

วิธีคัดแยกเชตัล์ตัวอ่อนหนูยาส์ระยะก่อนฝังตัวและการเพิ่มขยายปริมาณเขินส์ที่จำเป็น
ของไครโโนไซน์เอ็กซ์เดราวช ในบตาส ไดเมียร์เดียวเพื่อการกำหนดเหตุ

นางสาวพรกิต ตั้งชัยศิน



สถาบันวิทยบริการ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต^๑
สาขาวิชาสรีรวิทยา

บัณฑิตวิชาลัย จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2540

ISBN 974-638-379-5

ลิขสิทธิ์ของบัณฑิตวิชาลัย จุฬาลงกรณ์มหาวิทยาลัย

**PREIMPLANTATION MOUSE EMBRYO BIOPSY METHODS AND
AMPLIFICATION OF X- AND Y-CHROMOSOME SPECIFIC GENES IN
SINGLE BLASTOMERES FOR SEX DETERMINATION**

Miss Portpimon Tangchaisin

**สถาบันวิทยบริการ
A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Physiology**

**Graduate School
Chulalongkorn University
Academic Year 1997
ISBN 974-638-379-5**

Thesis Title Preimplantation Mouse Embryo Biopsy Methods and Amplification of X-
and Y-Chromosome Specific Genes in Single Blastomere for Sex Determination

By Miss Pornpimon Tangchaisin

Interdepartment Physiology

Principle Advisor Professor Dr. Pramuan Virutamasen

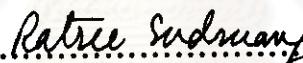
Co-advisor Dr. Thaweesak Tirawatnapong

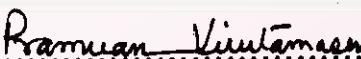
Associate Professor Dr. Vithaya Yodyingyoud

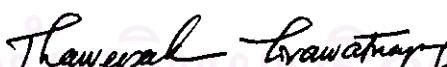
Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of
the Requirements for the Degree of Doctor of Philosophy

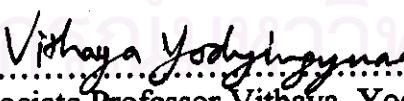

..... Dean of Graduate School
(Professor Supawat Chutivongse, M.D.)

Thesis Committee


..... Chairman
(Professor Ratree Sudsuang, Ph.D.)


..... Thesis Advisor
(Professor Pramuan Virutamasen, M.D.)


..... Member
(Thaweesak Tirawatnapong, Ph.D.)


..... Member
(Associate Professor Vithaya Yodyingyoud, Ph.D.)


..... Member
(Professor Kanok Pavasuthipaisit, M.D.)


..... Member
(Associate Professor Mongkol Techakumpu, Ph.D.)

C645731 : MAJOR PHYSIOLOGY

KEY WORD: PREIMPLANTATION MOUSE EMBRYO / EMBRYO BIOPSY / PCR / SINGLE BLASTOMERE / SEX DETERMINATION

PORNPIMON TANGCHAINSIN : PREIMPLANTATION MOUSE EMBRYO BIOPSY METHODS AND AMPLIFICATION OF X- AND Y-CHROMOSOME SPECIFIC GENES IN SINGLE BLASTOMERE FOR SEX DETERMINATION. THESIS ADVUSOR : PROF.PRAMUAN VIRUTAMASEN, M.D. THESIS CO-ADVISOR : Dr. THAWEESAK TERAWATANAPONG, Ph.D.; ASSOC. PROF. VITTHAYA YODYINGYOU, Ph.D. 198 pp. ISBN 974-638-379-5

The purpose of the present study was to compare the efficiency of two different embryo biopsy methods regarding the development in vitro and in vivo of biopsied embryos at three different cleavage stages in the mouse model and to assess the reliability of sex determination in mouse preimplantation embryos using the two-step polymerase chain reaction method. In a comparative study, ICR preimplantation mouse embryos at different three stages (four-, eight-cell and early morula stages) were randomly allocated into four groups: (1) a control group, (2) a solution control group, (3) a PZD-push biopsy group, and (4) a direct aspiration biopsy group. The rates of normal blastocyst formation and hatching blastocysts of the biopsy embryos (including PZD-push and direct aspiration) were not significantly different from those of the control and solution control embryos. However, in the direct aspiration group, the rates of completely hatched blastocysts (4-cell: 67.4%, 8-Cell: 72.8%, morula: 71.2% VS control embryos at the 4-cell: 86.7%, 8-cell: 86.4%, and morula: 87.6% and solution control group at the 4-cell: 85.4%, 8-cell: 85.2%, and morula: 85.2%) and live-births at the 4-cell (11.5% VS 27.3%) and 8-cell stages (24.2% VS 41.2%) were significantly ($p<0.01$) lower than those observed in the control and solution control embryos, whereas embryo biopsy at the 8-cell and morula stages with PZD-push technique were not significantly different from the controls. No grossly anatomical abnormalities were found in body weights or in subsequent ability to reproduce a second generation of mice derived from both biopsied and non-biopsy control embryos. Embryos biopsied at the morula stage had, however, more difficulties than at the 8-cell stage. Therefore, embryo biopsy at the 8-cell stage using PZD-push technique is the most suitable stage for embryo biopsy. For sex determination, the Sry and Zfy genes, known to be presented in the sex-determining region of the mouse Y chromosome, were selected for Y-specific target sequences and the DXNDs 3 locus located on the mouse X chromosome served as the internal control sequence. DNAs extracted from heart blood of male and female mice were used to test the accuracy and specificity of the selected primers using the two-step PCR method. The same experimental conditions were then applied to amplify the single copy genes in single mouse blastomere with two pairs of primers for each of the target sequences. The sex-determined embryos were transferred to the uteri of pseudopregnant recipients to test the consistency of the assay system. All male and female blood DNA sample results confirmed the correct sex identification of the origin (100%). Nineteen of 20 single blastomeres showed the accurate diagnosis when compared with their 7/8 embryos. The sex of 36 out of 37 mouse pups born from biopsied male and female embryos agreed with the predicted sex. Reliable genetic analysis of sex chromosome-specific sequences in single cells is possible by two-step PCR and may be applied for sex determination of human embryos and the diagnosis of defective genes in human preimplantation embryos derived from the in vitro fertilization program.



Acknowledgment

All experiments in this thesis were carried out in the Reproductive Medicine Unit, Department of Obstetric and Gynecology, Faculty of Medicine Chulalongkorn University during the academic period of 1993-1997.

Throughout the process of my research I received a lot of help, friendship, encouragement and different supports from many colleagues and friends in this unit. I would like to give my sincerely thanks to: Prof. Dr. Pramuan Virutamasen for providing me the opportunity to do research here and his thoughtful advice and supports in many aspects throughout my graduate career; Dr. Thaweesak Tirawatnapong for his help and close collaboration and for valuable advice in molecular genetics and PCR techniques; Associate Prof. Dr. Vithaya Yodyingyoud for his valuable advice and support; Prof. Dr. Ratree Sudsuang, Prof. Dr. Kanok Pavasuthipaisit, and Associate Prof. Dr. Mongkol Techakumpu for their helpful and critical proof-reading of my thesis; Assistant Prof. Dr. Khamtorn Pruksananonda and Assistant Prof. Dr. Somchai Suwajanakorn and all members of the IVF laboratory unit for their helpful and collaboration; Ms. Saowarat Propadith for help in typing some parts of this thesis and preparation of slides; MS. Petra Hirch who corrected my English language in my articles and in this thesis.

Finally, I would like to give my deepest appreciation to my parents, my sisters for their love, patience and understanding.

TABLE OF CONTENTS

ABSTRACT (THAI).....	iv
ABSTRACT (ENGLISH).....	v
ACKNOWLEDGMENTS.....	vi
TABLE OF CONTENTS.....	vii
LIST OF TABLES.....	xii
LIST OF FIGURES.....	xiii
LIST OF ABBREVIATIONS.....	xv
CHAPTER I. INTRODUCTION.....	1
A. General background.....	1
B. Development of preimplantation diagnosis.....	4
C. Methods for preimplantation diagnosis of genetic diseases.....	5
1. Micromanipulation of gamete and embryo.....	6
2. Preconception diagnosis.....	6
3. Embryo biopsy.....	9
3.1 Cleavage stage biopsy.....	10
3.2 Blastocyst biopsy.....	14
4. Genetic diagnostic techniques.....	16
4.1 Embryo sexing to avoid X-linked disease.....	18
a. The polymerase chain reaction.....	19
b. In situ hybridization.....	21
c. Other procedures.....	23
4.2 PCR-based diagnosis of single gene defects.....	26

D. Genes involved in sex determination.....	27
E. Aim of the study.....	30
F. Experimental design.....	31
CHAPTER II. MATERIALS AND METHODS.....	32
A. Materials.....	32
1. Instrument for embryo culture and biopsy.....	32
2. Instrument for DNA analysis.....	33
3. Chemical reagents.....	33
B. Methods for mouse embryo manipulation.....	34
1. Animals.....	34
2. Superovulation.....	34
3. Media.....	37
a. Flushing and washing medium.....	37
b. Biopsy medium.....	37
c. Culture medium.....	40
4. Embryo collection.....	40
5. Micromanipulation.....	41
a. Apparatus.....	41
b. Construction of microtools.....	41
c. Embryo biopsy procedure.....	42
1. Direct aspiration method.....	42
2. PZD-push.....	43
6. In vitro culture and assessment of viability of biopsy embryos....	43
7. Assessment of post-implantation development in vivo.....	50

a. Vasectomy and preparation of pseudopregnant female.....	50
b. Transfer of blastocysts to the pseudopregnant recipients.....	51
8. Assessment of postnatal development.....	52
C. Methods for DNA analysis.....	52
1. Selection of oligonucleotide primers.....	52
2. System testing.....	53
a. Preparation of DNA from whole blood.....	53
b. Blastomere collection for PCR.....	58
c. PCR methodology.....	59
d. Electrophoretic analysis of PCR products.....	60
e. Prevention of contamination.....	61
D. Sex determination of mouse embryo.....	61
E. Statistics.....	63
CHAPTER III. RESULTS.....	64
A. Effects of biopsy procedure on embryo development.....	64
1. In vitro development.....	64
2. In vivo development.....	75
3. Postnatal development.....	76
B. PCR amplification of sex-specific genes on mouse embryo.....	81
1. Blood testing system.....	81
2. Single blastomere PCR testing.....	81
3. Sex determination of mouse embryos.....	82
CHAPTER IV. DISCUSSION.....	88
A. Effects of biopsy procedure on embryo development.....	88

B. Sex determination in single blastomere.....	103
C. Summary and conclusion.....	113
REFERENCES.....	117
APPENDIX.....	152
A. Compositions of Modified T6 and HEPES-T6 medium.....	153
B. Embryo biopsy medium.....	154
C. Acidic Tyrode's solution for removing the zonae.....	155
D. TE buffer.....	156
E. Lysis buffer.....	156
F. 10X TBE buffer stock solution.....	156
G. Control of embryo biopsy at the 4-cell stage.....	157
H. Solution control of embryo biopsy at the 4-cell stage.....	158
I. Embryo biopsy at the 4-cell stage by PZD-push technique.....	159
J. Embryo biopsy at the 4-cell stage by direct aspiration technique.....	160
K. Control of embryo biopsy at the 8-cell stage.....	161
L. Solution control of embryo biopsy at the 8-cell stage.....	162
M. Embryo biopsy at the 8-cell stage by PZD-push technique.....	163
N. Embryo biopsy at the 8-cell stage by direct aspiration technique.....	164
O. Control of embryo biopsy at the morula stage.....	165
P. Solution control of embryo biopsy at the morula stage.....	166
Q. Embryo biopsy at the morula stage by PZD-push technique.....	167
R. Embryo biopsy at the morula stage by direct aspiration technique.....	168
S. Body weight of mouse pups derived from control embryos.....	169
T. Body weight of mouse pups derived from biopsied embryos.....	170

U. Reproductive capacity of adult mice derived from biopsied embryos...	171
V. Nucleotide sequence of mouse Y-specific gene, Sry.....	172
W. Sequence of cDNA of mouse Y-specific gene, Zfy.....	174
X. DNA sequence of the enhancer containing fragment, DXNds3.....	178
Y. Statistical analysis method.....	180
Curriculum Vitae.....	196
Publications.....	197

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table 1	Advantages and disadvantages of different stages of embryo biopsy for preimplantation genetic diagnosis.....	17
Table 2	Characteristic of mice.....	35
Table 3	Timing of developmental events of mouse embryo.....	39
Table 4	In vitro development of mouse embryos after biopsy at the 4-cell stage compared to their respective controls.....	70
Table 5	In vitro development of mouse embryos after biopsy at the 8-cell stage compared to their respective controls.....	71
Table 6	In vitro development of mouse embryos after biopsy at the morula stage compared to their respective controls.....	72
Table 7	In vitro development of mouse embryos after biopsy (including PZD-push and direct aspiration) at different cell stage.....	73
Table 8	In vivo development of biopsied mouse embryos after transfer to pseudopregnant recipients.....	78
Table 9	Body weight of mouse pups derived from control and biopsied embryos.....	79
Table 10	Reproductive normalcy of mating pairs derived from control and biopsied embryos as determined by litter size.....	79
Table 11	Reliability of sex determination in 1/8 blastomeres in comparison with the remaining 7/8 embryos.....	86
Table 12	Births of sex determined mouse pups after transfer to pseudopregnant recipients.....	87

LIST OF FIGURES

Figure 1	Estimated frequency of chromosome abnormalities in unfertilized oocytes, sperm cells, fertilized oocytes, and various stages of prenatal development.....	3
Figure 2	Methods considered for preimplantation genetic diagnosis.....	11
Figure 3	A schematic representation of the polymerase chain reaction (PCR).....	20
Figure 4	Localization of the sex-determining region, Sry and the zinc finger gene, Zfy on the Y chromosome.....	28
Figure 5	Morphology of normal preimplantation mouse development....	38
Figure 6	Arrangement of the micromanipulation set.....	44
Figure 7	Direct aspiration procedure for single-blastomere biopsy from the 4-cell mouse embryo.....	45
Figure 8	Direct aspiration procedure for single-blastomere biopsy from the 8-cell mouse embryo.....	46
Figure 9	The PZD-push procedure for single blastomere biopsy from the 4-cell mouse embryo.....	47
Figure 10	The PZD-push procedure for single blastomere biopsy from the 8-cell mouse embryo.....	48
Figure 11	The PZD-push procedure for single blastomere biopsy from the mouse morula embryo.....	49

Figure 12	Diagram showing the sequence of DNA denaturation, annealing of primers, and primer extension during successive cycles of the two-step PCR.....	54
Figure 13	Details of primer sets used during PCR reaction.....	55
Figure 14	Location of various oligonucleotide primers on Sry, Zfy, and DXNds3 sequences and sizes of fragments amplified with the primers.....	57
Figure 15	Examples of the three categories of groups of mouse embryo development in vitro after embryo biopsy.....	68
Figure 16	Blastocysts from biopsied mouse embryos on day 10 post hCG exhibiting the abnormal hatching morphology.....	69
Figure 17	Comparison of the ZP shed by normally hatched mouse blastocysts.....	74
Figure 18	The normal features of mouse pups derived from embryo following single-cell biopsy and non-biopsied embryo.....	80
Figure 19	Multiplex two-step PCR of genomic DNAs from male and female mouse blood.....	84
Figure 20	Multiplex two-step PCR of genomic DNAs from single mouse blastomere.....	85

LIST OF ABBREVIATIONS

APRT.....	adenine phosphoribosyl transferase
ART.....	Assisted reproductive technology
ATP.....	adenosine triphosphate
BSA.....	bovine serum albumin
bp.....	base pair
Ca ²⁺	calcium ion
cDNA.....	complementary DNA
CF.....	Cystic Fibrosis
cM.....	centimorgan
CO ₂	carbondioxide
CVS.....	Chorionic villi sampling
dATP.....	deoxyadenosine triphosphate
dCTP.....	deoxycytidine triphosphate
dGTP.....	deoxyguanosine triphosphate
dNTP.....	deoxynucleotide triphosphate
dTTP.....	deoxythymidine triphosphate
DMD.....	Duchenne muscular dystrophy
DNA.....	deoxyribonucleic acid
EDTA.....	ethylenediaminetetraacetic acid
FISH.....	fluorescent in situ hybridization
FITC.....	fluorescein isothiocyanate
hCG.....	human chorionic gonadotropin

HCl.....	hydrochloric acid
HPRT.....	hypoxanthine phosphoribosyl transferase
hr.....	hour (s)
ICM.....	inner cell mass
ICSI.....	Intracytoplasmic sperm injection
ISH.....	In situ hybridization
IU.....	International unit
IVF.....	In vitro fertilization
kb.....	kilobase or 1000 base pair
KCl.....	potassium chloride
LN ₂	liquid nitrogen
M.....	molar
Mg ²⁺	magnesium ion
MgCl ₂	magnesium chloride
mg.....	milligram
min.....	minute(s)
ml.....	milliliter
mM.....	millimolar
PAR.....	Pseudoautosomal region
PBS.....	phosphate-buffered saline
PCR.....	Polymerase chain reaction
PEP.....	Primer extension preamplification
pH.....	power of hydrogen
PID.....	Preimplantation genetic diagnosis

pmol.....	picomolar
PMSG.....	pregnant mares' serum gonadotropin
PZD.....	partial zona dissection
SRY.....	Sex determining region of Y
T6.....	Tyrode's solution 6
TDF.....	Testis-determining factor
Tdy.....	Testis-determining Y of mouse
TE.....	trophectoderm
UV.....	ultraviolet light
μ g.....	microgram
μ l.....	microlitter
μ m.....	micromolar
μ M.....	micrometer
V.....	volt (s)
VS.....	versus
ZFX.....	Zinc finger X gene
ZFY.....	Zinc finger Y gene
ZP.....	zona pellucida