CHAPTER III

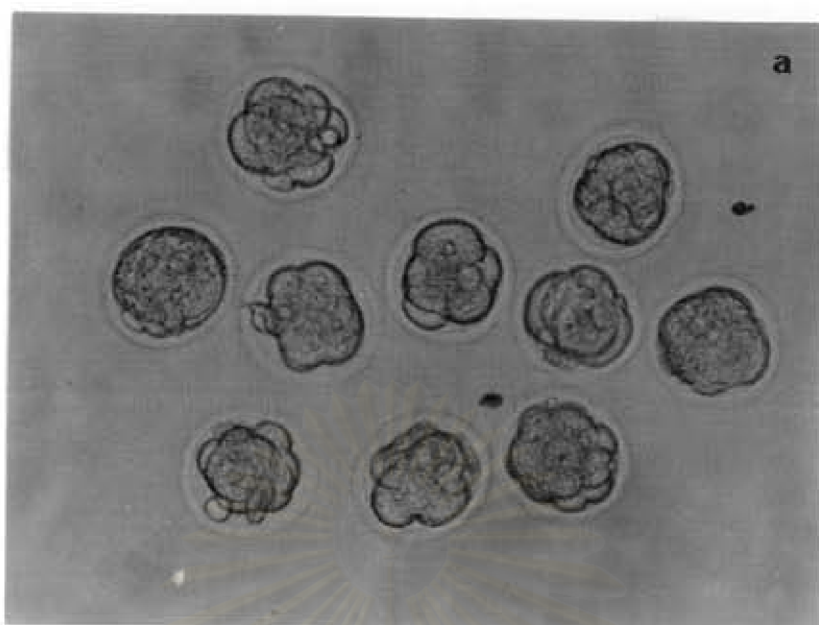
Results

A. Effects of biopsy procedure on embryo development

1. In vitro development

A total of 843 four-cell, 1027 eight-cell, and 870 morula stage samples of mouse embryos were evaluated as to their capacity to sustain in vitro development with and without biopsy using two mechanical biopsy techniques, PZD-push and direct aspiration biopsy techniques. At the 4-cell stage (Table 4), the potential to develop in vitro into early blastocysts (day 5 post hCG and figure 15a) and hatching blastocysts (day 6 post hCG, figure 15b) was similar between both control and biopsied embryos. The percentages of embryos that reached the early blastocyst stage in the control, solution control, PZD-push, and direct aspiration groups were 83.3%, 81.3%, 79.0%, and 77.2%, respectively, and the percentages of the early blastocysts that continued their development to the hatching blastocyst stage were 70.0%, 70.6%, 72.2%, and 70.1%, respectively. Although, the blastocyst formation and hatching rates of the direct aspiration biopsied embryos were not significantly lower than those of the control embryos, the direct aspiration technique lead to a significant ($P<0.01$) reduction in the rates of those

biopsy was performed by PZD-push technique at all three embryonic stages. There were also no significant differences in the rates of in vitro development when biopsy was performed at neither the 4-, 8-cell nor morula stages (table 7). The hatching processes in the control and solution control embryos exhibited expansion of the blastocyst, thinning of the ZP, extrusion of a cellular projection that penetrated the ZP, and rupture of the ZP. In the biopsied embryo groups, the blastocysts did hatch without the expansion of the ZP and the ZP did not become thinner (figure 15c and 16). Moreover, hatching commences earlier than those of the control embryos. In most of the biopsied embryos in which zona shedding is incomplete, the trophectobiast outgrowth was abnormal when further cultured for one or two days after hatching as shown in figure 17.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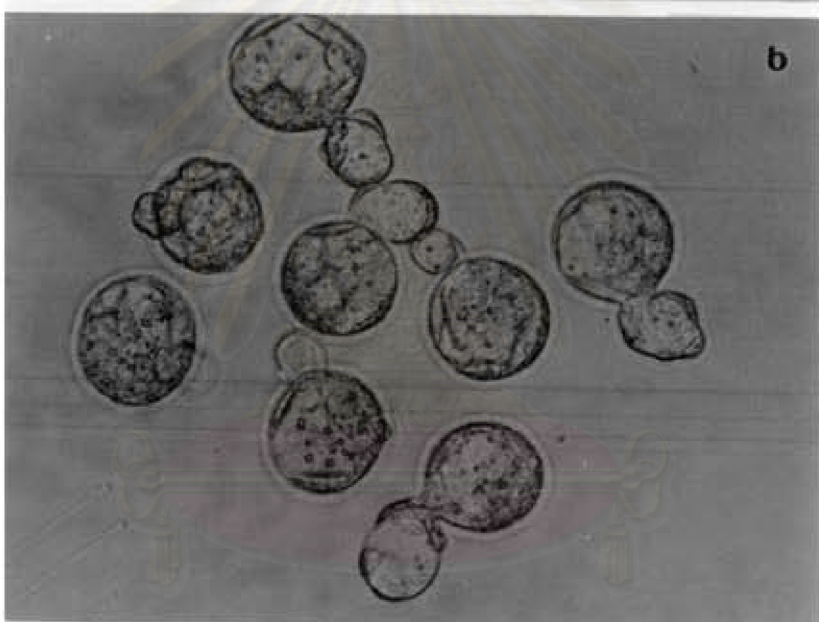
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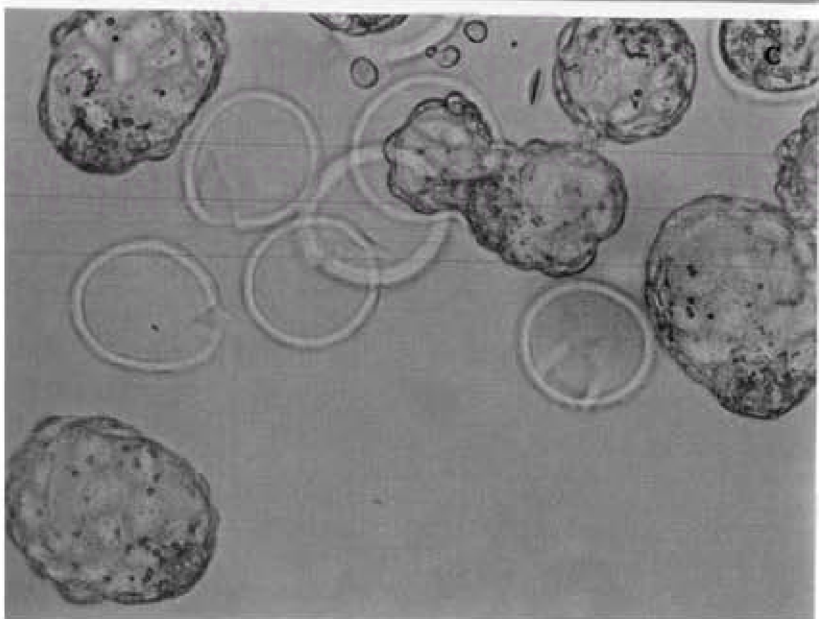
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Figure 15 Examples of the three categories of groups of mouse embryo development in vitro after embryo biopsy. (a) morula and early blastocyst stages development with blastocoele cavity formation in some embryos at day 5 post hCG; (b) hatching blastocyst development at day 6 post hCG; (c and d) completely hatched blastocysts with their empty zona pellucida at day 7 post hCG. Optical magnification: x200.

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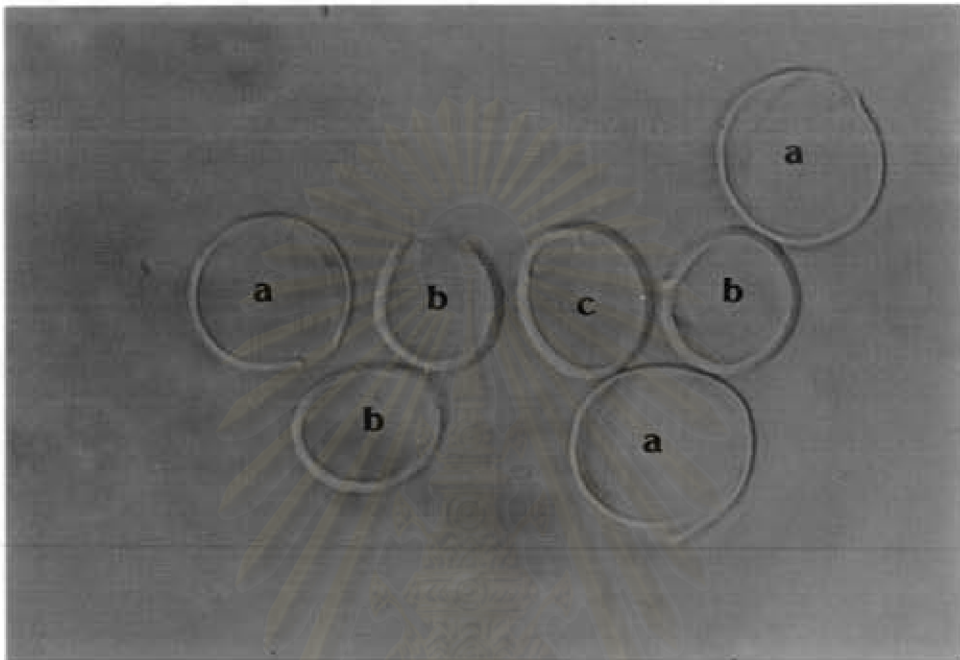

Figure 16 Comparison of the ZP shed by normally hatched mouse blastocyst (a) and by blastocyst that developed from PZD-push (b) and aspiration (c) biopsied embryos. The artificial holes in the shed zonae made by PZD-push are large and wedge shaped, as same as,but shorter than the holes in spontaneously shed zonae ,whereas the holes in aspirated embryos were round and small. The spontaneously shed zonae are thinner and larger than that of biopsied. Optical magnification: x200.

Table 4 In vitro development of mouse embryos after biopsy at the 4-cell stage as compared to their respective controls

Treatment	No. of biopsied embryo	No. of embryo developed into		Rate of complete hatched blastocyst (%)
		early blastocyst (%)	hatching blastocyst (%)	
Control	227	189 (83.3)	159 (70.0)	138/159 (86.7)
Solution control	214	174 (81.3)	151 (70.6)	129/151 (85.4)
PZD-Push	205	162 (79.0)	148 (72.2)	116/148 (78.4)
Aspiration	197	152 (77.2)	138 (70.1)	93/ 138 (67.4)[a,b]

Values in parentheses are percent

[a] P< .01 , compared to control and solution control (x^2 = 16.0891 and 13.1752)

[b] P< .05 , compared to PZD- push (x^2 = 4.3816)

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Table 5 In vitro development of mouse embryos after biopsy at the 8-cell stage as compared to their respective controls

Treatment	No. of biopsied embryo	No. of embryo developed into		Rate of complete hatched blastocyst (%)
		early blastocyst (%)	hatching blastocyst (%)	
Control	256	222 (86.7)	191 (74.6)	165/191 (86.4)
Solution control	206	174 (84.5)	149 (72.3)	127/149 (85.2)
PZD-Push	294	240 (81.6)	229 (77.9)	184/229 (80.3)
Aspiration	271	219 (80.8)	195 (71.9)	142/195 (72.8) [a]

Values in parentheses are percent

[a] $P < .01$, compared to control and solution control ($x^2 = 10.9107$ and 7.6351)

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Table 6 In vitro development of mouse embryos after biopsy at the morula stage as compared to their respective controls

Treatment	No. of biopsied embryo	No. of embryo developed to		Rate of complete hatched blastocyst (%)
		early blastocyst (%)	hatching blastocyst (%)	
Control	220	193 (87.7)	170 (77.3)	149/170 (87.6)
Solution control	194	167 (86.1)	149 (76.8)	127/149 (85.2)
PZD-Push	238	194 (81.5)	187 (78.6)	152/187 (81.3)
Aspiration	218	177 (81.2)	163 (74.8)	116/163 (71.2)[a,b]

Values in parentheses are percent

[a] $P < .01$, compare to control and solution control ($x^2 = 13.9096$ and 8.9457)

[b] $P < .05$, compared to PZD-push ($x^2 = 4.9695$)

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Table 7 In vitro development of mouse embryos after biopsy (including PZD-push and direct aspiration) at different cell stages

Biopsy procedure	Stage of embryo	No. of biopsied embryo	No. of embryo developed into		Rate of complete hatched blastocyst (%)
			early blastocyst (%)	hatching blastocyst (%)	
PZD-push	Four-cell	205	162 (79.0)	148(72.2)	116/148(78.4)
	Eight-cell	294	240 (81.6)	229(77.9)	184/229(80.3)
	Morula	238	194(81.5)	187(78.6)	152/187(81.3)
Aspiration	Four-cell	197	152(77.2)	138(70.1)	93/138(67.4)
	Eight-cell	271	219(80.8)	195(71.9)	142/195(72.8)
	Morula	218	177(81.2)	163(74.8)	116/163(71.2)

Values in parentheses are percentages

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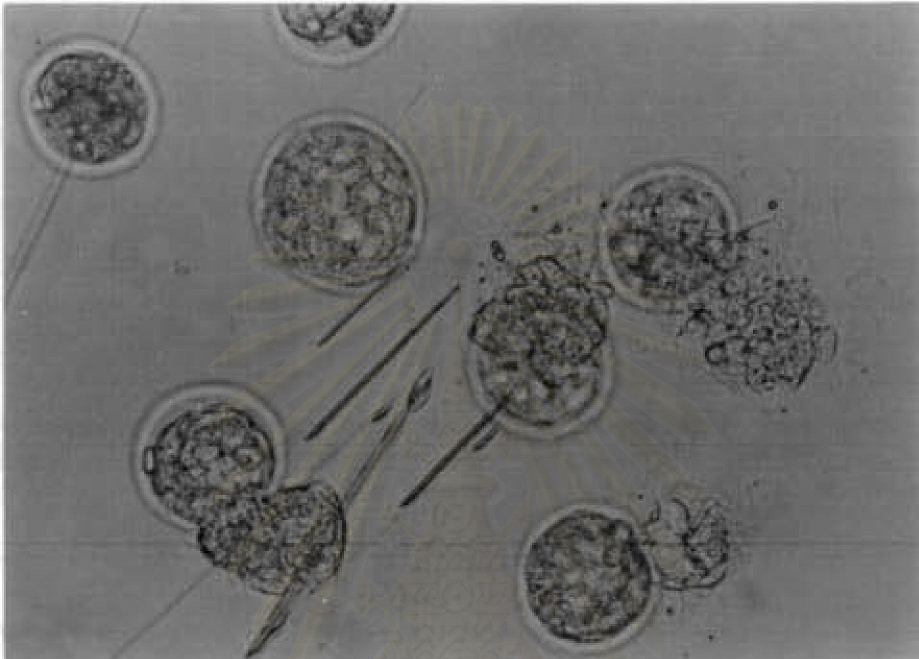

Figure 17 Blastocysts from biopsied mouse embryos on day 10 post hCG exhibiting the abnormal hatching morphology. Hatching is occurring through the opening with the thick ZP, resulting in the constricted 'figure-8' appearance. This blastocyst degenerated and did not shed theirs zona completely resulting in partial outgrowth of trophoblastic tissue. Optical magnification: x200.

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2. In vivo development

Table 8 presents the data as to the in vivo development of a total of 284 four-cell (96 controls, 102 PZD-push, and 86 aspiration groups), 300 eight-cell (104 controls, 100 PZD-push, and 96 aspiration groups) and 298 morula (106 controls, 102 PZD-push, and 90 aspiration groups) mouse embryos after transfer into the uteri of pseudopregnent mice at the early blastocyst stage. Ten days after transfer some recipients were sacrificed and implantation was evaluated. Due to the fact that some female mice may not have been suitable recipients, the implantation rate was expressed as the number of implantation sites divided by the number of embryos transferred only into recipients which achieved at least one implanting embryo (implantation rate in pregnant mice). With 4-cell stage embryos, a total of 22 (22%) implantation sites was noted after transfer of 100 embryos. There was no significant difference (P> 0.05) in the implantation rates for control (30%), PZD-push (22.2%), and aspiration (14.7%) groups. A total of 36 (19.6%) live-birth pups were noted after transfer of 184 embryos. Live-births of 18 (27.3%) pups from the control group, 12 (18.2%) after the PZD-push, and 6 (11.5%) after the aspiration technique, respectively were observed. The live-birth rate in the aspiration group was somewhat lower than that of the controls (P< 0.05), whereas the PZD-push technique was not significantly (P>0.05) different from the control group. With the 8-cell stage embryos, a total of 32 (32.7%) implantation sites was found after transfer of 98 embryos. There was no significant difference (P>0.05) in the implantation rate for the controls (41.7%), PZD-push (34.4%), and aspiration (20.0%) groups. A total of 202 biopsied and

control blastocysts (68 control, 68 PZD-push, and 66 aspiration embryos) was transferred into the uteri of pseudopregnant recipients and allowed to be carried to term. The rate of live birth pups from the embryos biopsied by the aspiration method (24.2%) was significantly ($P < 0.05$) lower than that of the controls (41.2%). However, no significant difference was found in the rate of live births from the embryos biopsied by PZD-push technique (33.8% VS 41.2% control). There was a slight decrease in the live birth rate but no significant difference between direct aspiration (24.2%) and PZD-push (33.8%). With morula stage embryos, a total of 36 implantation sites was found after transfer of 118 embryos. There was no significant difference with regard to the implantation rate after biopsy by PZD-push (30.9%) or aspiration (25.0%) technique when compared with the control group (35.0%). A total of 51 live-births (28.3%) was noted after transfer of 180 embryos. There was no significant difference in the live-birth rates between control (33.3%), PZD-push (30%), and aspiration (20.4%) groups.

There was no significant difference in the total implantation rates between 4-cell, 8-cell and morula stages. However, the live-birth rates of embryos biopsied at the 4-cell stage were significantly lower than that of the 8-cell ($P<0.01$) and morula ($P<0.05$) stages.

3. Postnatal development

Table 9 presents the body weight of mouse pups derived from control and biopsied embryos. There was no significant difference as to the body

weights (mean ± S.D.) registered at 24 hr after birth, weaning (3 weeks of age) and for fully mature (6 weeks of age) pups from control (1.65±0.23, 24.30±2.16, and 36.98±2.17gm., respectively) and biopsied embryos (1.67±0.17, 24.50±2.03, and 36.90±2.44 gm., respectively). No gross morphologic developmental abnormalities were observed in any of the control and biopsied groups (figure 18). After sexual maturity (6 weeks of age), the adults were assessed for their reproductive performance in producing second generation pups by paired mating between 3 pairs of control versus control, 5 pairs of control versus biopsied, and 5 pairs of biopsied versus biopsied. The litter size for the biopsied x biopsied group (10.8±1.9) were not significantly different from those of control x control (10.7±1.5) and control x biopsied (10.6±1.8) groups.

Table 8 In vivo development of biopsied mouse embryos after transfer to pseudopregnant recipients

Embryo stage	Treatment	No. of transferred embryos	Rate of implantation (%)	No. of live-birth fetuses (%)
Four-cell	Control	96	9/30 (30.0)	18/66 (27.3)
	PZD-Push	102	8/36 (22.2)	12/66 (18.2)
	Aspiration	86	5/34 (14.7)	6/52 (11.5)[a]
Eight-cell	Control	104	15/36 (41.7)	28/68 (41.2)
	PZD-Push	100	11/32 (34.4)	23/68 (33.8)
	Aspiration	96	6/30 (20.0)	16/66 (24.2)[b]
Morula	Control	106	14/40 (35.0)	22/66 (33.3)
	PZD-Push	102	13/42 (30.9)	18/60 (30.0)
	Aspiration	90	9/36 (25.0)	11/54 (20.4)

[a,b] $P<0.05$ compare to control (for a, $x^2= 4.4441$; for b, $x^2= 4.3550$)

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Table 9 Body weight of pups derived from control and biopsied embryos

Treatment	No. of mouse pups	Body weight (gm) at		
		birth	3 wk.	6 wk
Control	12	1.65 ± 0.23	24.30 ± 2.16	36.98 ± 2.17
Biopsied	12	1.67 ± 0.17	24.50 ± 2.03	36.90 ± 2.44

Data are presented as mean ± S.D.

Table 10 Reproductive normalcy of mating pairs derived from control and biopsied embryos as determined by litter size

Group	No. of couple	Litter size
Control x control	3	10.7 ± 1.5
Control x biopsied	5	10.6 ± 1.8
Biopsied x biopsied	5	10.8 ± 1.9

Data are presented as mean ± S.D.

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Figure 18 The normal features of mouse pups derived from embryos following single-cell embryo biopsy (left) and non-biopsied control embryo (right).

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B. PCR amplification of sex-specific genes on mouse embryos

1. Blood testing system

To ensure the correctness and specificity of the primers, the male and female blood DNAs were subjected to PCR amplification. As shown in figure 19, after amplification all male DNA samples were positive for the Sry, Zfy, and DXNds 3 genes, while the female DNA samples were positive for the DXNds 3 gene but negative for Sry and Zfy genes. No specific band appeared in the negative control lanes (lane 5). The sizes of the amplified fragments corresponded to those expected. These results indicate that the selected primers can bc used to determine the sex of mouse embryo.

2. Single blastomere PCR testing

To determine whether the multiplex two-step PCR method could detect the target sequences from a single DNA copy, the single blastomeres (1/8 embryos) isolated from 8-cell mouse embryos and the 7/8 embryos they originated from were amplified simultaneously. As shown in table 11, twenty blastomeres were obtained from 20 eight-cell stage embryos after removal of the ZP and incubation in Ca^{2+}- and Mg^{2+}-free biopsy medium. Although 1 of the 20 cases tested did not show amplification of the sequences of interest in the single blastomere (1/8) but only in the embryo it had originated from (7/8), sex determination was possible in all mouse embryos. The PCR results from the 1/8 and 7/8 blastomeres of the same embryos were always concordant. The

results shown in figure 20 represent a single trial in which five 1/8 embryos and five 7/8 embryos were analyzed. The positive control for male (lane M) showed amplified fragments of the Sry, Zfy, and DXNds 3 sequences, while the female control DNA (lane F) exhibited only a fragment of the DXNds 3 sequence. Three out of five of the 1/8 and 7/8 embryos showed all three amplified fragments. The other two single blastomeres and 7/8 embryos showed only the DXNds 3 fragment. Therefore, the embryos yielding fragments of the Sry, Zfy, and DXNds 3 sequences were predicted to be male (lane 3, 4, 5, 3′, 4′, and 5′) and those exhibiting only the DXNds 3 sequence to be female(lanes 1, 2, 1′, and 2′). It was noted that one of the 7/8 embryo (lane 4 ′) showed a faint band of the Zfy fragment. This result indicates that sporadic amplification failure can occur. The weak bands present in Sry fragments may be due to non-specific amplification with the outer primers (Sry1 and Sry4).

3. Sex determination of mouse embryos

To further confirm the accuracy of sex determination of embryos assayed by the multiplex two-step PCR method, another batch of single blastomeres isolated from mouse embryos was subjected to PCR amplification and putative male and female embryos at the blastocyst stage were transferred separately into the uteri of pseudopregnant recipients and allowed to be carried to term. In total, 188 embryos were biopsied, with 180 (95.7%) single blastomeres successfully removed in an intact condition, 4 single blastomeres with some

cell membrane damage, 2 single blastomeres lost during the washing procedure, and 2 single blastomeres with a naked nucleus and hence not included in PCR amplification. The single blastomeres were placed in 184 PCR reaction tubes and subjected to PCR amplification. As shown in table 12, of a total of 184 single blastomeres isolated from 184 biopsied embryos, 99 were predicted to be males and 79 females. The amplification failure occurred in 6 blastomere samples in which 4 samples were from the cell membrane damaged blastomeres. In ten negative control samples which contained water or medium where no template DNA was added to the tube, no amplification signals were detected. Only 56 putative male and 22 female embryos were selectively transferred to pseudopregnant recipients. The sex of 36 out of 37 mouse pups (97 % accuracy) agreed with the predicted sex. Of the 37 mouse pups, 21 were found to be male and 16 female. The ratio of male : female was 1.3 : 1.

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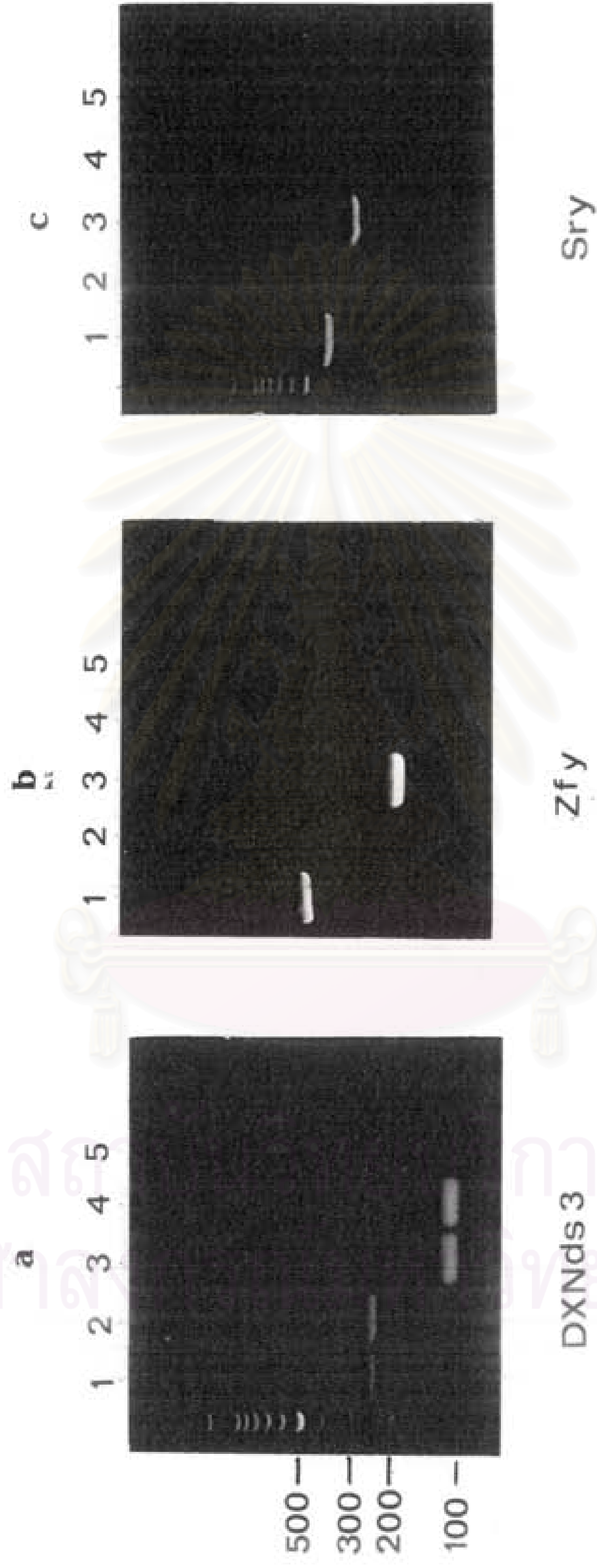
a
b
c
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
500
300
200
100
DXNds 3
Zfy
Sry

Figure 19 Multiplex two-step PCR of genomic DNAs from male and female mouse blood. The samples are amplified with various primers. Agarose gel electrophoresis and visualization of DNA amplification products by Ethidium bromide staining and UV light. (a) Products of PCR with pairs of DXNds3-specific primers: Nds1 and Nds4 (lanes 1&2), and Nds2 and Nds3 (lanes 3&4); (b) Products of PCR with pairs of Zfy-specific primers: Zfy1 and Zfy4 (lanes 1&2), and Zfy2 and Zfy3 (lanes 3&4); (c) Products of PCR with pairs of Sry-specific primers: Sry1 and Sry4 (lanes 1&2), and Sry2 and Sry3 (lanes 3&4). Lanes 1&3: purified DNAs from male mouse; lanes 2&4: purified DNAs from male mouse. Lane 5 : negative control. Left lane contains DNA size marker of 100 bp DNA Ladder.

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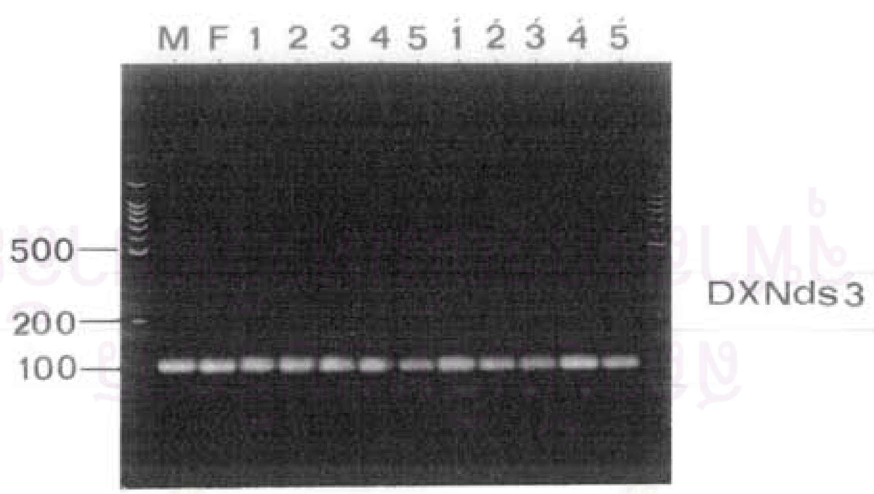
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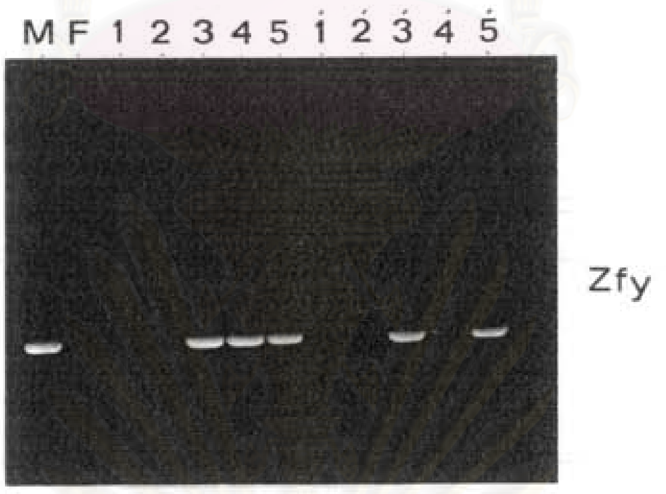
500
200
100
DXNds3


M F 1 2 3 4 5 1́ 2́ 3́ 4́ 5́
Zfy

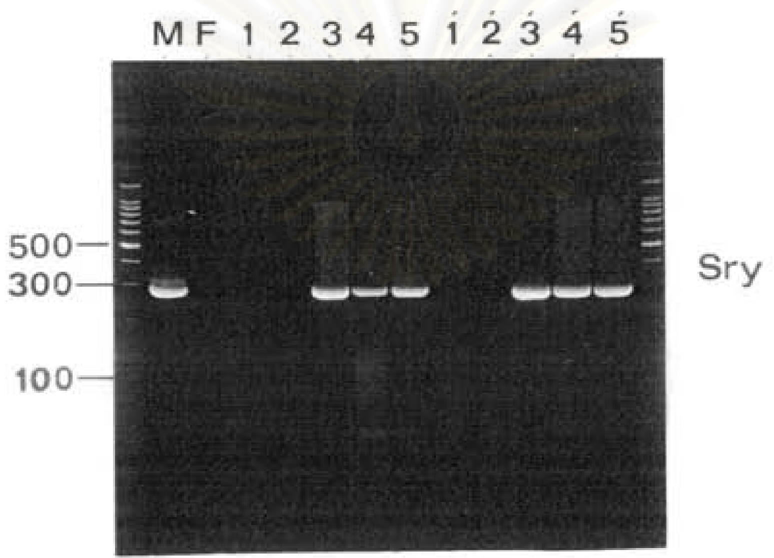

M F 1 2 3 4 5 1́ 2́ 3́ 4́ 5́
500
300
100
Sry

Figure 20 Products of two-step PCR method amplified from single mouse blastomere (lane 1-5) comparison with theirs 7/8 embryos (lane 1'-5'). Lane M : Male blood DNA, Lane F : Female blood DNA and left contrins DNA size marker of 10 bp DNA ladder.

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Table 11 Reliablility of sex determination in 1/7 blastomeres in comparison with the remaining 7/8 embryos.

Embryo	1/8 blastomere			7/8 embryo			Predicted
No.	DXNds3	Sry	Zfy	DXNds3	Sry	Zfy	Sex
1.	+	+	+	+	+	+	M
2.	+	+	+	+	+	+	M
3.	+	+	+	+	+	+	M
4.	+	-	-	+	-	-	F
5.	+	-	-	+	-	-	F
6.	+	+	+	+	+	+	M
7.	+	+	+	+	+	+	M
8.	+	-	-	+	-	-	F
9.	+	+	+	+	+	+	M
10.	+	+	+	+	+	+	M
11.	+	+	+	+	+	+	M
12.	+	+	+	+	+	+	M
13.	+	-	-	+	-	-	F
14.	+	-	-	+	-	-	F
15.	+	+	+	+	+	+	M
16.	+	+	+	+	+	+	M
17.	+	-	-	+	-	-	F
18.	+	+	+	+	+	+	M
19.	+	-	-	+	-	-	F
20.	-	-	-	+	+	+	M

F : Female, M : Male

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Table 12 Births of sex determined mouse pups after transfer to pseudopregnant recipients.

Expt.	No. of single blastomere	No. of embryos predicted (M/F)	No. of embryo transferred	No. of mouse pups	Phenotype sex
1.	15	9/6	8(XY)	4	4M
2.	21	11/8	8(XY)	1	1M
3.	18	8/10	6(XX)	3	3F
4.	20	13/6	8(XY)	4	3M, 1F
5.	20	11/9	8(XY)	2	2M
6.	15	8/7	6(XY)	1	1M
7.	18	7/11	8(XX)	6	6F
8.	20	10/8	10(XY)	5	5M
9.	18	13/5	8(XY)	5	5M
10.	19	9/9	8(XX)	6	6F
Total	184	99/79	56(XY)	37	21M
			22(XX)		16F

*M/F; putative male/female

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