SEXING OF *IN VITRO*-FERTILIZED PREIMPLANTATION MOUSE EMBRYOS BY THE PCR METHOD

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Summary   The applicability of the dual PCR method to embryo sexing was examined with the aim of establishing a noninvasive method of pre-implantation diagnosis for human genetic disorders. Mouse pre-embryos obtained by *in vitro* fertilization were studied. The *Sry* gene sequence and the myogenin sequence were amplified as the Y-specific and internal control sequences, respectively. Amplification of as little as 10 pg of mouse genomic DNA was possible with the dual PCR method, the sensitivity being 10-fold greater than that of the single PCR method. The sex was identified in 100% (24/24) and 96% (23/24) of the pre-embryos tested at the 16- and 4-cell stages, respectively. In addition, the sex of all four single blastomeres dissociated from 4-cell pre-embryos agreed in 76% (16/21) of the specimens tested and 94% (79/84) of dissociated blastomeres could be sexed. The sex of single blastomeres biopsied from pre-embryos at the 8-cell stage could be identified. After transfer of 13 male and 25 female sexed pre-embryos, six viable fetuses were obtained. Histological examination showed that all these fetuses were of the pre-dicted sex.

   Sexing of biopsied single blastomeres by the dual PCR method was rapid and reliable, suggesting its feasibility for preimplantation diagnosis of *in vitro* fertilized human pre-embryos.

Key Words   dual PCR, preimplantation diagnosis, mouse embryo

INTRODUCTION

   With the recent rapid progress in the field of molecular biology, the molecular basis of a number of genetic diseases has been identified and their prenatal diagnosis has become possible. At present, chorionic villus sampling and amniocentesis are

performed at about 10 and 16 weeks of gestation, respectively, and the fetal materials obtained by these methods can be used for DNA analysis to detect Duchenne's muscular dystrophy (Ubagai and Katayama, 1991) and hemophilia A (Takeshita, 1992). Since these methods can only be performed after pregnancy is established, obstetrically and psychologically undesirable consequences may arise if the fetus has genetic defects and the parents request termination.

In the place of invasive intrauterine diagnosis, the noninvasive preimplantation diagnosis of genetic diseases has been considered. If the diagnosis of genetic defects could be performed before implantation, unaffected embryos could then be transferred to the uterus. Also, the patient could avoid the potentially traumatic termination of an established pregnancy. In the case of certain X-linked genetic diseases, a specific method of diagnosis is not available, and determination of the sex of the embryo is the only alternative. Sex determination is also an important first step in the diagnosis of Duchenne's muscular dystrophy and hemophilia A or B, for which methods of DNA analysis have been established, since a female embryo will not be affected by these diseases.

Progress with *in vitro* fertilization has provided access to preimplantation embryos and micromanipulative techniques have been refined so that a single blastomere can be obtained without damaging the biopsied pre-embryo (Hardy *et al.*, 1990; Krzyminska *et al.*, 1990; Takeuchi *et al.*, 1991; Wilton and Trounson, 1989). Together with development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988), which can amplify a target sequence from a small amount of deoxyribonucleic acid (DNA) in a short time, one or more blastomeres biopsied from human pre-embryo have been used for sex determination by the PCR method (Handyside *et al.*, 1989, 1990). The method of amplifying target sequences from a single blastomere by the PCR can be applied not only to sex determination but also to the diagnosis of diseases such as sickle cell anemia and Tay-Sachs disease (Morsy *et al.*, 1992), which result from point mutations, or β-thalassaemia and cystic fibrosis (Hardy and Handyside, 1992) which result from deletions. However, the clinical application of such a new diagnostic method to human pre-embryos requires the prior examination of its reliability and safety in an experimental model.

In the present study, the feasibility of preimplantation diagnosis in humans was accordingly examined using mouse pre-embryos obtained by *in vitro* fertilization.

MATERIALS AND METHODS

Crj:CD-1 (ICR) mice were used in all the experiments.

1. Target sequences and primers. The single and dual (nested) PCR methods were performed using one set and two sets of primers, respectively, for amplification of the target sequences. The mouse *Sry* gene (Gubbay *et al.*, 1990) and myogenin

(Wright *et al.*, 1989), the muscle-specific regulatory factor, were chosen as the Y-specific target sequence and the internal control sequence, respectively. For each of these genes, outer and inner sets of oligonucleotide primers were synthesized, with the inner sets of primers being located inside the segments amplified by the corresponding outer sets of primers. The sequences of these primers were as follows:

    SRY-1 5' GTGAGAGGCACAAGTTGGC 3'
    SRY-2 5' TCTTAAACTCTGAAGAAGAGAC 3'
    SRY-3 5' CTCTGTGTAGGATCTTCAATC 3'
    SRY-4 5' GTCTTGCCTGTATGTCATGG 3'
    MYO-1 5' TTACGTCCATCGTGGACAGC 3'
    MYO-2 5' ATTTGCTCGCAGCTGACCCTA 3'
    MYO-3 5' TGGGCTGGGTGTTAGTCTTA 3'
    MYO-4 5' GGATACTCTCTGCTTTAAGG 3'

SRY-2, SRY-4, MYO-2, and MYO-4 were the outer primers, while SRY-1, SRY-3, MYO-1, and MYO-3 were the inner primers. SRY-1 to SRY-4 as well as MYO-1 and MYO-3 were the same primers as those used by Kunieda *et al.* (1992) and Koopman *et al.* (1991), respectively. Primers MYO-2 and MYO-4 were designed on the basis of the reported sequence (Wright *et al.*, 1989). All the primers were synthesized by a firm.

II. Sensitivity of the single and dual PCR methods. The sensitivity of amplification was compared between the single and dual PCR methods using serial dilutions of male and female mouse genomic DNA (10 ng, 1 ng, 100 pg, and 10 pg). Male and female mouse genomic DNA were prepared from skeletal muscle using a DNA extraction kit (Stratagene) according to the manufacturer's directions. As the positive and negative controls, 100 pg of male and female DNA were used, respectively. The single PCR method was performed as described below, using two outer sets of primers (SRY-2 and SRY-4, and MYO-2 and MYO-4). For serial dilutions of mouse genomic DNA, the reaction mixture was added. This contained reaction buffer (10 mM Tris-HCl, pH 8.9, 1.5 mM $MgCl_2$, 80 mM KCl, 500 µg/ml BSA, 0.1% sodium cholate, 0.1% Triton X-100), 200 µM of each dNTP, 20 pmol of each primer, and 2.5 U of *Thermus thermophilus* DNA polymerase (Tth polymerase, Toyobo). The PCR was performed in a Zymoreactor (Atto Corp.) for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 60 sec. After these 30 cycles, the tubes were kept at 72°C for a further 5 min. For the dual PCR method, 10 µl of the PCR products from the first PCR were dispensed into two tubes with a reaction mixture containing the corresponding inner set of primers (SRY-1 and SRY-3, MYO-1 and MYO-3) and amplified for an additional 30 cycles. The positive and negative controls as well as a blank (100 pg of male mouse DNA, 100 pg of female mouse DNA, and no DNA, respectively) were also subjected to dual PCR amplification. After amplification, 5 µl of the amplified product from each corresponding tube was

mixed and subjected to electrophoresis on 6% polyacrylamide gel at 16 mA and 140 V for 40 min. The amplified fragments were visualized by ethidium bromide (4 μg/ml in dH_2O) staining and ultraviolet illumination, and photographed with a Polaroid camera.

III. Sexing of whole embryos and single blastmeres obtained by in vitro fertilization. The sex of pre-embryos at the 4- and 16-cell stages was determined by the PCR method. At 8 to 10 weeks of age, Crj:CD-1(ICR) female mice were superovulated by the intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMS) (Serotropin, Teikokuzoki), followed 48 hr later with 5 IU of human chorionic gonadotropin (hCG) (Gonadotropin, Teikokuzoki). Oocytes were collected at 14 hr after hCG injection and inseminated with epididymal sperm, which was collected from male ICR mice and preincubated for 1 to 2 hr in TYH medium. Six hours after insemination, the fertilized eggs were transferred to Whitten's medium containing 100 μM EDTA and cultured at 37°C in an atmosphere of 5% CO_2 in air. Pre-embryos were collected at the 4- and 16-cell stages, and each pre-embryo was placed in Whitten's medium containing 0.5% pronase (Sigma Chemical) for 3 to 5 min to remove the zona pellucida. After removing the zona pellucida, several pre-embryos at the 4-cell stage were individually incubated for 30 min in Ca^{2+}- and Mg^{2+}-free Whitten's medium to dissociate the blastomeres from each other. The tubes containing whole embryos and single blastomeres were frozen to −70°C for 20–30 min and then immediately heated to 94°C for 20 min to destroy endogeneous deoxyribonucleases. Then the dual PCR method was performed as described above.

IV. Biopsy of single blastomeres from mouse pre-embryos and transfer of biopsied pre-embryos to pseudopregnant recipients. Pre-embryos at the 8-cell stage were collected as described above, washed twice, and incubated in Ca^{2+}- and Mg^{2+}-free Whitten's medium containing 270 μM EDTA at 37°C in an atmosphere of 5% CO_2 in air for 30 min to reduce adhesion contact. Pre-embryo biopsy was performed by the expulsion method of Sasabe (1993) using a micromanipulator. Then the biopsied blastomeres were individually transferred in a 5-μl volume to 10 μl of autoclaved double-distilled water under oil in 500-μl Eppendorf tubes (Quality). The tubes were frozen to −70°C for 20–30 min, heated to 94°C for 20 min, and then subjected to the dual PCR as described above. After biopsy, the pre-embryos were incubated in Whitten's medium containing 100 μM EDTA at 37°C in an atmosphere of 5% CO_2 in air for 8–10 hr until the results of PCR amplification were obtained.

After sexing of the biopsied blastomeres by PCR amplification, the male or female pre-embryos were transferred to the oviducts of separate pseudopregnant recipient females on the morning of day 2 of pseudopregnancy. On day 18 of pseudopregnancy, the recipients were sacrificed by cervical dislocation and Caesarian section was performed to obtain viable fetuses. Each fetus was fixed in 10% formaldehyde, dehydrated in ethanol, and embedded in paraffin. Sections were stained

with haematoxylin and eosin and examined for ovaries and testes under a light microscope.

V. *Precautions against contamination.* Contamination of the PCR mainly arises from the final amplification product and leads to false-positive results. To avoid contamination, the measures recommended by Kwok and Higuchi (1989) were taken, including the use of separate rooms for preparation of the PCR re-action mixture and processing of the final amplification products, and the use of positive displacement pipettes (Gilson) and aerosol-resistant tips (Quality).

RESULTS

I. *Sensitivity of the single and dual PCR methods*

With the single PCR method, amplification of the Y-specific *Sry* gene and the myogenin sequence were detected down to 100 pg in male genomic DNA. In-creasing the number of cycles to 40 did not alter the sensitivity of this method (Fig. 1). With the dual PCR method, however, the amplification products of *Sry* (147 bp) and myogenin (245 bp) were detected in serial dilutions of male DNA down to 10 pg, the equivalent amount in a single cell (Fig. 2). Amplification of the myogenin sequence was detected down to 10 pg in female DNA, while the *Sry* sequence was not found (Fig. 2).
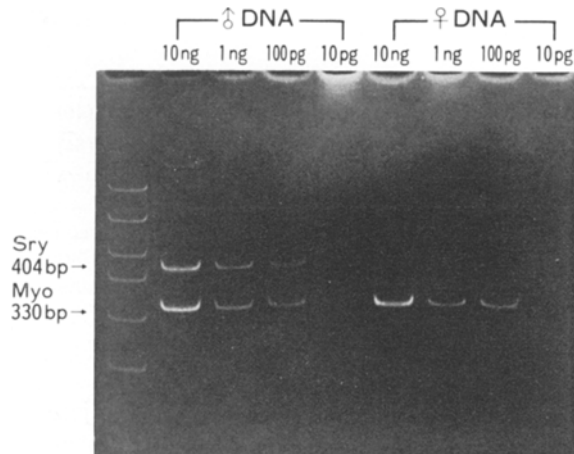

Fig. 1.   Detection of the *Sry* and myogenin sequences by the single PCR method in serial dilutions of purified mouse DNA (10 ng, 1 ng, 100 pg, and 10 pg). Amplification was performed using the outer primers for each sequence and 40 PCR cycles. Both the *Sry* and myogenin sequences were detected down to 100 pg of male mouse DNA. The myogenin sequence was detected down to 100 pg of female mouse DNA, while the *Sry* sequence was not detected. The left lane shows DNA size markers (BioMarker, BioVentures, Inc.) of 1,000, 700, 500, 400, 300 200, 100, and 50 bp.
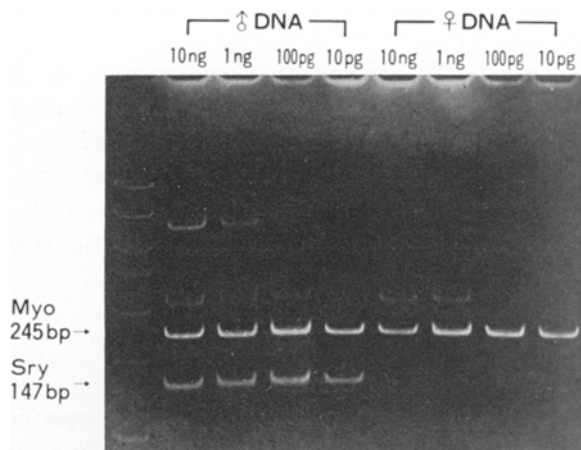
Fig. 2. Detection of the *Sry* and myogenin sequences by the dual PCR method in serial dilutions of purified mouse DNA (10 ng, 1 ng, 100 pg, and 10 pg). The amplified product of the *Sry* sequence was detected down to 10 pg of male DNA, while the product of the myogenin sequence was detected down to 10 pg in both male and female mouse DNA. The left lane shows DNA size markers.

Table 1. Results obtained by dual PCR of mouse pre-embryos at the 4- and 16-cell stages.

Embryo stage	No. of pre-embryos	Males[a] (%)	Females[a] (%)	Undetermined[b] (%)
4-cell	24	12 (50)	11 (46)	1 (4)
16-cell	24	11 (46)	13 (54)	0 (0)
Total	48	23 (48)	24 (50)	1 (2)

[a] Pre-embryos showing amplified products of the *Sry* and myogenin sequences were identified as males, while those showing only myogenin products were identified as females. [b] The sex of one 4-cell pre-embryo could not be determined because of unclear amplification of the *Sry* sequence.

II. Sexing of whole pre-embryos

When pre-embryos at the 4- and 16-cell stages were subjected to the dual PCR method (Table 1), amplification products were detected in all 24 (100%) 16-cell pre-embryos. In 11 (46%) pre-embryos, two bands of amplification products, one for *Sry* (147 bp) and one for myogenin (245 bp), were detected, which identified them as male pre-embryos. In 13 (54%) pre-embryos, only one band (myogenin 245 bp) was detected, which identified them as female pre-embryos. Amplification products were also detected in 23 out of 24 4-cell pre-embryos (96%). Twelve of these pre-embryos (50%) were identified as male and 11 (46%) as female, while one pre-embryo (4%) could not be sexed due to unclear amplification of *Sry*.

III. Sexing of single blastomeres

Twenty-one 4-cell pre-embryos were incubated in Ca^{2+}- and Mg^{2+}-free Whitten's medium for 30 min to dissociate the blastomeres from each other and a total of 84 blastomeres were obtained (Table 2). Out of these 84 blastomeres, 44 (52%) were identified as male and 35 (42%) as female, while five blastomeres (6%) were not identified because of unclear or no amplification products. Sixteen out of 21 pre-embryos (76%) showed agreement in sex with the result of amplification of each blastomere (8 male and 8 female pre-embryos) (Fig. 3). In two pre-embryos

Table 2. Results obtained by dual PCR of single blastomeres dissociated from 4-cell pre-embryos.

	No, of pre-embryos/ blastomere	Males[a] (%)	Females[a] (%)	Undetermined (%)
Pre-embryos	21	8 (38)	8 (38)	5 (24)[b]
Blastomeres	84	44 (52)	35 (42)	5 (6)[c]

[a] Pre-embryos/blastomeres showing amplified products of both the Sry and myogenin sequences were identified as males, while those showing only myogenin products were identified as females. [b] Pre-embryo sex was not determined because the amplified product of one of the four blastomeres did not agree with that of the other three blastomeres. [c] Blastomere sex was not determined because of unclear amplification or no amplification of either the Sry or myogenin sequences.
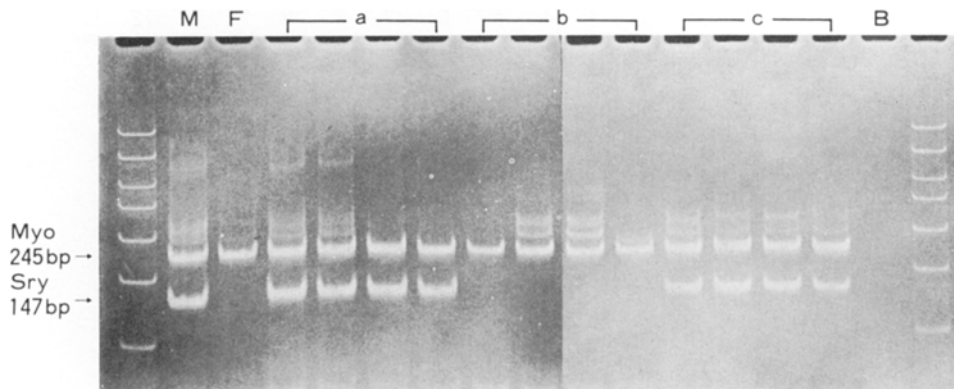

Fig. 3. Dual PCR amplification of Sry and myogenin sequences from single blastomeres dissociated from 4-cell pre-embryos. Results for three pre-embryos are shown. Pre-embryos a and c were identified as males, because there were two product bands (Sry at 147 bp and myogenin at 245 bp). Pre-embryo b was identified as female, since there was only one product band (myogenin at 245 bp). The amplified products of all four blastomeres from each pre-embryo were identical in these three specimens. Lane M, 100 pg of purified male mouse DNA; lane F, 100 pg of purified female mouse DNA; lane B, sample without DNA. The extreme left and right lanes show DNA size markers.

(10%), both the *Sry* and myogenin sequences were not amplified in one of four blastomeres. In three pre-embryos (14%), the amplification product from one blastomere did not agree with that of other three blastomeres.

IV. Sexing of biopsied single blastomeres and pre-embryo transfer

A total of 60 single blastomeres were biopsied from 60 8-cell pre-embryos and their sex was determined by the dual PCR method (Table 3, Fig. 4). Thirty per-

Table 3. Dual PCR of single blastomeres biopsied from 8-cell pre-embryos and outcome of the transfer of biopsied pre-embryos.

	No. of single blastomeres biopsied (%)	No. of pre-embryos transferred	No. of recipient mice	No. of fetuses[c]	Sex of fetuses[d]
Male[a]	18 (30)	13	3	3	3
Female[a]	25 (42)	25	5	3	3
Undetermined[b]	17 (28)	—	—	—	0
Total	60(100)	38	8	6	6

[a] Blatomeres showing amplified products of both the *Sry* and myogenin sequences were identified as males, while those showing only myogenin products were identified as females. [b] Blatsomere sex was not determined because no amplification products were detectable. [c] Fetuses were obtained by Caesarian section on day 18 of pseudopregnancy. [d] Fetuses were sexed by histological examination of the gonadal tissues.
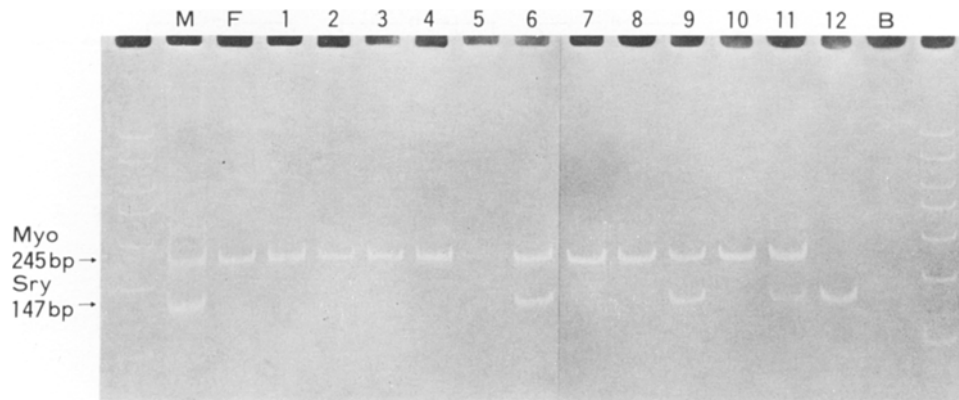

Fig. 4. Dual PCR amplification of *Sry* and myogenin sequences from single blastomeres biopsied from 8-cell pre-embryos. Results for 12 single blastomeres are shown. Blastomeres 6, 9, and 11 showed two product bands (*Sry* at 147 bp and myogenin at 245 bp) and were identified as males. Blastomeres 1–4, 7, 8, and 10, showed one product band (myogenin a 245 bp) and were identified as females. Blastomere 12 was identified as male from amplification of the *Sry* sequence. Lane M, 100 pg of purified male mouse DNA; lane F, 100 pg of purified female mouse DNA; lane B, sample without DNA. The extreme left and right lanes show DNA size markers.

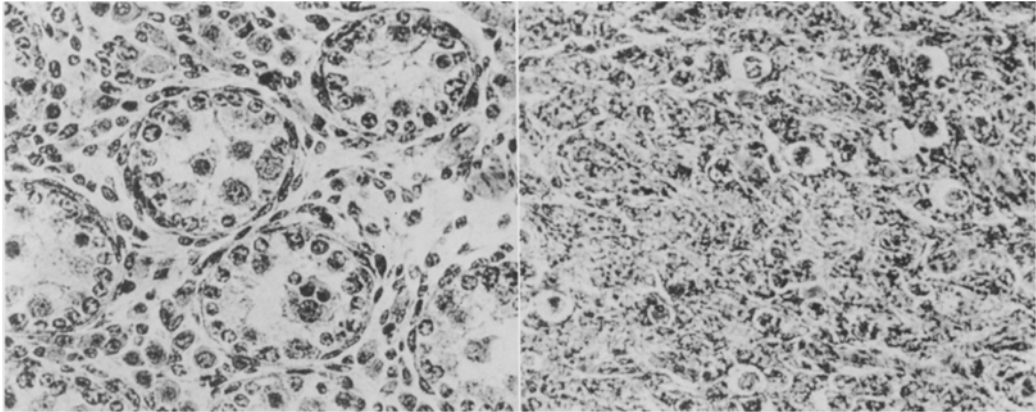(a)   (b)

Fig. 5. Histology of mouse fetal gonads. (a) The testicular tissue of a fetus obtained
by pre-embryo transfer: its sex was determined as male by the dual PCR method.
(HE, ×100) (b) Ovarian tissue of a fetus obtained by pre-embryo transfer:
its sex was determined as female by the dual PCR method. (HE, ×100)

cent were identified as male and 42% as female, while 28% could not be identified.
Of these pre-embryos, 13 male and 25 female specimens were transferred to three
and five pseudopregnant recipients, respectively. Caesarian section was performed
on day 18 of pseudopregnancy and six viable fetuses were obtained from three
mice. The sex of each fetus was determined by histological examination of the
gonadal tissues (Fig. 5), and all fetuses were of the predicted sex.

DISCUSSION

The sexing of preimplantation embryos has been done by karyotyping (Roberts
et al., 1990) and by measuring X chromosome-linked enzyme activity in single
blastomeres (Monk *et al.*, 1990). However, the application of these methods in
preimplantation diagnosis is limited, as it is difficult to obtain adequate metaphase
preparations from a limited number of cells in the former method and target gene
expression only occurs at certain stages of embryonic development in the latter
method. To replace these methods, some molecular biological techniques have
been developed. One of them is *in situ* hybridization using fluorescent-labeled Y
chromosome-specific DNA probes (Griffin *et al.*, 1991).

Bradbury *et al.* (1990) sexed biopsied single blastomeres of mouse pre-embryos
obtained by *in vivo* fertilization using PCR amplification of a Y-specific repetitive
sequence. Since this sequence has many copies, its amplification does not require
an additional amplification. However, some segments of the repeated sequence
of the human Y chromosome are homologous with sequences on certain autosomal

chromosomes (Nakagome *et al.*, 1991). Thus, amplification of the repetitive sequence in human pre-embryos may result in inaccurate sexing. In the present study, therefore, the mouse *Sry* gene was used as the target sequence for identifying male sex. The sex-determining region of the Y-chromosome (*Sry*) is a Y chromosome-specific single-copy gene that induces male development in the mouse (Gubbay *et al.*, 1990). Since this gene is not a repetitive sequence, the single PCR proved insufficient to amplify the DNA from a single cell and an additional amplification, such as the dual PCR was required. The sex of single mouse blastomeres has previously been determined by the dual PCR amplification of two Y chromosome-specific single-copy genes, *Sry* and *Zfy* (Kunieda *et al.*, 1992). Detection of the *Sry* and *Zfy* sequences completely corresponds to the Y chromosome in karyotyping studies, but amplification of these sequences in biopsied single blastomeres has not previously been examined.

In the present study, whole 4- and 16-cell embryos were easily sexed by the present dual PCR method, since they contain approximately 25 pg and 100 pg of DNA, respectively. In the sexing of single blastomeres dissociated from 21 4-cell pre-embryos, 79 out of 84 blastomeres (94%) were identified as either male or female. In terms of pre-embryo sexing, however, the sex of all four blastomeres only agreed in 76% of the embryos. Both the *Sry* and myogenin sequences were not amplified in one of the blastomeres from two embryos (Table 2). When neither of the *Sry* and myogenin are amplified by the present method, a sex is not assigned to avoid the possibility of misdiagnosis. In three embryos, however, only myogenin sequences was detected in one of blastomeres while both *Sry* and myogenin sequences were amplified in other three blastomeres. If the sex of these blastomeres was assigned, male embryos could be mistaken for female embryos. Where no amplification was detected, the possibility of sample loss during blastomere handling or degeneration during processing by freezing and thawing has to be considered.

When single blastomeres for sexing were biopsied from 8-cell mouse pre-embryos by the expulsion method, amplification products were detected in 43 (72%) out of 60 biopsied blastomeres and were not detected in 17 (28%). The expulsion method of biopsy has been shown to cause less damage to blastomeres compared with aspiration and other methods (Sasabe, 1993). Also, expulsion biopsy of mouse pre-embryos at the 4- or 8-cell stage has no effect on their *in vitro* development to the blastocyst stage. Moreover, following the transfer of biopsied mouse pre-embryos to pseudopregnant recipients, the rate of fetal development is similar to that for control pre-embryos (Sasabe, 1993). No effect on their development to blastocyst stage has been reported in biopsy of two blastomeres from 8-cell pre-embryos (Roudebush *et al.*, 1990).

Biopsied pre-embryos were transferred to separate pseudopregnant recipients after sexing by the dual PCR method and six fetuses were obtained. Histological examination of the gonadal tissues showed that all these fetuses were of the predicted sex. Thus, sexing by *Sry* gene amplification appears to be highly reliable. Since

blastomere biopsy and sexing by the dual PCR method can be completed within 8 hr, biopsied pre-embryos can be transferred without any need for freezing.   Thus, the feasibility of applying the dual PCR method for preimplantation sexing of humans has been suggested by the present findings in mouse pre-embryos.

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