CHAPTER II

Materials and Methods

A. Materials

1. Instrument for embryo culture and biopsy

1. CO_2 water-jacket incubator	Forma Scientific, Marietta, Ohio, USA
2. Laminar flow hood	Pravit Engineering Ltd. Part., Thailand
3. Stereo microscope	Olympus, Tokyo, Japan
4. Inverted microscope with Hoffman lens system	Diaphot-TMD, Nikon, Tokyo, Japan
5. Micromanipulator	Narishige, Tokyo, Japan
6. Osmometer	Model 3WZ, Advanced Instruments inc., Massachusetts, USA
7. pH meter	Model SA 520, Orion Research Incorporated Laboratory Products, Boston, USA
8. Micropipette puller	P-87, The Sutter Instruments, USA
9. Microforge	MF-9, Narishige, Tokyo, Japan
10.Microgrinder	MG-4, Narishige, Tokyo, Japan
11.Glass capillary tubing	The Sutter Instruments, USA
12.Balance	Model 2004MP, Sartorius, West Germany

2. Instruments for DNA analysis

1. DNA Thermal cycler	Perkin Elmer Cetus Instrument, USA
2. Gel electrophoresis apparatus	Hoefer, USA
3. High speed centrifuge	Sigma, B. Brawn Scientific International, Germany
4. Shaking water bath	Shel-lab USA
5. Positive-displacement pipette (20 ml)	Eppendorf, Hamburg, Germany
6. Adjustable pipettor (0.5- 1000 ml)	Pipetman, Gilson , France
7. Vortex	Fisher Scientific Industries, USA
8. Electrophoresis power supply	Hoefer, USA
9. UV Illuminator	Ultralum, Ultra-lum Inc., USA
10. Polaroid camera	
11. Polaroid film type 667	
12. Microcentrifuge tube (0.2, 0. 5 ml)	
13. Disposable glove, parafilm	

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3. Chemical Reagents

All chemical substances for preparation of culture media, mineral oil, pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Sigma Co, St Louis, MO, USA. Taq DNA polymerase, 25 mM $MgCl_2$, four deoxynucleotide triphosphates (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), and loading buffer were from Promega Co., Madison, W.I., USA. The 100 base-pair ladder DNA

marker, and agarose gel were from Gibco BRL, Life Technologies, Inc., MO., USA. Ethidium bromide used for staining the gel was from Pharmacia. Oligonucleotide primers were synthesized by using Gene Assembler Pharmacia and purified by Oligonucleotide Purification Catridge, ABI USA. and were diluted to a 50 mM concentration.

B. Methods for mouse embryo manipulation

1. Animals

Male and female random-bred ICR mice were obtained from The Animal Unit, Department of Medical Science, Ministry of Public Health, Thailand. The animals were maintained on a 14L:10D cycle (light on, 04:00; light off, 18:00). Groups of 5 young adult females were kept in the same cages and single males were kept in individual cages. Newly arriving mice should be acclimatized for a few days before use. As embryo sources, females of 6-8 weeks of age were used and males should be 12 weeks of age or older, up to 18 months. Some general information about mice is summarized in table 2.

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2. Superovulation

Superovulation was used for producing larger batches of embryos than natural ovulation. The process was started by intraperitoneal injection of 5 IU of PMSG between 1 p.m. to 2 p.m. of the first day, followed by an intraperitoneal injection of 5 IU of hCG 48 hr later.

Table 2 Characteristics of mice.

Characteristics	Approximate value
Life span	1.5 years
Gestation	19-21 days
Estrous cycle	4-5 days
Duration of sex receptivity	12 hr
Pseudopregnancy	12 days
Breeding age	
Male	60 days
No. of sperm/ejaculation	5×10^7
Female	55 days
No. of egg ovulated	
Natural	10
Superovulation	30 (< 15 to> 50)
Breeding life	
Male	1.5 years
Female	8 litters
Litter size	9 pups
Weaning age	19 days
Weight	
Birth	1.25 gm
Adult male	35 gm
Adult female	30 gm

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Table 2 Characteristics of mice (continue).

Characteristics	Approximate value
Blood volume (adult)	2 ml
Food consumption (daily)	4.5 gm
Water consumption (daily)	6 ml
Chromosome	
Diploid number	40
Total DNA	6 pg
Genes	10^5
Recombination units	1.6×10^3 cM

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For administration, PMSG and hCG were resuspended at a concentration of 50 IU/ml in sterile distilled water and then divided into aliquots so that a dose of 0.1 ml was injected into each animal. The aliquots were stored at -20 ^{o}C and used within one month. Immediately after hCG injection, three to five injected females were added to each male's cage and checked for a copulation plug the next morning (indicated day 1 of pregnancy). The sequence of events of preimplantation development of mouse embryos is illustrated in figure 5 and table 3.

3. Media

All media used in this study were in-house prepared. The constituents of each medium are shown in appendix A and B.

a. Flushing and washing medium : For retrieval of mouse embryos, HEPES-T6 medium (Quinn et al., 1984) supplemented with 0.5% bovine serum albumin (BSA; fraction V) was used to flush the embryos from the oviducts and to wash the embryos before placing them into the biopsy medium. This medium was also used for washing the isolated blastomeres.

b. Biopsy medium : The biopsy medium consisted of a Ca^{2+}- and Mg^{2+}-free buffered salt solution containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 100 mM sucrose (Gordon and Gang, 1990).

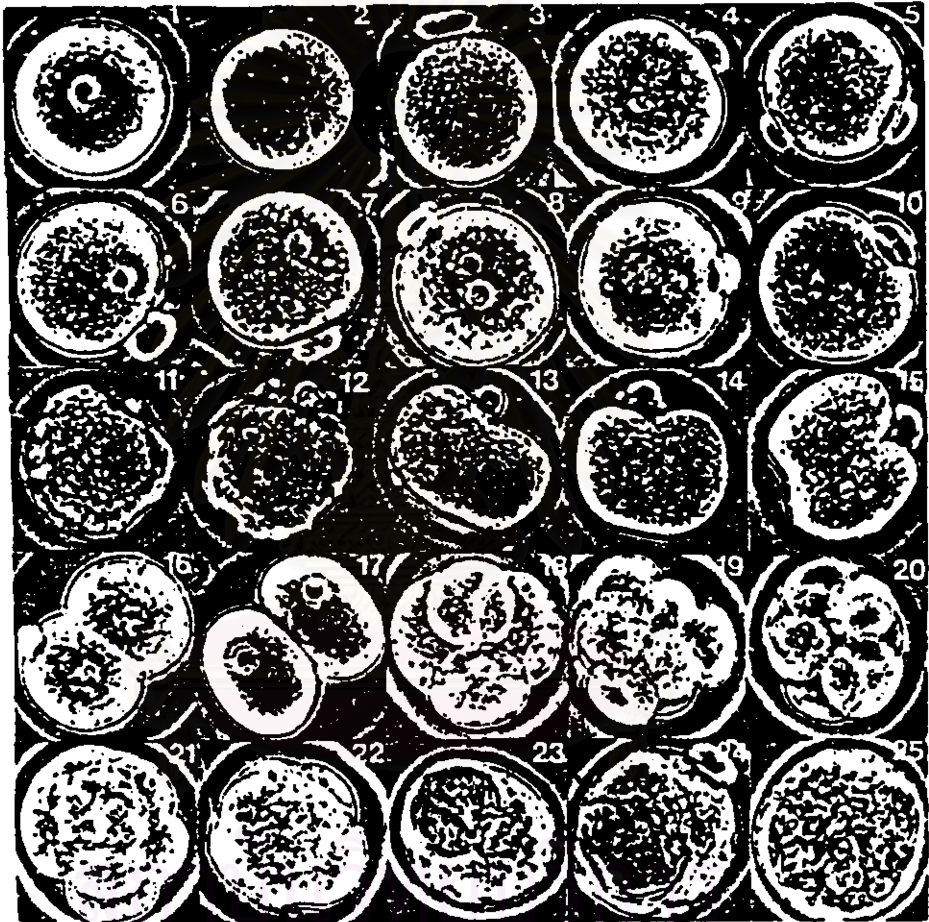

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Figure 5 Morphology of normal preimplantation mouse development . (1) Preovulatory oocyte with germinal vesicle intact. (2) Preovulatory oocyte showing breakdown of germinal vesicle. (3 and 4) Extrusion of first polar body follow by ovulation and fertilization. (5) Resumption of meiosis by female set of chromosomes and extrusion of second polar body. (6) Decondensation of sperm nucleus and formation of male pronucleus. (7) Formation of nuclear membrane around haploid set of female chromosomes to form female pronucleus. (8-10) Migration of pronuclei to center of egg. DNA replication. (11 and 12) Breakdown of pronuclear membrane . (13) Elongation of embryo. (14 and 15) Formation of 'waist'. (16) Newly formed 2-cell embryo with visible nuclei. (17) Later stage 2-cell embryo. (18-25) Later stage of preimplantation development. 18: 4-cell embryo 19-20: 6- to 8-cell embryo. 21: compacting 8-cell embryo. 22: compacted 16-cell embryo (morula). 23 and 24 early blastocyst. 25: fully expanded blastocyst. (Reproduced from Pratt et al., 1983).

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Table 3 Timing of developmental events of mouse embryo[a]

cell cycle	Stage of embryo after complete each cell cycle	Period of event (hr post hCG injection)
First cell cycle	2-cells	~32
Second cell cycle	4-cells	~48-50
Third cell cycle	8-cells	~58-60
Fourth cell cycle	16-cells	~72
Fifth cell cycle	32-cells	~84
Sixth cell cycle	Fully expanded blastocyst	~90-98
Subsequent cell cycles	Hatched blastocyst	~110
		~120

[a] This table lists the sequence of morphological events during pre-implantation mouse development in vitro in relation to phases of individual cell cycles and time of hCG injection (adapted from Pratt, 1989).

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c. **Culture medium** : All embryos were cultured for evaluation of their in vitro development in the modified T6 medium (Nasr-Esfahani et al., 1990) supplemented with 0.5% BSA.

All the prepared media were filtered through a 0.22 μ Millipore filter into small sterile containers, gas the air space with 5% CO_2 in air, and capped tightly to maintain a pH of 7.2-7.4. The media can be kept at 4 °C and routinely used for 1 month. The working media were incubated at 37 °C in a CO_2 incubator for 2-3 hr before used.

4. **Embryo collection**

To obtain 4- cell stage mouse embryos, mated females were sacrificed by cervical dislocation on the evening of day 2 (54-58 hr. post hCG). The oviducts were removed and placed in a warm (37°C) flushing medium. Embryos were released from the oviducts by inserting a 30 gauge needle in the ostium of the oviduct and injecting approximately 0.1-0.5 ml of flushing medium. Embryos pooled from 3-5 donors were washed off debris and white blood cells by repeated passage of the embryos through drops of washing medium using a flame-pulled glass Pasteur pipette attached to a mouth piece. Embryos were finally transferred into drops of modified T6 medium (~ 50 μl) under paraffin oil in plastic petri dishes , and incubated at 37 °C in a 5 % CO_2 atmosphere for 1-2 hr before the experiment. The 8-cell and morula stage embryos were obtained by flushing them from both the oviducts and uterine horns in the morning of day 3 of pregnancy (72-80 hr post hCG). Only embryos assessed

(2) A fine, sharp glass needle used for cutting the small part of the zona pellucida which was pulled by a horizontal micropipette puller (Sutter, P97. U.S.A.)

(3) The blunt-ended biopsy pipette is made by cutting the tip of a glass needle to yield a diameter of 30-40 μm.

(4) The bevelled biopsy pipette is created by grinding down the tip of a glass needle for a few seconds on a microgrinder (Narishige, Tokyo, Japan) The resulting pipette has a bevelled angle of 45° and a diameter of 20-30 μm.

c) Embryo biopsy procedure

Embryos at 4-cell, 8-cell, and morula stages were randomly divided into three groups: control; direct aspiration; PZD-push. All embryos were incubated in the biopsy medium at 37 ^{O}C for 60-90 minutes before biopsy. Nine 10-μl droplets of biopsy medium were set up under light-weight mineral oil in a small Petri dish (Falcon # 1006). Embryos randomly selected for biopsy were individually transferred to each droplet and then placed on the warm stage of the microscope for micromanipulation. One (at the 4-cell and 8-cell stages) or two (at the morula stage) blastomeres were removed from the embryos by means of two methods:

(1) Direct aspiration method (Wilson and Trounson,1989)

The embryo was held in place by suction pressure through the holding pipette at the 9 o'clock position. The tip of the bevelled biopsy pipette was

pushed directly through the zona pellucida into the embryo and a single blastomere was gently aspirated into the biopsy pipette. The biopsy pipette was then withdrawn from the embryo and the blastomere was expelled into the surrounding medium (figure 7 and 8).

(2) PZD-push method

With the embryo held in place, the small part of the zona pellucida was dissected using a sharpened microneedle gently inserted at the 1 o'clock position and threaded through the ZP to the 11 o'clock position. Subsequently, the microneedle holding the embryo was placed under the holding pipette, which is then used to rub off the portion of the zona perforated by the microneedle until the embryo drops off. The embryo with the partly dissected zona pellucida (PZD) was gently squeezed with the blunt-ended pipette, until a single blastomere protruded from the embryo (figure 9, 10 and 11).

6. In vitro culture and assessment of viability of biopsy embryos

In the first series of experiments, to further study in vitro development, only surviving embryos with intact blastomeres remaining within an intact zona pellucida were washed twice and cultured up to day 7 in groups of ten in 50 μl droplets of modified T6 medium supplemented with 0.5% BSA under lightweight mineral oil at 37 ^{o}C in 5% CO_2 with 100% humidity. The effect of cell loss on embryo development was evaluated by scoring for in vitro blastocyst formation and complete hatching from the zona pellucida.

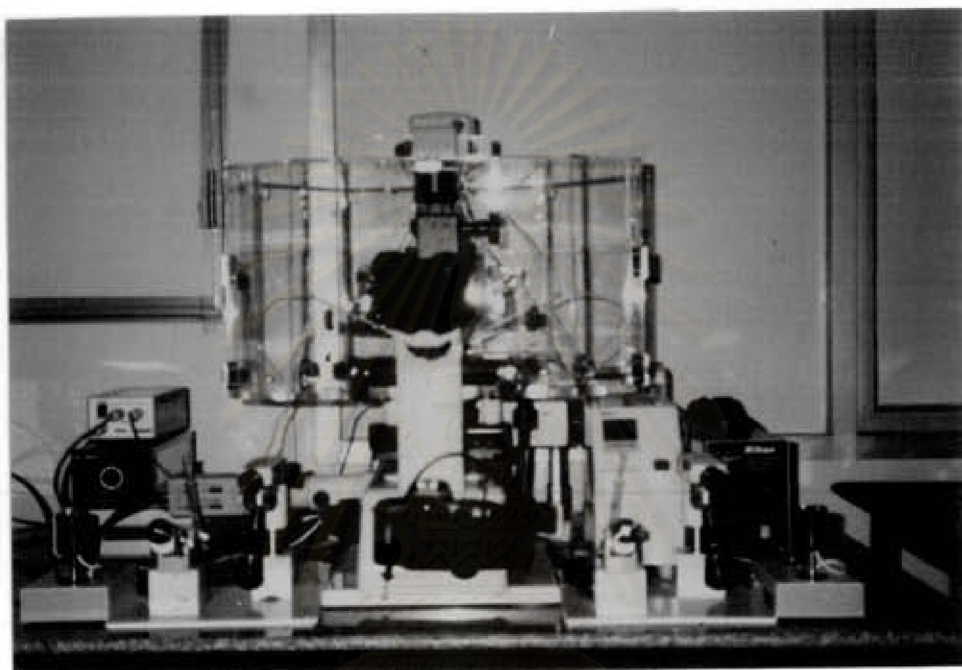

Figure 6 Arrangement of the micromanipulation set. The inverted microscope is flanked with two-side micromanipulators. Each set contains a 3D motor drive coarse control, 3D hydraulic remote fine control and a microinjector. The micromanipulator of the left is used for the holding pipette and the micromanipulator of the right is used for the biopsy pipette. The microscope is covered with a plastic incubator for environmental temperature and gas control.

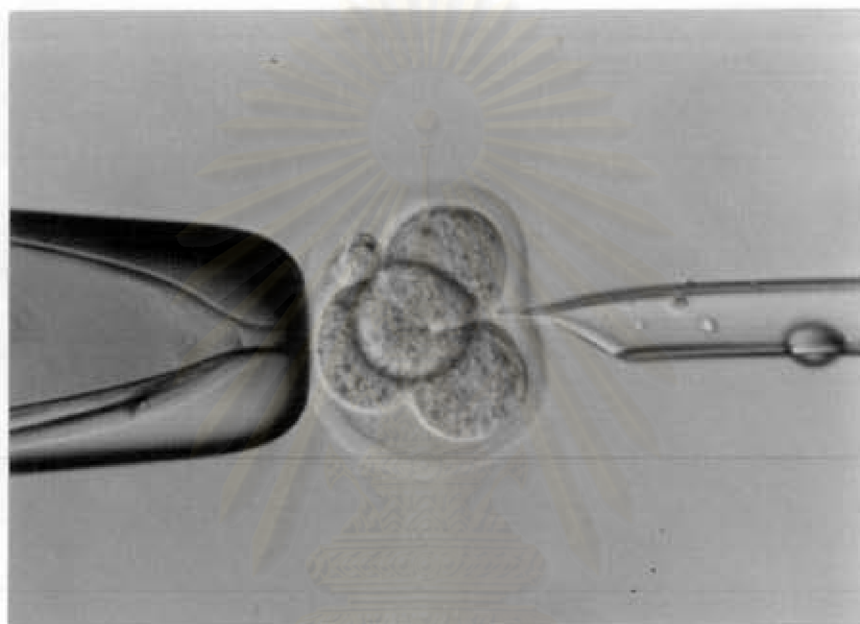

Figure 7 Direct aspiration procedure for single-blastomere biopsy from the 4-cell mouse embryo. Embryo held in position by suction on holding pipette. The tip of beveled biopsy pipette was pushed through the zona pellucida into the embryo and a single blastomere was removed from the embryo by aspiration pressure through the biopsy pipette. Optical magnification: x400.

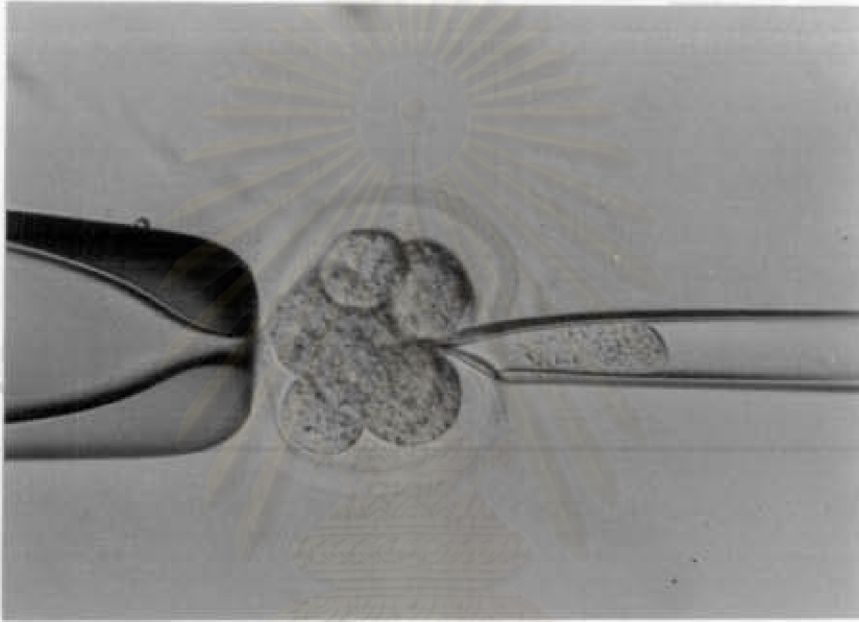

Figure 8 Direct aspiration procedure for single-blastomere biopsy from the 8-cell mouse embryo. Embryo held in position by suction on holding pipette. The tip of beveled biopsy pipette was pushed through the zona pellucida into the embryo and a single blastomere was removed from the embryo by aspiration pressure through the biopsy pipette. Optical magnification: x400.

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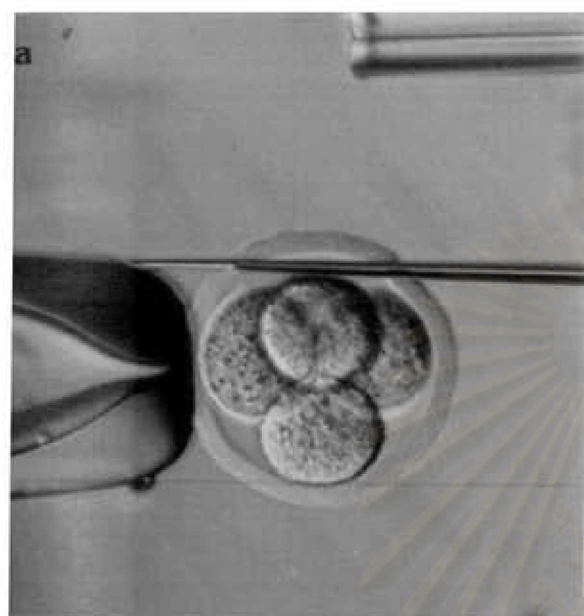
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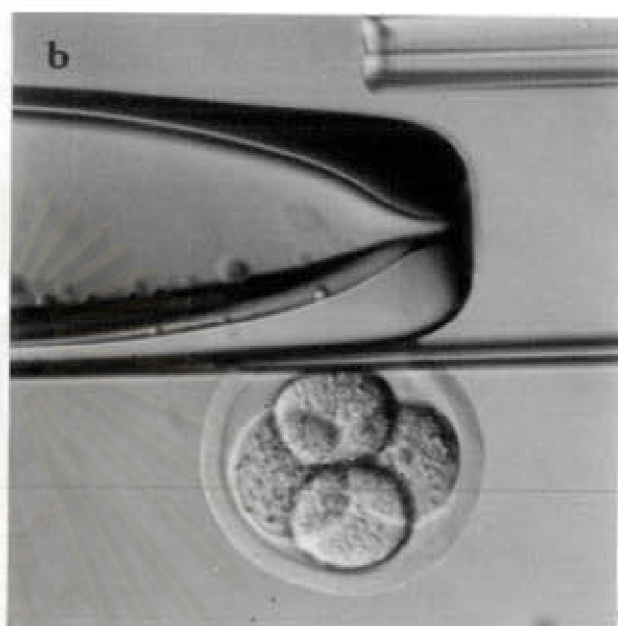
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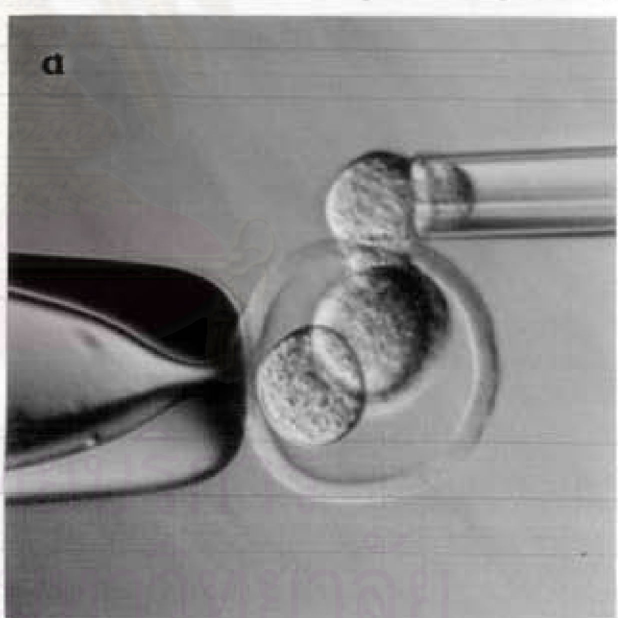
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Figure 9 The PZD-push procedure for single-blastomere biopsy from the 4-cell mouse embryo. (a) A fine glass needle is inserted into perivitelline space. (b) The needle is rubbed against the wall of the holding pipette to tear the zona. (c) One blastomere is pushed out of the embryo through the hole in the zona by a biopsy pipette. (d) The expelled blastomere is aspirated into a biopsy pipette. Optical magnification: x400.

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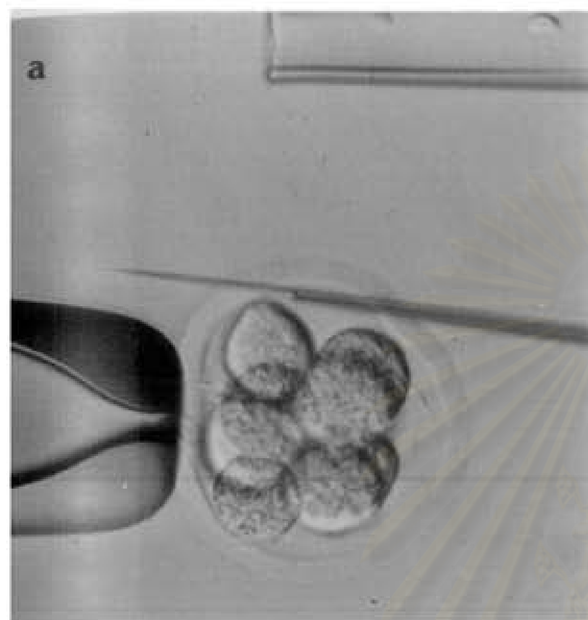
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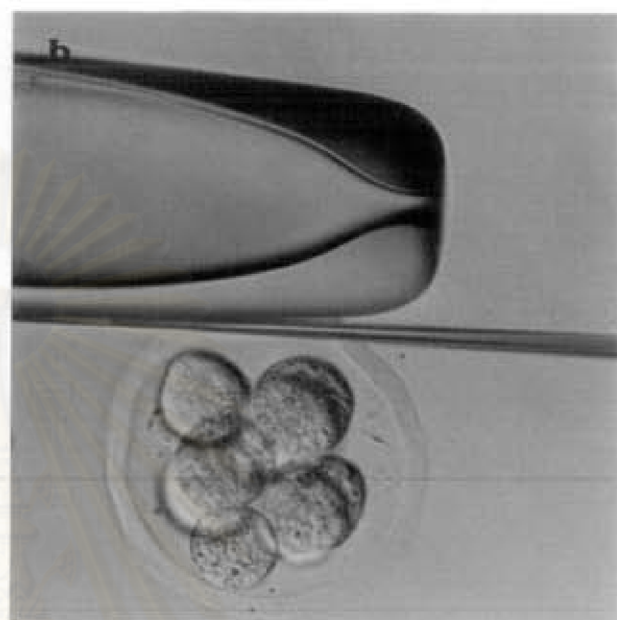
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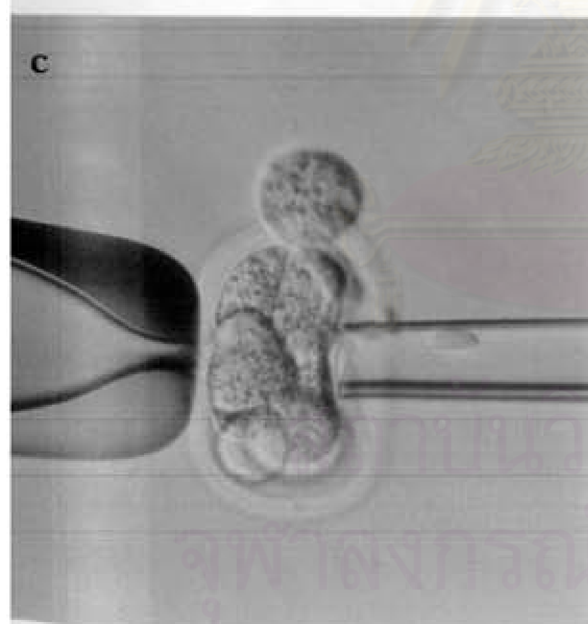
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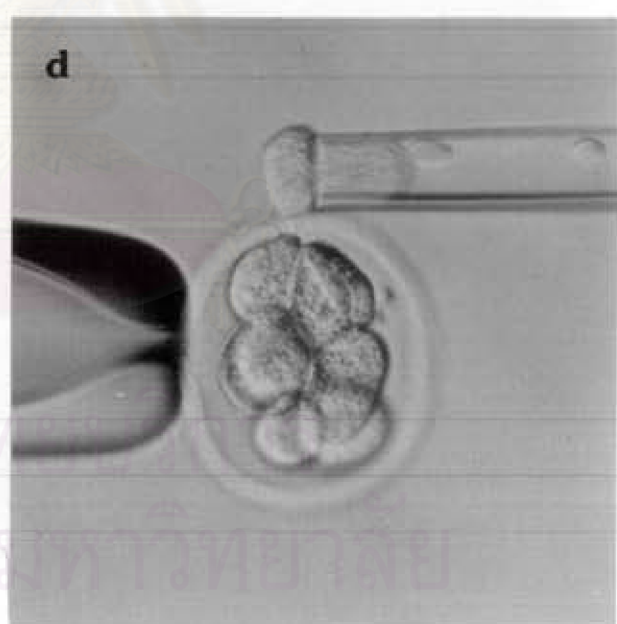
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Figure 10 The PZD-push procedure for single-blastomere biopsy from the 8-cell mouse embryo. (a) A fine glass needle is inserted into perivitelline space. (b) The needle is rubbed against the wall of the holding pipette to tear the zona. (c) One blastomere is pushed out of the embryo through the hole in the zona by a biopsy pipette. (d) The expelled blastomere is aspirated into a biopsy pipette. Optical magnification: x400.

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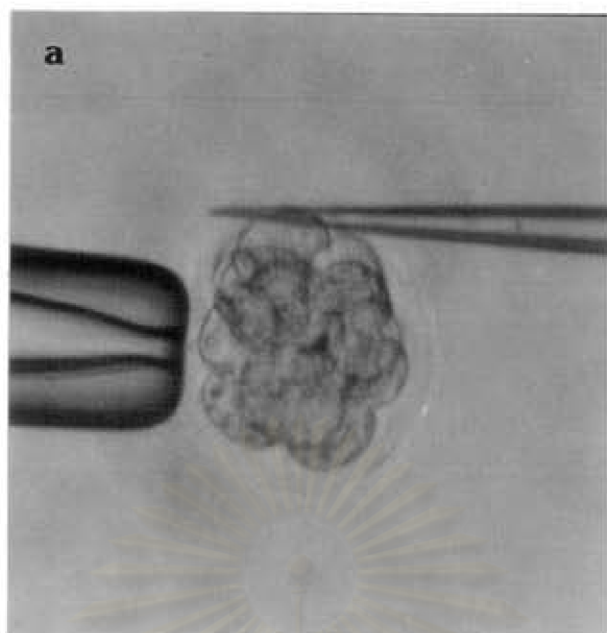

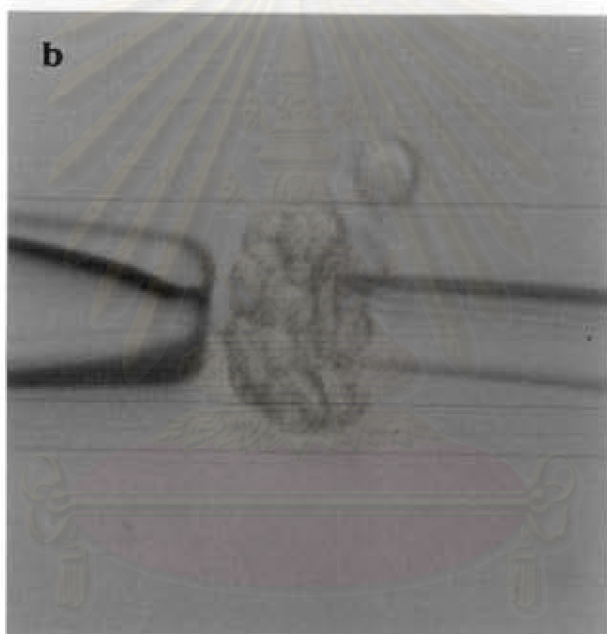

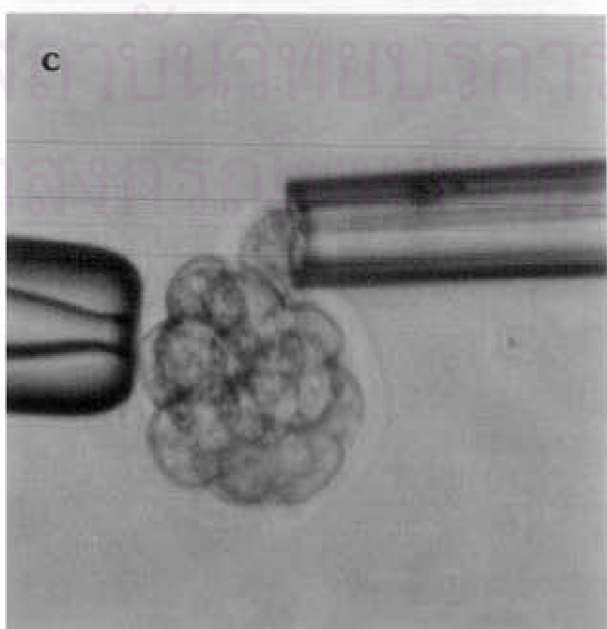

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Figure 11 The PZD-push procedure for single-blastomere biopsy from the mouse morula stage embryo. (a) A fine glass needle is inserted into perivitelline space. (b) One blastomere is pushed out of the embryo through the hole in the zona by a biopsy pipette. (d) The expelled blastomere is aspirated into a biopsy pipette. Optical magnification: x400.

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The in vitro development of biopsied and control embryos was evaluated every 24 hr, i.e., embryos biopsied at the 4-cell, 8-cell and morula stage were further cultured for 72, 56, and 48 hr, respectively. The rate of blastocyst formation and of complete hatching blastocysts were compared.

7. Assessment of post-implantation development in vivo

In the second series of experiments, the completion of in vivo development and recovery of live mice were evaluated by transferring both control and biopsied blastocysts to the uterus of pseudopregnant foster mothers.

a) Vasectomy and preparation of pseudopregnant females

Vasectomized males are used to produce pseudopregnant females for embryo transfer. Male mice were anesthetized with ether and restrained in the supine position. Abdomen and scrotum were wiped with 70% alcohol. Using forceps and dissection scissors, a 1.5-cm transverse incision opens the body wall at a point level with the top of the legs. With the blunt-ended forceps, the fat pad on the one side was pulled out. The testis, vas deferens, and epididymis will come out with it. The vas deferens lies underneath the testis and can be identified by a blood vessel running along one side. Using a fine Watchmaker forceps it was dissected from the vessel. The vas deferens was held in a loop with one pair of forceps and the vas deferens loop was cauterized with the red-hot tips of a second pair of forceps. The testis and its associated parts were

carefully replaced inside the body wall and the operation repeated on the contralateral side. When the operation was completed, the abdominal wall incision was closed with one to two stitches. The vasectomized males were allowed to recover for a week (without access to females) before being used for pseudopregnant mating. Before mating with the vasectomized males, the recipient females were injected with 5 IU of PMSG and followed by 5 IU of hCG 48 hr apart in order to phase estrus and increase the number of plug-positive recipients.

b) Transfer of blastocysts to the pseudopregnant recipients

Biopsied and control embryos at the early blastocyst stage (day 5) were asynchronously transferred to the uterine horns (4 -6 embryos/horn) of day 3 pseudopregnant recipient females. The method of blastocyst transfer was modified from Hogan et al. (1986). The recipient female was anesthesized with ether and its back was wiped with 70 % alcohol. A small single midline incision (less than 1 cm) was made on the mid-dorsal skin at the level of the last rib. The opening was slid to either side until the incision was over the ovary or fat pad. The lateral abdominal wall was picked up and incised for less than 1 cm. Through the incision, the fat pad was seized and pulled out bringing the ovary and uterus with it. With the recipient's uterus already prepared, four to six blastocysts were loaded into the transfer pipette prepared from a fire-pulled pasteur pipette. The top of the uterus was gently held with a fine blunt forceps and a hole was made a few millimeters down the uterus with a 26-gauge needle. The transfer pipette was inserted about 2-3 mm into the hole and

the blastocysts were expelled in as small a volume as possible. Upon completion of the transfer, the uterus, oviduct and ovary were carefully replaced to the abdominal cavity. The operation was repeated on the contralateral side and the skin incision was closed with two suture stitches. The pregnant recipients were kept in individual cages. Ten days after transfer some pregnant recipients were killed and the uteri were excised for determining the implantation rates. The remaining pregnant recipients were allowed to deliver at term (19 to 22 days) and the numbers of live fetuses were compared.

8. Assessment of postnatal development

In the third series of experiments, postnatal growth and development of offspring from biopsied versus control embryos were compared. Body weight was measured at 24 hr. after birth and at 3-6 weeks of age. To test the normalcy, female mice developed from biopsied embryos were allowed to grow to sexual maturity (4-6 months of age) and mated with normal male mice to evaluate their capacities to produce a second generation by counting the litter size.

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C. Methods for DNA analysis

1. Selection of oligonucleotide primers

For DNA analysis, a multiplex two-step (nested) PCR system was used (figure 12). The DNA sequences of the Sry (Gubbay et al.,1990) and Zfy

(Mardon and Page, 1989; Asworth et al., 1989) genes, known to be present in the sex determining region of the human and mouse Y chromosome, were chosen for the Y-specific target sequences , and DXNds 3 locus , which is a polymorphic microsatellite locus located on mouse X chromosome (Love et al., 1990), served as the internal control sequence. DNA sequences of all three genes were shown in appendix G-I. For every locus ,two sets (an outer and an inner set) of primers were used as shown in figure 13. The locations of each primer are summarized in figure 14.

2. System testing

To test the accuracy and specificity of these primers, DNA from male and female mouse leukocytes was subjected to the multiplex two-step PCR system. To investigate the sensitivity and reliability of the PCR procedure at the single cell level, single mouse blastomeres (1/8 blastomeres) were subjected to PCR analysis simultaneously with the remaining 7/8 embryos. In this way it was possible to compare the PCR results of two separated cells originating from the same embryo.

a) Preparation of DNA from whole blood

Five male and female mice were anesthesized with ether and 0.5 ml of heart blood was collected individually into separate heparinized tubes. DNA from whole blood was extracted using the method of Kawasaki (1990).

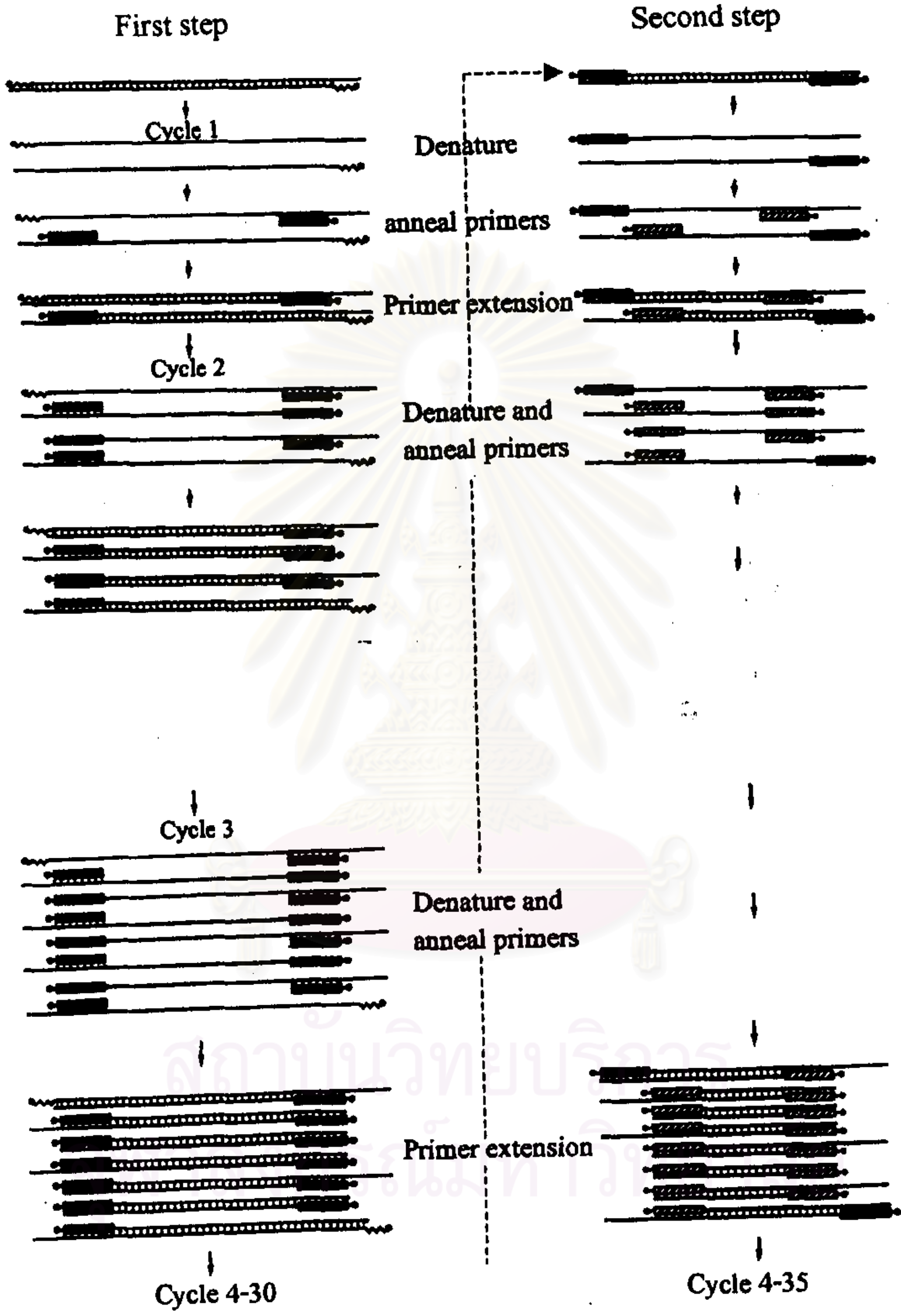
First step
Second step
Cycle 1
Denature
anneal primers
Primer extension
Cycle 2
Denature and
anneal primers
Cycle 3
Denature and
anneal primers
Primer extension
Cycle 4-30
Cycle 4-35

Figure 12 Diagram showing the sequence of DNA denaturation, annealing of primers, and primer extension during successive cycles of the two-step PCR. In the second step or nested reaction, the amplification product is smaller than in the first-step. (Reproduced from Dziadek and Bakker, 1993).

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The outer primer sets

Sry outer primers :

Sry 1 : 5' TCTTAAACTTCTGAAGAAGAGAC 3'

Sry 4 : 5' GTCTTGCCTGTATGTGATGG 3'

These primers amplify a 404 bp DNA fragment

Zfy outer primers :

Zfy 1: 5' AAGATAAGCTTACATAATCACATGGA 3'

Zfy 4: 5' TTTTTGAGTGCTGATGGGTGACGG 3'

These primers amplify a 617 bp DNA fragment

DXNds3 outer primers :

Nds 1: 5' GAGTGCCTCATCTATACTTACAG 3'

Nds 4: 5' TCTAGTTCATTGTTGATTAGTTGC 3'

These primers amplify a 244 bp DNA fragment

The inner (nested) primer sets

Sry inner primers :

Sry 2 : 5' GTGAGAGGCACAAGTTGGC 3'

Sry 3 : 5' CTCTGTGTAGGATCTTCAATC 3'

These primers amplify a 299 bp DNA fragment

Figure 13 Details of primer sets used during PCR reaction.

Zfy inner primers :

Zfy 2: 5' CCTATGAAATCCTTTGCTGCACATGT 3'

Zfy 3: 5' GTAGGAAGAATCTTTCTCATGCTGG 3'

These primers amplify a 217 bp DNA fragment

DXNds inner primers :

Nds 2: 5' ATGCTTGGCCAGTGTACATAG 3'

Nds 3: 5' TCCGGA AAG CAG CCA TTGGAGA 3'

These primers amplify a 111 bp DNA fragment

Figure 13 Details of primer sets used during PCR reaction (continue).

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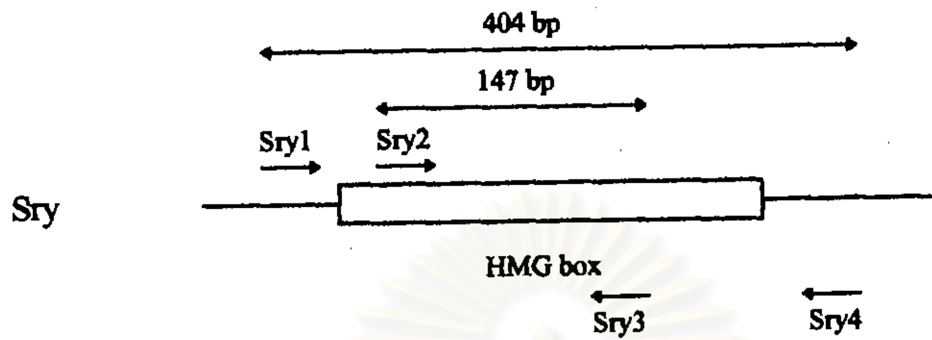

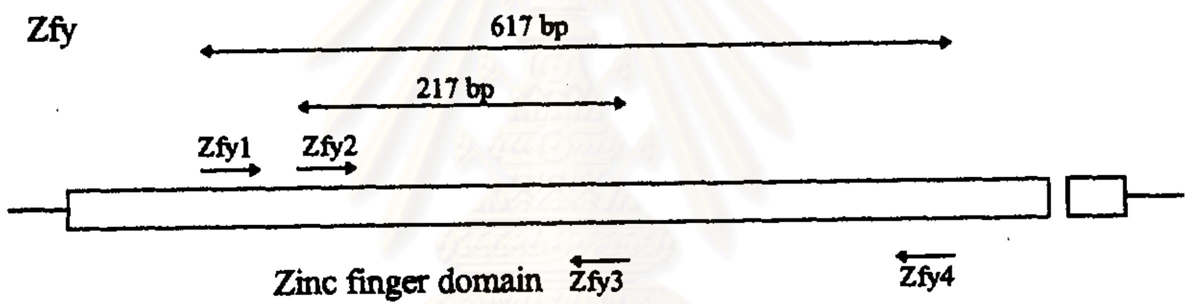

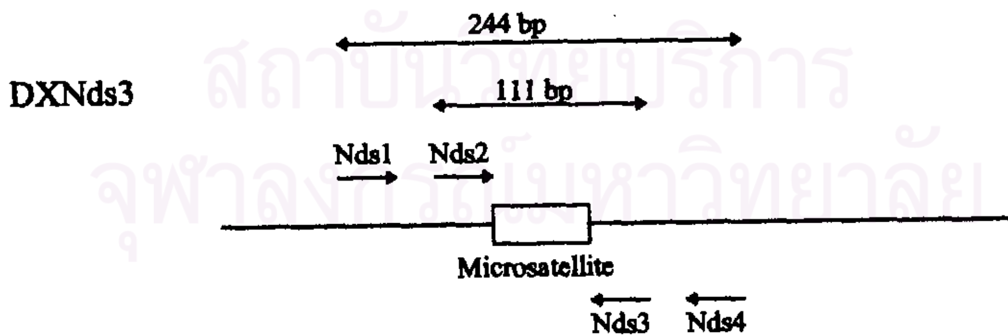

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Figure 14 Location of various oligonucleotide primers on Sry, Zfy, and DXNds3 sequences and sizes of fragments amplified with the primers. Single-headed arrows indicate position of the primers and double-headed arrows indicate the regions amplified with the primers. Open boxes represent characteristic regions of the sequences. HMG box and Zinc finger domain are putative DNA-binding domains of the Sry and Zfy genes, respectively, and microsatellite is a polymorphic region consisting of a dinucleotide repetitive sequence.

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Briefly, 50 μl of whole blood was mixed with 0.5 ml of TE buffer (appendix D) in a 1.5 ml microfuge tube, and then spun for 10 seconds at 14,000 x g.The pellet was resuspended in 0.5 ml TE buffer by vortexing and pelleted as before. The procedure was repeated twice more and then the final pellet was resuspended in 100 μl of lysis-K buffer (appendix E) and incubated for 45 min at 56°C, then for 10 min at 95°C. This DNA suspension was used as the DNA template for testing the PCR system and as a positive control.

b) Blastomere collection for PCR

Some batches of the 8-cell mouse embryos were used for testing the PCR system. The zona pellucida of the embryos was first removed by incubation in acidic Tyrode's solution (appendix C) for 2-3 min. The disappearance of the zona was continuously observed under the stereomicroscope. The embryos without zona were then washed in HEPES-T6 washing medium several times. The Zona-free embryos were incubated in the biopsy medium for 30-60 min to loosen the intercellular contacts between the blastomeres. Free single blastomeres were isolated by flushing them through the mouth-controlled drawn pasteur pipette several times. Single blastomeres (1/8 blastomeres) and the remaining embryos (7/8 blastomeres) were then placed directly into 30 μl of HEPES-T6 medium and washed three times, changing drops between washes. After the last wash, the 1/8 and 7/8 blastomeres were individually transferred to 10 μl of autoclaved double distilled water in 0.2 μl thin PCR tubes. This step was performed under the dissecting stereomicroscope to ensure that the cells were present in the PCR tube. These samples were left at

room temperature for a minimum of 10 min to ensure complete cell lysis and then subjected to three cycles of freezing at -70 ^{O}C and thawing at 37°C prior to amplification. Thawed samples were heated at 95 ^{O}C for 15 min and cooled immediately on ice. Following brief centrifugation, the sample tubes were returned to the ice box for further PCR analysis.

c) PCR methodology

A multiplex two-step PCR was performed for simultaneous amplification of Zfy, Sry and DXNds3 sequences using two pairs of primers for each target sequence. PCR reaction mixtures were freshly prepared containing the following compositions: 1X PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 ^{O}C; 50 mM KCl; 1% Triton X-100); 1.5 mM $MgCl_2$; 200 µM each of the four deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP); 40 pmol of each of the oligonucleotide primers and of Taq polymerase (5 IU/ ml). For the first-step PCR reaction, 40 µl of reaction solution which contains a mixture of all of the outer pairs of primers was pipetted into the sample tubes containing 1/8 or 7/8 blastomere under the layer of mineral oil. Amplification was carried out in a DNA Thermal cycler 2400 (Perkin-Elmer Cetus Instruments) employed the following conditions: 4 min denaturation at 94 ^{O}C followed by 30 cycles of denaturation, annealing and extension for 1 min each, at 94, 60, and 72 ^{O}C, respectively. Then a final extension step at 72 ^{O}C for 5 min and cooled to 4 ^{O}C.

For the second-step PCR reaction, 2 µl portions of the first-step products were dispensed separately into PCR tubes with 48 µl of a fresh mixture containing the inner pairs of primers for each individual locus. Amplification was performed for 35 cycles as in the same conditions as the first-step PCR reaction. Positive and negative controls (including 10 µl of male DNA, 10 µl of female DNA and 10 µl of distilled water or the last drop of washing medium, respectively) were also subjected to the multiplex two-step PCR procedure together with the embryo samples.

d) Electrophoretic analysis of PCR product

Electrophoretic analysis was performed on 10 µl aliquots of the second-step PCR products diluted with 2 µl of 0.1 M Na_2EDTA, 1% sodium lauryl sulfate, 0.25% bromophenol blue, 20% ficoll-400. Using a minigel apparatus, horizontal tandem gels of 3 % Nusieve 3:1 agarose (FMC BioProducts) were run for 2 hr at 80 V. in Tris-borate-EDTA running buffer (appendix F). Ethidium bromide was added directly to the gel to a concentration of 1 µg/ml gel. A 5 µl of a low-molecular-weight marker fragment ,100 base-pair ladder DNA, was run adjacent to the test samples to confirm the correct size of the amplified bands. After complete electrophoresis, amplified fragments were visualized by ultraviolet illumination and Polaroid photography on a transilluminator.

e) Prevention of contamination

Because the PCR can generate trillions of DNA copies from a template sequence, contamination of the amplification reaction with products of a previous PCR reaction, exogenous DNA, or other cellular material can create problems in diagnostic results. To control these problems, four separated room were used. Embryos culture and blastomere preparation were performed in a Hepa filter sterile room. Reaction tube preparation was performed in a laminar flow hood in a different room from that where test sample was added. Another room was used for PCR product analysis. It is essential to use strict sterile techniques in all experiments. Disposable glove were used at all times and frequently changed. Hair covers and face masks were worn for setting up all experiments. A separate sets of supplies and positive displacement pipettes were used for sample preparation and for setting up reactions. Ultraviolet lamps inside hood were kept on overnight and at least 30 min before initiating all polymerase chain reaction experiments, thus exposing dishes, pipette tips, pipette devices, tubes, oil, and water to irradiation until initiating an experiment. All reagents were placed in separate small aliquots, so that if contamination occur, a new bottle can be used immediately.

D. Sex determination of mouse embryo

To collect the 8-cell stage mouse embryos, female ICR mice were superovulated and mated with fertile males and were sacrificed on day 3 of pregnancy. The 8-cell stage embryos were flushed from the reproductive tract

and cultured for 15 min in modified T6 medium supplemented with 0.5% BSA. All the normal morphological appearance embryos were incubated in biopsy medium for 60-90 min. Embryos were then individually transferred to a 10- μl droplet of biopsy medium for micromanipulation. Embryo biopsy was performed using the PZD-push technique as described before.

After biopsy, each embryo was separately transferred to individually labeled modified T6 droplets under mineral oil for continuing culture. All the isolated single blastomeres were separately transferred to a thin-wall PCR tube containing 10 μl of sterile distilled water, using a fine hand-drawn Pasteur pipette. This procedure was carry out under a dissecting stereomicroscope in a horizontal laminar flow hood to confirm that the blastomere had been successfully expelled. Following transfer into water, the blastomeres were left at room temperature for a minimum of 10 min to ensure complete cell lysis and then subjected to three cycles of freezing at -20 ^{O}C and thawing at 37 ^{O}C prior to amplification. Thawed samples were heated at 95 ^{O} C for 15 min and cooled immediately on ice. PCR amplification of X- and Y-chromosome specific sequences was performed as the same conditions as described in section C.2.c The time taken from biopsy to obtaining an embryo sex result was about 10 hr.

Embryo transfer was performed in the morning of day 4 when the biopsied embryos were at the blastocyst stage and began to hatch. All the putative male blastocysts determined by PCR amplification were collected into one labeled 30-ml droplet of modified T6 medium, and the putative female blastocysts into another droplet. Six to eight of the same putative sex

were transferred to day 3 pseudopregnant recipient mouse. The recipients were individually caged and allowed to deliver offspring developed from the putative male and female blastocysts. Sex of mouse pups was phenotypically identified on the day of birth and again at 6-10 weeks of age.

E. Statistics

The results of in vitro and in vivo development of biopsied embryos were analysed by a χ^2 two-by-two contingency table test and unpaired student t-test. Significance was considered as $P < 0.05$.