanipulator so as to create a sudden vibration. Therefore the time required for the process could be prolonged to 3 min, depending upon the status of the protruding blastomere attached to other blastomeres. However, in the PZD-push method, a single blastomere clinging to other blastomeres could be easily detached from the embryo by sucking it into the biopsy micropipette and then expelling it into the surrounding medium. Consequently, embryo biopsy by this method could be accomplished in less than 1 min per embryo as is the case with direct aspiration. The high rate of subsequent development of biopsied embryos also reflects the minimal damage of the embryos. It has also been reported that the PZD method was employed for opening the ZP of human oocytes to circumvent the problem of acid Tyrode's solution (Garrisi et al., 1990; Malter and Cohen, 1989).

Since the purpose of embryo biopsy is to obtain an intact blastomere for DNA analysis, this is an important consideration. Direct aspiration results in a much greater incidence of traumatic damage to the blastomeres that are being removed (data not shown). Blastomere damage during biopsy could be attributed to secondary contact between the blastomere membrane and the glass micropipette (Hendl, 1987). With the PZD push technique the blastomeres are pushed through a hole in the ZP and subsequently aspirated by means of a small suction in the tip of the biopsy pipette, which appears to be less traumatic than direct aspiration.

Because the development of mammalian embryos is characterized by the commitment of cells to a specific pattern of differentiation, followed by execution of a genetic program that leads to establishment and maintenance of a specific, terminally differentiated phenotype. The first and most important of such developmental sequences leads to the formation of the blastocyst (blastulation). Blastocyst formation is especially significant for two reasons. First, commitment of cells to the ICM and TE can occur normally during in vitro culture. Second, if blastocyst formation does not proceed correctly and within the appropriate time frame, implantation cannot occur and the embryo dies. In the mouse, the allocation of embryonic cells to the ICM and TE occur during the fourth (Handyside, 1981) and fifth cleavage divisions (Balakier and Pedersen, 1982). So far, the vigorous timing of blastocyst formation allows embryos to survive when one or two blastomeres have been removed during the early cleavage stage for PID.

Several studies have reported the rate of normal blastocyst formation as the observed end point of in vitro development after removal of a single blastomere from preimplantation embryos (Wilton et al., 1989: Kryzyminska et al., 1990; Takeuchi et al., 1992). In this study, no significant difference was found with regard to normal blastocyst formation and hatching blastocysts in embryos that were biopsied at either the 4-cell, 8-cell or the morula stage by both embryo biopsy techniques when compared with their untreated controls. The results imply that neither PZD-push nor direct aspiration biopsy procedures are necessarily deleterious to in vitro development in the mouse.

However, the rates of those completely hatched from the ZP of the hatching blastocyst after biopsy had been performed by direct aspiration were significantly lower than those of the control groups at all three stages of biopsied embryos. These findings are in agreement with those of Cui et al. (1993) and thus the rate of completely hatched blastocysts was considered a sensitive end point for the discrimination of in vitro developmental potency of biopsied mouse embryos. The ability of biopsied embryos to develop into blastocysts after the PZD-push technique was not significantly different from that observed after application of the direct aspiration technique (table 7). Although not statistically significant, a higher percentage of embryos did survive biopsy at the 4-cell, 8-cell and morula stages and developed to the blastocyst stage when the biopsy procedure was performed with the PZD-push technique. The trend towards a lower rate of blastocyst development in the direct aspiration group of embryos suggests that this may be a more traumatic method of biopsy than zona slitting. Since the direct aspiration requires the beveled biopsy pipette with a spike on its tip for puncturing through the ZP into the embryo, this spike may pierce the plasma membrane of adjacent blastomeres and disturb its development. However, the rates of completely hatched ones in the direct aspiration group were also significantly reduced, implying that the hatching process may be more abnormal following embryo biopsy with the direct aspiration technique and thus leading to a significant decrease in the implantation and live birth rates after transfer of these biopsied embryos into the uteri of foster mothers.

Naturally, the ZP, or outer glycoprotein layer surrounding the mammalian oocyte and embryo, is of structural and functional importance during fertilization and preimplantation development. The function of the ZP during embryo cleavage seems to be mostly of a physical nature: to prevent dispersal of the blastomeres and avoid direct contact between the embryo and foreign cells whether these be the epithelial lining of the reproductive tract, leukocytes, spermatozoa, or the cells of other embryos (Moore et al., 1968; Bronson and McLaren, 1970; Modlinski, 1970). The presence of the ZP is clearly not essential for cleavage, since denuded embryos can form viable morulae and blastocysts in vitro (Bronson and McLaren 1970; Trounson and Moore, 1974; Rottmann and Lampeter, 1981). Nevertheless, its removal from early cleaving embryos prior to their transfer to the oviduct, has been found to result in the complete failure of development in vivo (Moore et al., 1968; Modlinski, 1970). All mammalian embryos must hatch from the ZP in order to be able to implant in the uterus at the blastocyst stage of development (McLaren, 1982; Renfree, 1982). Two factors have been identified as possible mediators of hatching: lysis of the ZP by substances secreted either form the embryo or female reproductive tract and pressure exerted on the ZP by expansion of the blastocyst. The role of lysins in the hatching process is supported by the findings of Perona and Wassarman (1986) that a trypsin-like proteinase termed strypsin is secreted by the mural tropectoderm of the blastocyst immediately before hatching. Moreover, a trypsin-like protease can be identified in culture medium in which mouse embryos have been allowed to hatch (Sawada et al.,

1990) and addition of protease inhibitors to culture medium can inhibit hatching in vitro (Dabich, 1981; Yamazaki et al., 1985).

The concept of mechanical pressure exerted on the zona by the expanding blastocyst is supported largely by cinematographic studies performed by Cole (1967) and by the documentation of enzymatic mechanisms actively pumping fluid into the developing blastocoele cavity (Wiley, 1984). Cole showed that mouse blastocysts contract and reexpand in cycles. Such cyclic changes have also been observed in sheep, cows, and humans. In the mouse, contraction is achieved in 4-5 min, while expansion requires several hours. Due to the elasticity of the ZP, it becomes extremely expanded, attenuated and almost invisible during the contraction-reexpansion cycle (Cole, 1967). This dynamic activity, which could clearly exert mechanical force against the ZP at the time when hatching is imminent, has convinced most investigators that the pressure of blastocyst expansion plays at least some role in the hatching process (Cohen, 1991; Wiley, 1984). The successful hatching of the embryo is thought to be a key event in the implantation process. Among the numerous reasons that could explain the low implantation rate of embryos used for in vitro fertilization, an impairment of in vivo hatching has been proposed as a possible cause of implantation failure (Cohen et al., 1990). The present study found that the ZP of biopsied embryos does not thin as it normally does during blastocyst expansion and hatching occurs earlier and at a higher frequency. The premature hatching of biopsied embryos usually preceded the hatching of nonbiopsied embryos by approximately 24 hr. This finding is in agreement

with the results of Malter and Cohen (1989) who performed assisted hatching by breaching of the ZP using PZD or zona drilling with acid Tyrode's solution in mouse and human preimplantation embryos. However, many biopsied embryos exhibited incomplete hatching.

Due to lack of zona thinning and expansion during expulsion of the blastocyst through the artificial hole in the ZP, many biopsied embryos become trapped between the thick ZP ridges surrounding the hole and form a figure of eight configuration. Whether hatching is completed depends on the size of the opening and the type of procedure. PZD technique can produce a range of different hole sizes, depending on the position of the microneedle threaded through both sides of the ZP. Cohen and Feldberg (1991) found that only 16 % of PZD embryos migrating through narrow (<10 μm) holes hatched completely whereas 72 % of those migrating through the larger (11-25 μm) PZD holes hatched completely. In this study, the opening in the ZP made by direct aspiration shows a small round hole, whereas the PZD opening has the appearance of an elongated slit of the same shape as, yet shorter than the natural opening in the control groups (figure 16) and therefore more PZD-push biopsied embryos than those from the direct aspiration groups hatched completely from the ZP. While the appearance of the embryos during the hatching process itself was somewhat unusual, the blastocysts which did hatch completely from both biopsied (including PZD-push and direct aspiration methods) and control embryos exhibited normal morphology (figure 15c).

Although biopsy of preimplantation embryos has been carried out at a number of developmental stages with successful pregnancies after embryo transfer [mouse 2-cell embryos (Nijs et al., 1988), mouse 4-cell (Wilton et al., 1987), mouse 8-cell (Monk et al., 1987), mouse blastocyst (Monk et al., 1988), rabbit blastocyst (Gardner and Edwards, 1968), bovine blastocyst (Herr and Reed, 1991), monkey blastocyst (Summers et al., 1988) and human 8-cell stage (Handyside et al., 1992)], there have been few systematic studies on the efficiency of embryo biopsy procedure. These experiments also investigated whether successful biopsy can be performed at the 4-cell, 8-cell and morula stages in mice with subsequent successful in vitro and in vivo developments. The present result confirms that successful embryo biopsy can be performed at the 4-cell, 8-cell and morula stage in mice with subsequent successful pregnancy. It shows, however, that there were substantial differences in the developmental potential of embryos biopsied at different cell stages. Although, there was no statistically significant difference between the rate of in vitro development of embryos biopsied at the 4-cell stage and that of those at the 8-cell or morula stages, a higher percentage of embryos did develop to the blastocyst stage and the rate of live-birth was higher with biopsy performed at the 8-cell and morula stages. However, conflicting results have been published. Monk and Handyside (1988), Wilton and Trounson (1989) and Takeuchi et al. (1992) reported that single cell biopsy at the 4-cell stage had no deleterious effect on the percentage of embryos reaching the blastocyst stage; whereas Krzyminska et al. (1990) found a lower proportion of blastocysts in 3/4 embryos than in 6/8 embryos which have experienced a similar loss of

one-quarter of the embryo, suggesting that the subsequent developmental potential is affected by the cleavage stage at the time of biopsy. It was observed that, compared to the 8-cell or morula stage, more embryos biopsied at the 4-cell stage fail to implant (table 8). The reasons for such a loss of viability at the 4-cell but not the 8-cell stage are not clear. However, in mouse embryos, the second cleavage division (beginning of the 4-cell stage) is invariably asynchronous resulting in the transitory existence of a 3-cell stage embryo. The early-dividing cell distributes disproportionately more cells to the ICM than the late-dividing cell (Kelly et al., 1978; Surani and Barton, 1984). Up to the third cleavage (beginning of the 8-cell stage) cell divisions are equal, but take place in an irregular fashion according to the direction of the preceding division plane (Menezo and Renard, 1993). The decision of which cell will form the ICM and the TE is taken during the latter part of the fourth cleavage division, when an intact embryo has 12-16 cells (McLaren, 1985). According to the inside-outside or epigenetic hypothesis (Tarkowski and Wroblewska, 1967) different environmental conditions play a decisive role as to the differentiation of cells into either one of the two directions (ICM VS TE). Therefore, position and intercellular interactions of blastomeres in an embryo at this stage lead to a change in polarization of cells and determine whether a cell will contribute to either the ICM or the TE (Johnson, 1981, Ziomek and Johnson, 1982; Pickering et al., 1988). The peripheral layer of cells forms tight junction and develops into TE, while the cells positioned inside give rise to an ICM. The normally organized blastocyst can then be formed. It has been demonstrated that embryos with a disturbed allocation of cells to the ICM and

TE have lower developmental potential both in vitro (Tarkorski and Wroblewska, 1967) and in vivo (Rossant, 1976). Additionally, other workers have demonstrated that, at the 4-cell stage, the earlier-dividing blastomeres will preferentially contribute descendants to the ICM (Kelly et al., 1978; Garbutt et al., 1987). Somers et al. (1990) have found that there was an average of 4-5 inner cells at the 16 cell stage (after the 4th cleavage division) and allocation of cells to the ICM occurs twice during the 5th cleavage division (Pedersen 1986; Fleming, 1987), indicating the timing of blastulation to be constant and independent of cell number. Hence, the reduction of cellular mass during cleavage results in blastocysts with a reduced total cell mass (Smith and McLaren, 1977). In addition, Somers et al. (1990) have also found that the ICM : TE ratio in ¾ mouse embryos was significantly lower when compared with untreated control embryos. An altered ICM : TE ratio may result in an insufficient total number of ICM and a reduced capacity to form fetuses (Rands, 1985). Therefore, embryo biopsy at the 4-cell stage might result in fewer cells occupying an inside position at the time of blastocyst formation, causing reduced development in vitro, impaired implantation and capacity for live births of these embryos in comparison with their controls.

The 8-cell and morula stage embryo, containing more cells and exhibiting a higher division rate at the time of biopsy is not affected by cellular loss and its blastocyst formation can take place with a sufficient number of blastomeres developing into an ICM. The rates of implantation and fetal development after embryo biopsy at these stages were similar to their non-biopsied control

embryos. Also there was not any effect of biopsy on the birth weight of the living young (table 9). Embryo biopsy at the 8-cell and morula stage is technically easier because the blastomeres are smaller, and more than one blastomere can be removed without a detrimental effect on the developing embryo. We found that some of the 8-cell embryos from which two blastomeres had accidentally been removed by the biopsy procedure can develop into normal blastocysts (data not shown). Several studies have shown that up to two or three blastomeres can be removed from the 8-cell embryo without significantly affecting embryo development in mice (Roudebush et al., 1990) in vitro, as well as in vivo, and in humans (Hardy et al., 1990). In the mouse embryo, the blastomeres retain functional totipotency until the early morula stage (16-cells). (Rossant and Vijh, 1980; Ziomek, Johnson and Handyside, 1982). However, at the late morula stage (approximately 30 cells), when the blastocoele cavity is formed, only some of the cells in the ICM retain their totipotency (Rossant and Vijh, 1980). Therefore, it is impossible to judge whether any presumptive ICM or TE cell is impaired by biopsy at this stage of the embryo, because removal of a few cells from the embryo or damage during biopsy of the presumptive ICM may be directly reflected in a poor implantation rate (Krzyminska et al., 1990). Therefore, in this study, embryo biopsy at the early morula stage was chosen in order to avoid this problem and the results show that the biopsied morula can normally develop into blastocysts and proceed to live births at the same rate as non-biopsied control embryos. Krzyminska et al. (1990) have reported that embryo biopsy had the least impact on subsequent in vitro and in vivo

development when performed at the 8-cell stage and was most detrimental at the morula stage, reducing the implantation rate from 65 % for control to 21% for biopsies and fetal viability from 42 % to 26 % respectively. Their procedure involves removal of the ZP and slicing of five blastomeres from the embryo whereas this study used the techniques that affect the ZP minimally; Nevertheless, the results obtained were similar. Likewise, the retention of the ZP is beneficial if the embryos need to be frozen (Trounson, 1986; Wilton et al., 1989).

Biopsy procedures at the morula stage reported in the literature are mainly splitting techniques. Whereas these techniques perform well when applied to sheep and cattle (Williams and Moore, 1988) they may have a detrimental effect in the mouse (Lawitts and Graves, 1988). Indeed in mice, as in humans, the number of cleavages occuring before differentiation into ICM and TE, is much lower than in sheep and cattle (McLaren, 1985). Since the ICM of the mouse must contain at least five cells at the implantation stage to give rise to viable fetuses (Ansell and Snow, 1975), the halved embryo may have too few. Therefore, the removal of one or a few blastomeres is more advantageous.

Although it would be of interest to perform biopsy at the morula stage, morulae show some degree of compaction, and a relatively large number of lysed cells is induced when biopsy is attempted because desmosomes and tight junctions between blastomeres begin to form (Menezo and Renard, 1993). This problem may be solved by incubating the embryos in divalent-cation

deficient medium (Ca^{2+}-and Mg^{2+} -free PBS), a chelating mixture of ethylenediamine tetraacetic acid (EDTA) and glycine (Van Blerk et al., 1991; Dziadek, 1981), and/or cytochalasins (B or D) (Krzyminska, 1990). However, evidence suggests that the use of cytochalasins (B or D) that disrupt the organization of microfilaments and inhibit the formation of new filaments may be more detrimental than the use of a divalent cation deficient medium or an EDTA-glycine mixture. Cytochalasins (B or D) do not only disrupt and inhibit compaction but may result in the arrest of cleavage preventing further development (Surani et al., 1990). In this study, the use of a biopsy medium containing hypertonic sucrose (100 mM) and the chelating agent, EDTA (2.00 mM) to attenuate interblastomeric contacts seems to have no detrimental effect on the in vitro development of mouse embryos as the high percentages of normal development in the solution control groups have shown. Thus, the effect of the incubating solution on the in vitro and in vivo development of mouse embryos can be excluded. Any effect of biopsy of preimplantation embryos on the in vivo development or postimplantation development can be assessed by transfer of blastocysts from several experimental groups into the uteri of pseudopregnant foster mothers. The viability of the apparently morphologically normal biopsied embryos was slightly below that of the control groups, especially when the biopsy was performed at the 4-cell stage. This may be due to the reduction of the total cell mass at the time of cavitation and hence lead to a decline in implantation rate and live births. However, no macroscopic developmental abnormalities were observed at birth (figure 18) or

during the period before weaning and sexual maturation. Complete histologic evaluation was not performed.

The reproductive capacity of the offspring derived from biopsied embryos did not appear to be impaired by the biopsy procedures, although the sample size was limited. As with the first generation, no developmental abnormalities were observed at birth or during the period of weaning of the second generation. Although implantation, fetal development and births are the desired outcome of any assisted reproductive technique, these may occur at rates dependent on other factors that mask differences between biopsied and control embryos. Wilton et al. (1989) noted that the transfer protocol, rather than the biopsy itself, may have affected the implantation rate in their early studies.

B. Sex determination in single blastomere

Preimplantation diagnosis of inherited disease requires the detection of genetic defects in a small number of cells biopsied from each embryo (Handyside, 1992). Various techniques have been developed to detect genetic disorders during the preimplantation phase of embryonic development, such as the polymerase chain reaction (PCR)(Lynch and Brown, 1990), microbiochemical techniques for detection of known enzyme defects (Monk, 1987), and methods of karyotyping individual cells, including fluorescent in situ hybridization (FISH) (Grifo et al., 1990). However, biochemical methods are not completely reliable (Braude, 1991). DNA analysis by PCR was first described by Saiki et al. (1985) allowing genetic diagnosis to be performed on

DNA derived from a single cell, although thereby also increasing the chance of DNA contamination (Strom et al., 1991). Several important systems must be optimized to reliably amplify DNA from single cells. These include the elimination and/or prevention of contamination with extraneous DNA sequences, the optimization of cell lysis conditions, primer design, PCR specificity and appropriate internal controls (Strom and Rechitsky, 1992). Even with these precautions, PCR analysis can potentially lead to an error resulting from possible failure of amplification. The introduction of nested PCR amplification has recently made it possible to significantly increase the percentage of successful amplification of DNA from single cells (Mullis and Faloona, 1987; Li et al., 1991). A nested primer approach requires a two-step amplification (Li et al., 1990). In the first step, the target DNA fragment is amplified with two regular (outer) primers. Then an aliquot of the PCR product from the first round is amplified with nested (inner) primers, which are internal to the two regular primers (figure 14). The other potential problem associated with allele-specific PCR failure in single cells (Navidi and Arnheim, 1991), can be avoided by the use of multiplex PCR amplification with internal control for general PCR failure. Multiplex PCR makes it possible to amplify several DNA segments simultaneously using multiple pairs of primers (Chamberlain et al., 1988). Previous studies used the Y-repeated sequences for gender determination of preimplantation embryos by PCR in humans (Handyside et al., 1989; 1990), mice (Bradbury et al., 1990) , and cattle (Peura et al., 1991). In humans, a 3.4 kb Y-specific repeated sequence (DY21) (Nakaori et al., 1986) has been recognized. However, amplification of DYZ1

for PID has been identified as a misdiagnosis (Handyside et al., 1990). The susceptibility to misdiagnosis is understandable since there are more than 484 TTCCA and other nucleotide substitutions within the sequence and it is very similar to satellite III located on chromosome 1 and has been recovered from both male and female DNA (Nakahori et al., 1986). The presence of a small number of copies on some autosomal chromosomes has occasionally caused an amplification of the sequences in female DNA. It is noteworthy that similar problems were encountered when a mouse Y-repeated sequence (PY 353/B) was used to sex mouse embryos (Bradbury et al., 1990). The high frequency of non-specific templates and a large amount of non-specific products may have been responsible for the misdiagnosis (Winston et al., 1991). Thus, selection of a sequence highly specific to the Y-chromosome is critical for precision of diagnosis.

To overcome the possibility of misdiagnosis attributable to amplification failure or mispriming events, two sets of oligonucleotide primers for different sequences of the same gene were found to be beneficial (Kunieda et al., 1992) In this study, the multiplex two-step PCR amplification with separate tubes was used for sex determination of mouse embryos as a model to evaluate the performance and practicality of the PCR technique in PID. For the exclusion of genuine male embryos, diagnosis is based on the presence of an X-linked control signal (DXNds 3), combined with the presence of both Y-linked single copy gene signals, Zfy and Sry fragments. The initial setting of the nested PCR protocol in this study was performed on genomic DNA extracted from

male and female mouse blood cells using multiplex PCR for simultaneously amplifying the segments of two Y-chromosome specific single copy genes, Zfy and Sry, to prevent the false sexing resulting from the possible failure of detection of the single sequence in single blastomeres. In addition to the detection of Y-specific sequences, simultaneous detection of X-chromosomal sequence, DXNds 3, was carried out as an internal control to check the presence of the blastomere in the sample tube. The expected specific 217-bp, 147-bp, and 111-bp products corresponded to Zfy, Sry, and DXNds gene fragments, respectively, confirming the sex of origin (100 %) after PCR amplification as shown in figure 19. This finding indicates that each pair of primers used could specifically amplify the target sequences, since we detected the complete signal specific for males in blood samples derived from males. Although some related sequences of considerable homology to the Sry and Zfy genes are known to be present on the autosomes and X-chromosome (Nagamine et al., 1989; Mardon and Luoh, 1990; Mitchell et al., 1989), no male-specific fragment was detected in female DNA samples. This may have been due to a selection such that the sequences of the primers would be less homologous to the related sequences.

To further optimize the procedure for single-cell analysis, the same experimental conditions were then applied to amplify DNA segments from the mouse single blastomere isolated from the 8-cell stage embryos (1/8 blastomere) for comparison with the embryos (7/8) they originated from. The detection of target sequences on the 1/8 blastomeres perfectly corresponded to

the detection of these sequences on the 7/8 embryos they originated from, except for one 1/8 blastomere in which all three sequences failed to be detected (figure 20). However, PCR conditions for DNA analysis from single cells are entirely different from those for general DNA (blood) preparations, probably due to the very small number of DNA copies in the starting material. For most routine applications of PCR, a sample consisting of the amount of DNA purified from 150,000 diploid cells (1 μg) is typical. In this case, not all of the original 300,000 copies of the target are required to be amplified during every PCR cycle in order to determine the genotype of the DNA accurately. A single blastomere contains only two DNA molecules representing each single copy gene and therefore the accuracy of genotype determination is much more sensitive to random fluctuations in the efficiency with which each individual molecule is amplified during each PCR cycle.

The other problem associated with single-cell PCR analysis is the reliability of cell transfer into the PCR reaction tube. During collection, each single blastomere was carefully examined and only those with nuclei were selected for PCR amplification. Also, the transfer of each blastomere into the PCR tube containing sterile distilled water was confirmed by continuous observation under the dissecting microscope. Due to the collection method applied here, complete PCR failure in this case is unlikely due to the absence of the cell in the PCR reaction tube. With the majority of unique target sequences examined, however, amplification fails in 10-20 % of single cells irrespective of the cell type or sample preparation (Li et al. 1988; Boehnke et

al., 1989). Liu et al. (1994) showed that, in 16 % of cases, human blastomeres could not be sexed as either male or female because neither Y-nor X-specific fragments were amplified from the same single blastomere. A possible source of error in these cases could be the fact that the DNA is sticky and is taken out accidentally as soon as reaction mixtures are added to the reaction tubes.

Alternatively, sample preparation may constitute a critical step, for example, there was a report that blastomeres are remarkably resistant to lysis in distilled water (Gibbons et al., 1995) and this phenomenon may be, in part, the cause of amplification failure. Liquid nitrogen (LN_2) has been used to rupture the cell membrane, making the DNA more accessible to primers (Handyside et al., 1990; 1992). In the early part of this trial, the isolated balstomeres were snap-frozen in LN_2 to speed processing, however, amplification failure frequently occurred. It seems that, instead of osmotic swelling, cell lysis, and dissolution of cell contents, blastomeres remain as a "ghost" with an intact nucleus which can be visualized by fluorescent staining of the DNA (Kontogianni et al., 1996). In this situation, boiling prior to PCR to inactivate endogeneous proteinases and nucleases may simply fix the DNA in situ, which may then be relatively inaccessible to the primers, preventing successful amplification in the first critical cycle. However, when cell lysis was performed by three cycles of a slow freeze-thawing process, strong amplification of the target sequences was achieved with most single blastomeres (19 of 20 single blastomeres in table 11 and 178 of 184 single blastomeres in table 12). Sheardown et al. (1992) report that in the mouse,

amplification of target sequences during PCR could still be achieved despite lysis of the biopsied blastomere if the entire wash drop containing the lysed cell was collected and amplified. However, in this study, amplification was neither detected after PCR of lysed biopsied cell samples nor denucleated blastomeres. Thus, for application to human embryo preimplantation genetic diagnosis, an intact blastomere with a clearly discernible nucleus is recommended for a reasonable chance of achieving an accurate diagnosis.

The final proof of the reliability and accuracy of the two-step PCR method is dependent on the correct prediction of births. As shown in table 12, in 178 out of 184 single blastomeres (96.7%), DXNds3 (female blastomeres) or DXNds3, Sry and Zfy (male blastomeres) fragments were detected after the multiplex two-step PCR amplification. In none of the samples analysed was there a Y-specific fragment present without the simultaneous presence of an X-specific fragment. These data confirm the sensitivity of the multiplex two-step PCR as to detection of specific gene sequences in single blastomeres. Interestingly the male:female embryo sex ratio was higher than 50:50, similar to other reports (Lehtinen and Pelliniemi,1984; Bradbury et al., 1990; Cui et al., 1993). After transfer of a total of 78 sexed embryos into the uterine horn of pseudopregnant foster mothers, the sex of 36 of 37 (97% accuracy) mouse pups born from male and female embryos is in agreement with the predicted sex, except for the 4th experiment in which eight male-sexed embryos have been transferred but three male and one female mouse pups are born. The possible source of error in this case could be loss of DNA material before

starting the PCR procedure combined with amplification of contaminating DNAs leading to misdiagnosis of a single female blastomere as male. In addition to the DNA potentially missing, analysis of small numbers of target molecules is also highly susceptible to errors resulting from contamination (Navidi and Arnheim, 1991). Since the amount of the starting DNA material is limited, more than 40 PCR cycles are required for amplification of target sequences, However, such a number of PCR cycles are also sufficient to amplify minute DNA contamination and can lead to misdiagnosis. Contamination, including carryover of the amplified products to the samples by laboratory equipment, presents a serious problem in the detection of a specific sequence (especially in the single gene defects) from a small amount of DNA, since the PCR procedure produces a very large number of molecules that could be amplified by subsequent PCR to result in false-positives. The incidence of amplification of contaminating male DNA from female blastomeres is relatively high (Strom et al., 1991). Fortunately , such false-positives would not result in a serious misdiagnosis of sex since they would be identified as males and not selected for transfer. It would simply have an effect on pregnancy rates by reducing the number of female embryos identified for transfer. However, stringent precautions against such a contamination are essential in PID in order to prevent misdiagnosis. The well-documented methods developed in order to avoid carryover in general PCR reactions are inadequate for prevention of contamination in single-cell PCR. The precautions for decontamination in single cell PCR include: (1) the laboratory for embryo manipulation, preparation of PCR reaction mixtures, especially transfer of the

first-round PCR products to the second-round reaction mixture tubes, and analysis of PCR products should be set up in separate rooms, or at least locations; (2) the isolated blastomeres should be washed several times before placing them into the PCR tubes; (3) all reagents used in the embryo laboratory and for PCR preparation should be kept in separate small aliquots to minimize the necessity for repeated sampling, so that if contamination occurs, a new aliquot can be used immediately; (4) all PCR reagent containers are never to be touched without gloves; (5) gloves should always be worn and changed frequently during PCR preparation; (6) use of separate sets of pipetting devices specific for single-cell PCR, or use of positive displacement pipettes is crucial; (7) preparation of blastomeres and PCR reaction mixtures must be performed in separate laminar flow hoods; (8) a negative blank control should be simultaneously run with every PCR reaction. However, even the strictest application of the above precautions may not be sufficient in order to avoid contamination in all circumstances, all reagents, and/or pipetting devices and therefore, PCR tubes must be decontaminated by ultraviolet (UV) irradiation immediately prior to PCR.

The PCR reliability obtained with our method is as similar to the result on single human blastomeres obtained by Chong et al. (1993) but is in contrast to the 100% PCR amplification rate on single mouse blastomeres obtained by Cui et al.(1995). Sex determination in PID represents an alternative to gene-specific PCR analysis for avoiding X-linked recessive diseases. The obvious advantage of sexing in comparison with gene-specific PCR diagnosis is its applicability

for all X-linked diseases without the necessity to adapt the technology to each individual disease. The disadvantage is that, at least theoretically, 50 % of the discarded male embryos are healthy.

As noted earlier, the major challenge for PCR-based PID is to amplify DNA from single cells which frequently causes serious difficulties. An additional problem is encountered as soon as more than two mutations need to be analyzed in which case even a nested PCR would not be suitable. Recently, other applications of PCR were developed to improve the reliability and accuracy of the technique. One of these applications is a method called primer extension pre-amplification (PEP) has been described (Zhang et al., 1992). This new technique seems to be able to circumvent some of the above mentioned problems in that it increases the sensitivity of single-cell genetic diagnosis by pre-amplifying the whole genomic DNA using a 15-base random oligonucleotide in a modified PCR reaction and thereby increasing the number of templates for subsequent PCR. They demonstrated that at least 30 copies of any specific DNA fragment from > 78% of the genome can be produced from a single cell. However, PEP is time-consuming technique. Since the PEP process alone takes at least 10 hr, this should be optimized in order to meet the needs of PID in a clinical situation. The other improvement is fluorescent PCR, where PCR fragments are not detected on ethidium bromide-stained gels, as is done routinely, but are labeled with a fluorescent dye so that they can be detected when excited by the correct laser (Hattori et al.,1992; Findlay et al., 1996).

Recently, preimplantation sex determination by multiplex PCR has been independently evaluated by FISH for embryos that were not replaced (Munne et al., 1993b). The additional advantage of FISH over PCR was demonstrated by an embryo exhibiting only the signals characteristic for X chromosomes in the PCR results, yet which was found to be XO by FISH. Since on the basis of PCR alone, XO cannot be distinguished from XX or XXX, this embryo would have mistakenly been transferred as a normal female when it was in fact monosomic for chromosome X. The FISH technique has also been refined that up to five different chromosomes can now be detected in a single cell simultaneously, allowing screening for the commonest aneuploidies (e.g. trisomies 13,18 and 21)(Munne et al., 1994).

C. Summary and Conclusion

Based on the results obtained in the mouse model , the present study confirms that biopsy of preimplantation embryos applying micromanipulation is technically possible, but may have some impact on the development potential of the embryos. Detrimental effects varied according to the developmental stage and the method of biopsy. The use of intact controls, solution controls and biopsied embryos in the mouse model allows the following conclusions: (1) minimal trauma from biopsy of mouse embryos can be assured by applying the PZD-push rather than the direct aspiration technique, resulting in a high rate of normal post-biopsy development. This normalcy includes demonstration of the capacity to reproduce a second generation; (2) at the 4-cell stage, biopsy caused reduction in both blastocyst

formation and pregnancy potential. This may be due to insufficient reduction in the cellular mass at the time of blastulation; and (3) at the 8-cell and morula stage, biopsy had minimal effects on the subsequent development of mouse embryos.

The results of this study suggest that embryo biopsy at the 8-cell stage by means of the PZD-push technique is most likely suitable for PID. This stage is compatible with the logistics of the human in vitro fertilization (IVF) procedures. More than one blastomere can be removed from the 8-cell mouse embryos without being detrimental and thus, more material is available for any diagnostic procedure. Biopsy of embryos at the morula stage that previously treated with Ca^{2+}- and Mg^{2+}-free biopsy medium containing EDTA and sucrose may be feasible. However, at this stage with more cell compaction and intercellular junctions, biopsy is more difficult to perform than at the 8-cell stage. The rates of blastocyst formation and fetal development have been shown to be similar in embryos biopsied (at the 8-cell stage) to those not having undergone biopsy. Also, the biopsy procedures described did not have any adverse effect on the fetal weight and neither were there any phenotypic abnormalities noted with the offspring derived from biopsied embryos. Moreover, we demonstrated the reproductive potential of the biopsied animals not to have been compromised by the micromanipulations, since their respective offspring were healthy live-births. Hence, this technique can safely be applied for genetic diagnosis of hereditary disorders in humans, thereby carrying the promise to maybe eliminate some genetic diseases from the gene pool.

Our study demonstrate that an accurate and reliable gender analysis of single blastomeres biopsied from preimplantation embryos is feasible in the majority of cases by means of the multiplex two-step PCR reaction for simultaneous detection of fragments from two Y-specific sequences, Sry and Zfy, together with the X-chromosome specific sequence, DXNds3, in single blastomeres. In the first-step of PCR, all three fragments are simultaneously amplified from the outer primers specific for each DNA sequence. In the second step of PCR, amplification of each specific fragment is performed in separate tubes. Under these conditions, the reactions are independent of each other and reproducible. All three specific fragments are detected in male embryos and, due to absence of the Y chromosome, only the X-specific fragment in females. This assay allows sexing in the course of about 6-8 hr after embryo biopsy which is a sufficient period for transfer of the sexed embryos to recipients without freezing. The data obtained from single blastomere analysis support the assumption that the multiple-marker approach is inherently more reliable than one based solely on one type of Y-linked signal, since signals indicative of a genuine female genotype can unequivocally be identified and therefore differentiated from PCR failure or absence of cellular DNA.

Setting up a preimplantation diagnosis procedure is a complex problem. On the one hand, as much cellular material as possible has to be biosied to perform a reliable diagnosis. On the other hand, the biopsied embryo must retain its capacity for normal development and implantation. If the diagnostic

procedure takes longer than the embryo can be cultured in vitro, specific conditions for preservation of the embryo are required. Furthermore, all experimental preimplantation diagnostic procedures have to be confirmed in the human. Studies of biopsy methods of cells from other embryonic stages, such as blastocyst stage are needed, as well as improvements in culture conditions of the isolated blastomeres to increase the number of genetic material for diagnosis. To improve the efficiency of the diagnosis, further investigation and development of recombinant DNA, enzymatic and cytogenetic techniques on a minimum amount of biopsied material are absolutely necessary.

Considering the technical difficulties, the PCR-based PID technique is still in its infancy. Fundamental research is still needed and it will still take sometime for PID to be used widely in routine clinical practice. The first priority lies in increasing the reliability of the assay in order to reduce the rate of misdiagnoses to a clinically acceptable level. Although general optimization of PCR technology will improve PCR accuracy, investigation into the causes of the discrepancy observed between experimental and clinical studies is essential.