EMBRYONIC STEM CELL LINES ESTABLISHMENT FROM MOUSE SINGLE BLASTOMERE

Chanchao Lorthongpanich

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2007

การผลิตเซลล์ต้นกำเนิดตัวอ่อนหนูจากเซลล์ตัวอ่อนระยะก่อนฝังตัว

นางสาวจันทร์เจ้า ล้อทองพานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2550

EMBRYONIC STEM CELL LINES ESTABLISHMENT

FROM MOUSE SINGLE BLASTOMERE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

_____

(Asst. Prof. Dr. Chokchai Wanapu)

Chairperson

_____

(Dr. Rangsun Parnpai)

Member (Thesis Advisor)

_____

(Asst. Prof. Dr. Anthony W.S. Chan)

Member

_____

(Asst. Prof. Dr. Mariena Ketudat-Cairns)

Member

_____

(Assoc. Prof. Dr. Supat Srinawat)

Member

_____ _____

(Assoc. Prof. Dr. Saowanee Rattanaphani) (Asst. Prof. Dr. Suwayd Ningsanond)

Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

จันทร์เจ้า ล้อทองพานิชย์ : การผลิตเซลล์ต้นกำเนิดตัวอ่อนหนูจากเซลล์ตัวอ่อนระยะก่อนฝังตัว (EMBRYONIC STEM CELL LINES ESTABLISHMENT FROM MOUSE SINGLE BLASTOMERE) อาจารย์ที่ปรึกษา : อาจารย์ ดร.รังสรรค์ พาลพ่าย, 184 หน้า

งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตเซลล์ต้นกำเนิดตัวอ่อนที่มีระบบภูมิคุ้มกันเหมือนกับเจ้าของเซลล์ซึ่งในการทดลองนี้ได้ใช้ตัวอ่อนหนูเป็นตัวอย่าง ในการทดลองนี้ได้เริ่มจากการศึกษาระบบการเลี้ยงเซลล์ตัวอ่อนหนูในหลอดแก้วเพื่อให้เจริญสู่ระยะบลาสโตซีส จากการทดลองพบว่าเซลล์ตัวอ่อนหนูที่แยกจากตัวอ่อนระยะสองและสี่เซลล์สามารถเจริญสู่ระยะบลาสโตซีสและเจริญเป็น outgrowth ได้ดีในหลอดแก้ว จากนั้นได้นำตัวอ่อนระยะบลาสโตซีสที่เจริญมาจากตัวอ่อนตั้งต้นตัวเดียวกันมาศึกษาการแสดงออกของยีนที่แสดงออกถึงความเป็นเซลล์ต้นกำเนิด คือ Oct-4 และ Sox-2 และยีนที่แสดงถึงความเป็นเซลล์ trophectoderm คือ Cdx2 พบว่าเฉพาะบางตัวอ่อนและบาง outgrowth ที่ได้มาจากตัวอ่อนระยะสองและสี่เซลล์มีการแสดงออกของยีนแสดงความเป็นเซลล์ต้นกำเนิด จากการทดลองแรกพบว่าสามารถผลิตเซลล์ต้นกำเนิดซึ่งเป็นเซลล์ตัวอ่อนที่มาจากตัวอ่อนหนูระยะสองเซลล์ได้ 4 สายพันธุ์ (5.6%; 4/72) แต่ไม่สามารถผลิตได้จากเซลล์ตัวอ่อนที่มาจากตัวอ่อนหนูระยะสี่เซลล์ จากนั้นนำ MAPK inhibitor (I) สองตัวคือ P38MAPK (I) และ MEK-1 (I) และฮอร์โมน ACTH มาใช้ทดสอบเพื่อเพิ่มความสำเร็จในการผลิตเซลล์ต้นกำเนิดจากเซลล์ตัวอ่อนหนู จากการทดลองพบว่า MEK-1 (I) และ P38MAPK (I) มีผลยับยั้งการเจริญของตัวอ่อนปกติและตัวอ่อนที่เจริญมาจากเซลล์ตัวอ่อนระยะสองและสี่เซลล์ แต่ P38MAPK (I) จะมีผลในการยับยั้งการเจริญเติบโตของตัวอ่อนมากกว่า MEK-1 (I) จากการทดลองนี้ผลิตเซลล์ต้นกำเนิดได้ 17 สายพันธุ์ โดย 9 สายพันธุ์ ผลิตมาจากเซลล์ตัวอ่อนระยะสองเซลล์ที่เติม ACTH (12.5%; 9/72) 5 สายพันธุ์ผลิตมาจากตัวอ่อนระยะสองเซลล์ที่ไม่เติม ACTH (6.9%; 5/72) และ 3 สายพันธุ์ผลิตมาจากเซลล์ตัวอ่อนระยะสี่เซลล์ที่เติมด้วย ACTH (4.3%; 3/72) แต่ไม่สามารถผลิตเซลล์ต้นกำเนิดตัวอ่อนจากเซลล์ตัวอ่อนที่เลี้ยงใน MEK-1 (I) และ P38MAPK (I)ได้ จากการทดลองในตัวอ่อนหนูสามารถสรุปได้ว่าอัตราความสำเร็จในการผลิตเซลล์ต้นกำเนิดตัวอ่อนขึ้นอยู่กับอายุของตัวอ่อน คือถ้าตัวอ่อนอายุมากขึ้นจะมีอัตราความสำเร็จในการผลิตเซลล์ต้นกำเนิดลดลงเมื่อเทียบกับเซลล์ตัวอ่อนที่นำมาจากตัวอ่อนอายุน้อยกว่า และ ACTH มีผลในการเพิ่มอัตราความสำเร็จในการผลิตเซลล์ต้นกำเนิดจากเซลล์ตัวอ่อน หลังการทดลองในตัวอ่อนหนูประสบความสำเร็จโดยสามารถผลิตเซลล์ต้นกำเนิดตัวอ่อนหนูจากเซลล์ตัวอ่อนได้แล้ว จึงได้ทำการทดลองต่อไปในตัวอ่อนลิงวอก โดยนำตัวอ่อนลิงระยะสองและสี่เซลล์มาแยกเซลล์ตัวอ่อนออกเป็นเซลล์เดี่ยวก่อนที่จะเลี้ยงในหลอดแก้ว จากการทดลองพบว่าเซลล์ตัวอ่อนที่มาจากตัวอ่อนระยะสองเซลล์หยุดการเจริญเติบโตหลังจากการแยกเซลล์ แต่เซลล์ตัวอ่อนที่มาจากตัวอ่อนระยะสี่เซลล์สามารถเจริญเป็น

ตัวอ่อนระยะ บลาสโตซีส (7.9%; 6/76) จากนั้นได้นำตัวอ่อนระยะบลาสโตซีสมาใช้ผลิตเซลล์ต้นกำเนิด พบว่าเซลล์ที่ได้มีลักษณะภายนอกแตกต่างไปจากเซลล์ต้นกำเนิดตัวอ่อนลิงปกติ

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2550

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

CHANCHAO LORTHONGPANICH : EMBRYONIC STEM CELL LINES ESTABLISHMENT FROM MOUSE SINGLE BLASTOMERE. THESIS ADVISOR : RANGSUN PARNPAI, Ph.D. 184 PP.

SINGLE BLASTOMERES/ EMBRYONIC STEM CELL/ PERSONAL STEM CELL/ ACTH/MAPK INHIBITOR

The objective of this study was to establish personal embryonic stem cell by using mouse embryonic stem cells as a model. First the suitable culture system for single blastomeres was studied. The result indicated that single blastomeres could be cultured individually and could form blastocyst and ICM outgrowth. The expression of ES cell markers was determined from each blastomeres which derived from the same embryo. The expression patterns of ES cells markers (Sox-2 and Oct-4) and trophectoderm marker (Cdx2) showed that only some but not all sister blastomeres derived from embryos and embryonic outgrowth expressed ES cell markers when two and four cell stage embryonic blastomeres were individually cultured. There were four ES cell lines (5.6%; 4/72) established from single blastomere derived from two-cell stage embryo (2CBD) while no ES cell line could be established from four-cell stage embryo. Two MAPK inhibitors (I), P38MAPK (I) and MEK-1 (I), and ACTH were individually used for the embryonic stem cell enhancement from single blastomeres derived from two and four-cell stage embryo. The results demonstrated that both MEK-1 (I) and P38MAPK (I) delay early development of normal embryos and inhibited the development of single blastomere derived embryos. The P38MAPK (I) had stronger inhibitory effect when compared to MEK-1 (I). As a result, a total of

seventeen ES cell lines were established. Among these ES cell lines, nine (12.5%; 9/72) and five (6.9%; 5/72) ES cell lines were established from single blastomere derived from two-cell embryo with and without the supplement of ACTH, respectively. In addition to 2CBD, three (4.3%; 3/72) ES cell lines were established from blastomere of four-cell embryo only with the supplement of ACTH. However, no ES cell line was established from culture supplemented with either MEK-1 (I) or P38MAPK (I). Based on this study, the success rate of establishing ES cells from single blastomere is influenced by embryonic stage. The more advance stage embryo resulted in lower success rate in establishing ES cells from single blastomere. After mouse ES cell lines were successfully established from single blastomere, two and four cell monkey embryos were then used for the establishment of ES cells from single blastomere. The results demonstrated that only blastomeres derived from four-cell embryos could develop to blastocyst (7.9%; 6/76) and four blastocysts were used for ES cell establishment. The results showed that colonies derived from those outgrowths had different morphology from normal monkey ES cell.

School of Biotechnology

Academic Year 2007

Student's Signature _____

Advisor's Signature _____

Co-advisor's Signature _____

ACKNOWLEDGEMENTS

This work was supported by the Royal Golden Jubilee (RGJ) Scholarship Program, Thailand, and part of the work done at the Yerkes National Primate Research Center was supported by the base grant RR-00165 awarded by the Animal Resources Program of the NIH.

The completion of this dissertation would not have been possible without the inspiration, support and advice received from many sources.

I would like first to express my deepest gratitude and my sincerest appreciation to my supervisor Dr. Rangsun Parnpai for his guidance and gave me a good scholarship that led me to valuable experiences throughout the entire Ph.D study. I am also profoundly indebted to my co-advisor, Asst. Prof. Dr. Anthony W. S. Chan, for his guidance, making many extensive and valuable advice and many stimulating discussions throughout my thesis work. My appreciation is also to Asst. Prof. Dr. Mariena Ketudat-Cairns for her advice, warm wishes and encouragement. I am very grateful for all the lecturers who have taught my graduate courses in the School of Biotechnology at SUT.

I gratefully acknowledge Shang Hsun Yang, Pei-Hsun Cheng, Karolina Piotrowska Niches, Jin-Jing Yang, Eric Ching-Hsun Cheng and Liu Jun at the Yerkes National Primate Research Center (YNPRC); the veterinary staff and the animal resources at the YNPRC for their thoughtful comments, assistance and troubleshooting lab experiments. My appreciation is also to Leslee Sinclair for her warm wishes and encouragement. She is one of the people who made I feel warm

during stay in Atlanta, GA, USA.

I believe that million thanks would be insufficient to express my appreciation to my friends and colleagues in the Embryo Technology and Stem Cell research center for their helps and suggestions. Finally, I extend my thanks to Chuti Laowtammathron for his warm wishes and encouragement, Tanwalee Sooksa-nguan for her kind help in my presentation material preparations and Bussayarat Mai-khuntod for her warm wishes and valuable advice for statistic analytical data.

I am greatly indebted to my parents and my siblings for good care of my life, their constant love, understanding and encouragement, without their support and cooperation none of this would have been possible.

Chanchao Lorthongpanich

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ES cell	=	Embryonic Stem cell
ml	=	milliliter
IU	=	international unit
μg	=	microgram
μl	=	microlitre
μm^2	=	Square micrometer
STO	=	Sim 6-thioguanine and ouabain resistant
G1	=	First gab of interphase
S	=	Synthesis phase of interphase
G2	=	Second gab of interphase
M	=	Mitosis phase
Na^+	=	Sodium ion
K^+	=	Potassium ion
h	=	hour
IgG	=	Gamma immunoglobulin
mM	=	millimolar
SEM	=	Standard error means
2/4CBD	=	Two/four cells break down

CHAPTER I

INTRODUCTION

1.1 Introduction

Stem cells are unique cell type that is immortal and capable of self-renewal under the suitable condition. They are pluripotent and capable to differentiate to specialized cell types when induced by appropriate stimulants under suitable conditions (Bongso and Lee, 2005). Stem cell can be classified into two groups regarding to their source, adult stem cell and embryonic stem cell (ES cell) (Thomson et al., 1998). Adult stem cells have been described and isolated from various tissues and organs, which include the blood (heamatopoietic stem cell), bone marrow (mesenchymal stem cell), cord blood (cord blood stem cell), skin and hair (epidermal stem cell) and the latest on the list, amniotic stem cell (Coppi et al., 2007). Adult stem cell has limited differentiation capability in deriving cell types of its own origins such as oligodendrocyte precursors can reverse to the multi-lineage neural stem cells (Kondo and Raft, 2000). Several reports have suggested that the potency of adult stem cell retains that hematopoietic stem cells could repopulate liver hepatocyte population (Lagasse at al., 2000). However, the adult stem cell plasticity remains controversial and additional effort is necessary to confirm their plasticity and their stemness (Slayton and Spangrude, 2004). ES cell is more potent than adult stem cell because it is originated from the ICM of a blastocyst stage embryo which are capable to differentiate into all cell types of the three embryonic germ layers, ectoderm, mesoderm and endoderm, including germ

cells (Smith, 2001). ES cell was first isolated in 1981 from mouse blastocyst (Evansand Kaufman, 1981; Martin, 1981) and subsequently in different animal species such as rabbit (Graves and Moreadith, 1993), mink (Polejaeva et al., 1997), horse (Saito et al., 1992), porcine (Chen et al., 1999; Li et al., 2003; 2004), bovine (Wang et al., 2005), monkey (Thomson et al., 1995; Suemori et al., 2001; Vrana et al., 2003; Shoukhart et al., 2006), and human (Thomson et al., 1998; Heins et al., 2004; Cowan et al., 2004; Lee et al., 2005; Ludwig et al., 2006; Baharvand et al., 2006). ES cells from both animal and human sources are very useful tool for developmental studies *in vitro* and *in vivo*, and for the development of stem cell based therapies and drug assays (Barberi et al., 2003; Klein et al., 2006; Wang et al., 2007). Thousands of patient who suffer from diseases such as leukemia, Parkinson, Stroke, Huntington and Alzheimer are now waiting to be cured by using the stem cell based therapy. However, the ES cell based therapy is not yet practical because of its own unique characteristics, which include immortal and pluripotent. These might result in the development of tumor and most importantly is the ethical concern of the source of the ES cells. One of the major problems related to the source of ES cells is the immune reaction between patient and ES cell derived from unrelated donors. It would be useless if the patient has incompatible immune system with the ES cell. The incompatibility of the ES cell graft and patient may cause chronic or acute graft rejection that might be fatal. Therefore, the development of a personal ES cell line has been an active area of investigation. A personal stem cell will be histocompatible with patient and will not induce immune response against the ES cell graft. Thus a more successful stem cell based therapy could be achieved. Since the ES cells have been successfully established from early embryos, two cells to morula stage. Additionally, instead of using the inner cell mass

(ICM) of a blastocyst (Bradley et al., 1984; Eistetter, 1989; Strelchenko et al., 2004; Tesar, 2005), establishing ES cell from single biopsied blastomere of eight cell stage mouse and human embryo have also been reported (Chung et al., 2006; Klimanskaya et al., 2006, respectively). However, the success rates remain low and the key factor(s) that dictate the derivation of ES cell from a single blastomere has not yet to be determined. The use of supporting ES cell has shown to have supporting effect on the establishment of ES cell from single biopsied blastomere. Moreover, the biopsied blastomere itself might affect ES cell establishment rate because several reports have been suggested that blastomeres of early embryo have distinct fate of development (Piotrowska et al., 2001; Piotrowska-Nitsche et al., 2005). Therefore, the goal of this study is to determine if the blastomeres derived from early embryo, two and four cells stage, are equally competent to develop to ES cell, which will be determined base on the expression ES cell markers and if ES cell could be derived without the supporting ES cells.

1.2 References

Baharvand, H., Ashtiani, S.K., Taee, A., Massumi, M., Valojerdi, M.R., Yazdi, P.E., Moradi, S.Z., and Farrokhi, A. (2006). Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. **Develop. Growth. Differ.** 48: 117 – 128.

Barberi, T., Klivenyi, P., Calingasan, N.Y., Lee, H., Kawamata, H., Loonam, K., Perrier A.L., Bruses, J., Rubio, M.E., Topf, N., Tabar, V., Harrison, N.L., Beal, M.F., Moore, M.F., and Studer, L. (2003). Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in

parkinsonian mice. **Nat. Biotechnol.** 21: 1200 – 1207.

Bongso, A., and Lee, E.H. (2005). Stem Cells: Their definition, classification and sources: In A. Bongso and E.H. Lee (Eds). **Stem cells from bench to bedside** (pp. 1-13). Singapore: World scientific publishing Co. Pte.

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. **Nature** 309: 255-256.

Chen, L.R., Shiue, Y.L. Bertolini, L., Medrano, J.F., BonDurant, R.H., and Anderson, G.B. (1999). Establishment of pluripotent cell lines from porcine preimplantation embryos. **Theriogenology** 52: 195 – 212.

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L., and Lanza, R. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. **Nature** 439: 216-219.

Coppi, P.D., Bartsch, G., Siddiqui, M.M., Xu, T., Santos, C.C., Perin, L., Mostoslavsky, G., Serre, A.C., Snyder, E.Y., Yoo, J.J., Furth, M.E., Soker, S., and Atala, A. (2007). Isolation of amniotic stem cell lines with potential for therapy. **Nat. Biotechnol.** 25: 100 – 106.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang S., Morton, C.C., McMahon, A.P., Power, D., and Melton, D.A. (2004). Derivation of embryonic stem cell lines from human blastocyst. **N. Engl J Med.** 350: 1353 – 1356.

Eistetter, H. R. (1989). Pluripotent embryonal stem cells can be established from disaggregated mouse morulae. **Dev. Growth. Differ.** 31: 275-282.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. **Nature** 292: 154-156.

Graves, K.H., and Moreadith, R.W. (1993). Derivation and characterization of putative pluripotential stem cells from preimplantation rabbit embryos. **Mol. Reprod. Dev.** 36: 424 – 433.

Heins, N., Englund, M.C., Sjoblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, A., Hanson, C., and Semb, H. (2004). Derivation, characterization, and differentiation of human embryonic stem cells. **Stem Cells** 22: 367–376.

Klein, D., Schmandt, T., Muth-Kohne, E., Perez-Bouza, A., Segschneider, M., Gieselmann, V., and Brustle, O. (2006). Embryonic stem cell-based reduction of central nervous system sulfatide storage in an animal model of metachromatic leukodystrophy. **Gene Therapy** 13: 1686 – 1695.

Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. **Nature** 444: 481-485.

Kondo, T., and Raff, M. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. **Science** 289: 1754 – 1757.

Lagasse, E., Connors, H., Al-Dhalimy, M. Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. **Nat. Med.** 6: 1229 – 1234.

Lee, J.B., Lee, J.E., Park, J.H., Kim, S.J., Kim, M.K., Roh, S.I., and Yoon, H.S. (2005). Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum free condition. **Biol. Reprod.** 72: 42 – 49.

Li, M., Zhang, D., Hou, Y., Jiao, L., Zheng, X., and Wang, W.H. (2003). Isolation and culture of embryonic stem cells from porcine blastocysts. **Mol. Reprod.**

Dev. 65: 429 – 434.

Li, M., Li, Y.H., Hou, Y., Sun, X.F., Sun, Q., and Wang, W.H. (2004). Isolation and culture of pluripotent cells from *in vitro* produced porcine embryos. **Zygote** 12: 43 – 48.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggern, W.T., Mitchen, E.R., France, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S., Llanas, R.A., and Thomson, J.A. (2006). Derivation of human embryonic stem cells in defined conditions. **Nat. Biotechnol.** 24: 185 – 187.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. **Proc. Natl. Acad. Sci. U. S. A.** 78: 7634-7638.

Piotrowska, K., Wianny, F., Pedersen, R. A., and Zernicka-Goetz, M. (2001). Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. **Development** 128: 739 - 748.

Piotroska-Nitsche, K., Gomez, A.P., Haraguchi, S., and Goetz, M.Z. (2005). Four cell stage mouse blastomeres have different developmental properties. **Development** 132: 479 – 490.

Polejaeva, I.A., Reed, W.A., Bunch, T.D., Ellis, L.C., and White, K.L. (1997). Prolactin – induced termination of obligate diapause of mink *(Mustela vison)* blastocysts in vitro and sub-sequent establishment of embryonic stem like cells. **J. Reprod. Fertil.** 109: 229 – 236.

Saito, S., Ugai, H., Sawai, K., Marata, T., Obata, Y., and Yokoyama, K (2002). Isolation of embryonic stem cell lines from equine blastocysts and their differentiation *in vitro.* **FEBS. LETT.** 531: 389 – 396.

Slayton, W.B., and Spangrude, G.J. (2004). Adult stem cells plasticity: In K. Turksen

(Ed). **Adult stem cells** (pp. 1-18). Totowa, New Jersey: Humana press.

Shoukhart, M., Kuo, H.C., Byrne, J., Clepper, L., Meisner, L., Johnson, J., Zeier, R., and Wolf, D. (2006). Isolation and characterization of novel rhesus monkey embryonic stem cell lines. S**tem Cells** 24: 2177-2186.

Smith, A. (2001). Chapter 10: Embryonic stem cells. In D.R. Marshak R.L. Gardner and D. Gottlieb (Eds). **Stem cell biology** (pp. 205 – 230). New York: Cold Spring Harbor Laboratory Press, Cold Spring, Harbor.

Strelchenko, N., Verlinsky, O., Kukharenko, V., and Verlinsky, V. (2004). Morula derived human embryonic stem cells. . **Reprod. Biomed.** 9: 623-629.

Suemori, H., Tada, T., Torii, R., Hosoi, Y., Kobayashi, K., Imahie, H., Kondo, Y., Iritani, A., and Nakatsuji, N. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. **Dev. Dyn.** 222: 273–279.

Tesar, P. (2005). Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. **Proc. Natl. Acad. Sci. U. S. A.** 102: 8239-8244.

Thomson, J.A., Kalishman, J., Golos, T. G., Durning, M., Harris, C.P., Becker, R.A., and Hearn J.P. (1995). Isolation of a primate embryonic stem cell line. **Proc. Natl. Acad. Sci. U. S. A.** 92: 7844-7848.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. **Science** 282: 1145–1147.

Vrana, K., Hipp, D.J., Goss, A.M., McMool. B.A., Riddle D.R., Walker, S.J., Wettstein, P.J., Studer, L.P., Taber, V., Cunniff, K., Chapman, K., Vilner, L., West, M.D., Grant, K.A., and Cibelli, J.B. (2003). Nonhuman primate

parthenogenetic stem cells. **Proc. Natl. Acad. Sci. U. S. A.** 100: 11911 – 11916.

Wang, L., Duan, E., Sung, L.Y., Jeong, B.S., Yang, X., and Tian, C. (2005). Generation and characterization of pluripotent stem cells from cloned bovine embryos. **Biol. Reprod.** 73: 149 – 155.

Wang, Y., Chen, S., Yang, D., and Le. W.D. (2007). Stem cell transplantation: A promising therapy for parkinson's desease. **Journal of NeuroImmune Pharmacology** 2: 243 – 250.

CHAPTER II

REVIEW OF LITERATURES

2.1 Mouse embryo for experiments

The mammalian reproduction and embryology research were started in late 1950s. Much of the early experimental work in mammalian embryology was done in rabbit embryo (Chang, 1959; Browning and Wolf, 1981; Heape, 1980). Rabbits were used initially because eggs are relatively large and easy to handle, being surrounded by a thick mucin coat and the female ovulates only after mating, so that the age of the embryos could be timed quite precisely. However, the advantages of rabbit embryos were soon outweigh become of the well-defined reproductive physiology and genetic of the mouse. Mouse embryos have become the mammalian embryos of choice because the mouse has small size, large litter size, short life cycle and *in vitro* culture system of mouse embryo is well established. The first attempt to culture mouse embryos *in vitro* from 8 cell/morula stage to the blastocyst stage was done by Hammond in 1949. He had also cultured the two cell stage embryos but the embryos died soon after *in vitro* culture (Hammond, 1949). It was until 1956, Whitten and colleagues developed a defined medium, Krebs-ringer's-bicarbonate solution, supplemented with bovine serum albumin. By using this defined media, the mouse zygotes could pass the two cell block and develop to blastocyst *in vitro*. Then few years later, the precise nutritional requirements of the preimplantaion mouse embryo was reported (Whitten, 1957; Brinster, 1965; Bigger et al., 1965). The breakthroughs

in *in vitro* culture of mouse embryos have accelerated the development of important techniques in mammalian embryo research.

2.2 Mouse embryo development

Prior to fertilization, mouse oocytes wrapped in cumulus cells were released from the ovary and captured by the frimbriae of the oviduct. Then the oocytes will meet spermatozoa in the oviduct and fertilization occurs in the ampula. Then the developing embryos migrate along the female reproductive tract and implant in the uterus at approximately day 4-5 post-fertilization (Nagy, 1994).

First cleavage

After sperm enters an egg, the completion of meiosis is evidenced by the extrusion of the second polar body and the first cleavage begins approximately 22h post-fertilization. The first cleavage is a normal meridional division that divides evenly. A unique physiology requirement of the mouse zygote to undergo the first cleavage is the presence of pyruvate instead of glucose as an energy substrate because the early stage embryo may not contain the enzyme for glucose metabolism (Brinster, 1965; Kiessling and Anderson, 2003).

Second cleavage

The second cleavage, one of the two blastomeres divides meridionally and the other divides equatorially. This is called rotational cleavage (Gulyas, 1975).The mammalian cleavage has been marked as asynchrony blastomere division. Mammalian blastomeres do not divide at the same time, thus an odd number of blastomere during embryo development is commonly observed. In mouse, the second cleavage is relatively long, approximate 20h, whereas the third and subsequence

cleavages adopt more conventional cleavage cycle length of 12h as somatic cells (Eliot, 2003). In second cell cycle, G1 phase of the two cell embryo is short, approximate 1-2h, and S phase requires 6-8h. Half of the chromosomes resulting from S phase in both blastomere are comprised of double stranded DNA in which both strands have been replicated after egg activation. The G2 phase of the mouse two cells embryo is usually longer than other phases, about 12h. The long G2 mouse two cells is also a period which developmental arrested may occur, called two cells block. After G2, the M phase takes 1-2h to complete both karyokinesis and cytokinesis (Eliot, 2003).

The later cleavage

The blastomeres that do not under go cell cycle arrest will cleaved again in 18-24h to complete subsequent embryonic cleavage. However, each step of the later cleavage cycles is similar to the second cleavage except the G2 phase is shorter (Fig 2.1). Following the third-fourth cleavage, the blastomeres undergo a spectacular change in their behavior such as the expression of cell adhesion protein, E cadherin, and blastomeres begin to bundle together and from a compact ball of cells, called compact morula stage. This compact embryo is stabilized by tight junctions that are formed between the outer layer of cells of the ball, sealing off the surface of the sphere. The cells inside the sphere form gap junctions which enable small molecules and ions to pass through.

2.3 The blastocyst

After the compact-morula stage, the blastomeres would make a decision to become either inner cell mass (ICM) or trophectoderm cell (TE). Once the decision

is made, the membrane of the TE cells contains the sodium pumps (a Na^+/K^+ ATPase and a Na^+/H^+ exchanger) and will pump Na^+ into the central cavity.

Figure 2.1 The comparison of cell cycle length of the second and later cleavage. The G2 phase of the second cleavage mouse embryo takes longest time for completing cell cycle whereas the later and subsequence cleavages take about 6h shorter. Blastomeres of the two cell block embryo usually found at G2 phase of the second cleavage.

The consequence of Na^+ influx is drawn in water (H_2O) across the TE cells and accumulate in spaces among the inner blastomeres. Subsequently, cell morphology changes, the outer cell become elongated and appear as flattened epithelial cells align on one side of the embryo. As the cells pump water and ions inward, the fluid collects in the cavity on one side of the interior, called blastocoel, give rise to the blastocyst (Kiessling and Anderson, 2003). The fluid inside the blastocoel is thought to provide necessary nutrients for the expanding ICM. The cavity creates the polarity of an embryo, the abembryonic and embryonic pole. The abembryonic pole contains trophoblast cells that align on the opposite side of the embryonic pole, which contain a clump of ICM cells. Therefore, blastocyst is composed of two major cell types, the trophectoderm and the ICM cells (Fig 2.2). Trophectoderm will develop to placenta and ICM cells will develop to the three

embryonic germ layers and the fetus after implantation.

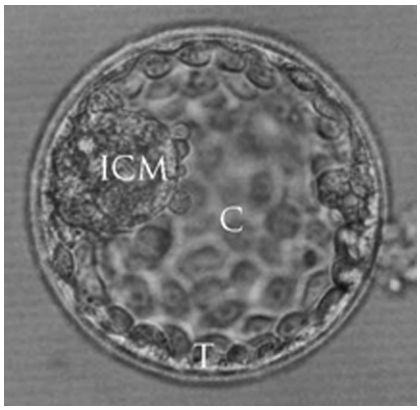


Figure 2.11 AC enzyme produce active cAMP whereas PDE catalyse the in active cAMP called 5'-AMP

Even todays' laboratory conditions could support the development of embryos from different species including mouse, bovine, rabbit, monkey and human, there are at least four characteristics that *in vitro* developed embryos are differed from *in utero* embryo development. First, the *in vitro* blastocyst contains markedly fewer cells than blastocyst developed *in utero.* This might due to the fact that *in vitro* developed two cell stage embryos takes longer time to staying in two cell stage (Fig 2.1) thus the accumulated lag time during early cleavage has led to lower cell number in the blastocyst, which usually contain only half or one-third the number of cells when compared to the *in utero* developed embryo. Second, the cultured blastocysts contain smaller ICM cells than embryos developed *in utero*. Third, blastocyst develop *in vitro* rarely develop an endoderm layer. It is because the *in vitro* derived blastocyst contain small number of ICM cells and it is insufficient to stimulate the secondary differentiation of the endoderm cells. Forth, the *in vitro* cultured embryos could reach blastocyst stage with higher rate than *in utero*. Several studies have demonstrated that

most of the fertilized eggs in a hormone treated mouse will develop to blastocysts in cultured but many of the *in utero* embryos arrested at the early morula stage in the uterus. It has been speculated if uterine signals stimulate the programmed cell death of unhealthy embryos (Kiessling and Anderson, 2003).

Maintaining lineage in the mouse blastocyst

Once the blastomeres committed to become either TE or ICM cells, the cell of these two regions will establish distinct gene expression profile that determine their cell fate (Fig 2.3). The TE cells synthesized the Eomesodermin and Cdx-2 transcription factors which responsible for down regulating Oct-4, Nanog and STAT3 (Strumpf et al., 2005). In blastocyst, Eomesodermin and Cdx-2 are expressed in the TE cells whereas Oct-4 is expressed in the ICM cells. The expression of Oct-4, Nanog and STAT3 transcription factors is a characteristic of ICM cell which are critical for the formation of the embryo and preserving pluripotent capability of the ICM. Oct-4 blocks cells toward TE fate. Nanog prevent the ICM cells become hypoblast cells. The phosphorylated STAT3 stimulate self-renewal of ICM blastomeres (Chamber et al., 2003; Mitsui et al., 2003). If the ICM was dissociated from TE and cultured under a proper condition that continue express and maintain Oct-4, Nanog and phosphorylated STAT3 proteins, these cells could become ES cell. The pluripotency of the ES cells is retained by the expression of these three transcription factors and some unknown factors that are not yet defined.

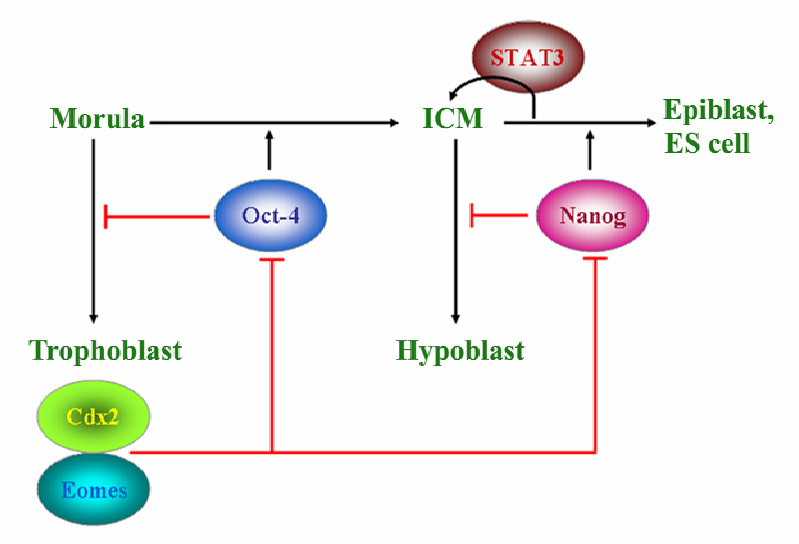


Figure 2.3 Function of Oct-4, Nanog and STAT3 transcription factors in retaining the uncommitted pluripotent fate of embryonic cells.

- Oct-4 stimulates morula cells retaining it to become ICM not TE cells
- Nanog works at the next differentiation event, preventing the ICM cells from becoming hypoblast and promoting their becoming the ES cell.
- STAT3 is involved in the self-renewal pathway of these pluripotent cells.
- Cdx2 and Eomesodermin prevent Oct-4 and Nanog expression, thereby stabilizing the TE lineage.

2.4 Embryonic Stem (ES) cell

Since ICM could develop to be a fetus, so, it is a valuable source for embryonic stem cell establishment. There are several techniques to isolate a clump of ICM cells such as blastocyst outgrowth (Tabar, 2005; Genbacev et al., 2005), mechanical dissection (Nagy, 2005; Wang et al., 2005), laser-assisted blastocyst dissection (Tanaka et al., 2006) and immunosurgery (Solter and Knowles, 1975; Reubinoff et al., 2000; Tanaka et al., 2005; Mateizel et al., 2006). Immunosurgery technique is one of the most efficient techniques used for isolating ICM cells. Once

isolated and cultured *in vitro* under appropriate conditions, the ICM cells could propagated as an ES cell line.

ES cell biology

ES cell has unique properties that capable of symmetric division and self-renewal for a long period if cultured under appropriate condition (Fig 2.4). Moreover, ES cells are pluripotent and are capable to give rise to specific cell types if they were induced by appropriate inducers and environment (Bongso and Lee, 2005).

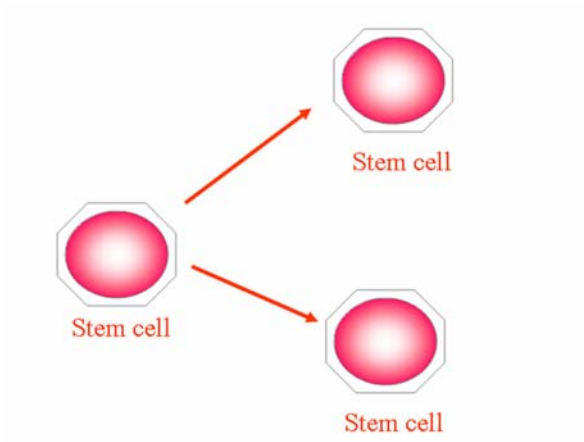


Figure 2.4 Stem cell has symmetrical dividing property. The symmetrical dividing could generate two daughter cells which absolutely identical to mother cell.

Another important property of stem cells is that of asymmetric division, yielding one differentiated progeny and one stem cell daughter (Cai et al., 1997; Lu et al., 2000) (Fig 2.5). This is when each stem cell produces two progeny cells that are initially the same as the parent, but one has potential to differentiate to be several cell types. Once a daughter cell has started the process of differentiation, it cannot dedifferentiate to its parent cell. The other progeny cell remains identical to the mother stem cell from the original niche and the self-renewal property could found in this progeny. Both self-renewal and asymmetric division are hallmarks of stem cells.

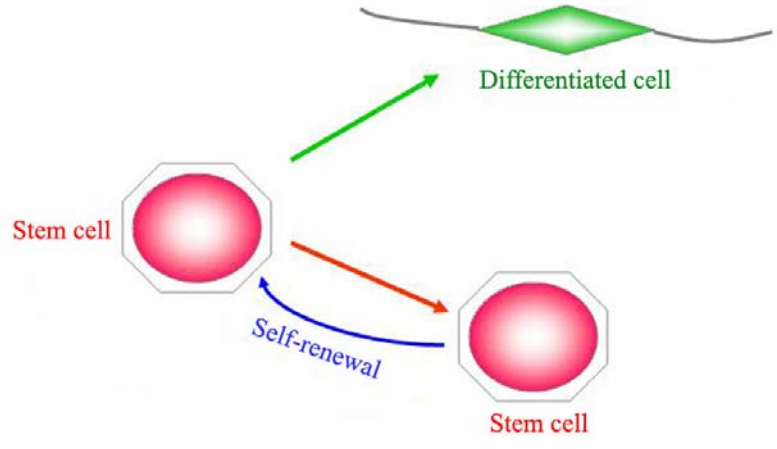


Figure 2.5 Stem cell has capacity to self-renew and asymmetric division. At each cell division, stem cells have to choose between self-renewal and differentiation. These two fates are influenced by several factors such as neighboring cells, growth factors and extra-cellular matrix components that adjust the balance between self-renewal and differentiation or even apoptosis.

The ES cells are considered pluripotent because they can differentiate into almost all tissues of the three embryonic germ layers after *in vitro* induced differentiation and are capable to form teratocarcinomas after subcutaneous injection into nude or severe compromised immune deficient (SCID) mice, except that ES cells could not differentiated to any cell types of the trophoblast lineage (Cavaleri and Schöler, 2004). The general features of ES cells were concluded as below:

- Derived from pre-implantation embryo
- Pluripotent: capable of differentiating into representative cells from all three embryonic germ layers
- Immortal: with prolonged proliferation at the undifferentiated stage (self renewal) and expression of high telomerase activity
- Maintaining normal karyotype after prolonged culture

- Ability to contribute to all embryonic germ layers including the germ line after injection into blastocyst
- Expressing unique pattern of ES markers such as Oct-4, Sox-2, Nanog and cell surface markers such as stage specific embryonic antigen (SSEA) – 1, SSEA-2, SSEA-3, SSEA-4, Tumor resistant antigen (TRA)-1-60 and TRA-1-81
- Clonogenic: each individual cell processing the above characteristics

The derivations of pluripotent cell lines from mouse blastocyst were reported in 1981 by two different groups (Evan and Kaufman, 1981; Martin, 1981). Then, the knowledge from murine ES cell establishment has been used for establish ES cells lines from different species including rabbit, hamster, pig, monkey and human (Gardner, 2004). Even such ES cell lines were claimed as pluripotent, they exhibit different properties from murine ES cell such as morphology of undifferentiated colonies and the expression profile of stem cell markers. Among the complications in assessing cell line in undifferentiated ES colonies of different species, the morphologically appearance of ES colonies vary in different animal species such as mouse ES undifferentiated colonies exhibit colonies with clear edge, dome shape and high nuclear – cytoplasmic ratio while the undifferentiated primate ES cells as appeared as flat colonies with high nuclear – cytoplasmic ratio (Thomson et al., 1995). Moreover, the primate ES cells are differed from the murine ES cell in the expression pattern of stem cell markers and cytokine factors that are important for maintaining at undifferentiated stage (Table 2.1). Recently, ES cells have been successfully established from early embryos, two cells to morula stage, instead of using the inner cell mass (ICM) of a blastocyst (Bradley et al., 1984; Eistetter, 1989; Strelchenko et al., 2004; Tesar, 2005). Furthermore, ES cells have been successfully established from a single biopsied blastomere of early pre-implantation stage mouse

and human embryo (Chung et al., 2006; Klimanskaya et al., 2006, Wakayama et al., 2007). These breakthroughs have demonstrated the feasibility of deriving personal ES cells.

Table 2.1 Comparision of murine, monkey and human ES cell

Pluripotent marker	**Murine ES cell**	**Monkey ES cell**	**Human ES cell**
AP	+	+	+
SSEA-1	+	-	-
SSEA-3	-	+	+
SSEA-4	-	+	+
TRA-1-60	-	+	+
TRA-1-81	-	+	+
Oct-4	+	+	+
Feeder cell	need	need	need
Cytokine factor control self-renew	LIF and some growth factors that work through GP130 receptor	Feeder cell and unidentified growth factor	Feeder cell and bFGF
Morphology of undifferentiated colony	Multi layers clump, dome shape, clear edge	Mono layer clump, loose, flat colony	Mono layer clump, loose, flat colony
EB formation	Yes	Yes	Yes
Teratoma formation	Yes	Yes	Yes
Chimera formation	Yes	N/A	Yes

2.5 Maintaining of ES cell in their undifferentiated state

The hallmark feature of ES cells is pluripotency. There are many defined and undefined signaling pathways involved in self-renewal and the proliferation of ES

cells such as LIF-STAT, Wnt and BMP signaling pathway. Since signal transductions are able to cross talk among other pathways, which make it even more difficult to understand the cascade of control. So far, only the leukemia inhibitory factor (LIF) receptor (LIFR) – STAT3 pathway has been defined in detail and the elucidation of the Wnt and BMP pathway is an ongoing effort.

Cytokine control pluripotent state

Cytokine is a polypeptide protein could control the undifferentiated state of ES cell. The important cytokine for ES cell maintaining self-renewal state is LIF which secreted from feeder cell and added into culture medium. Even thought, it is possible to culture ES cells in the absence of feeder cells in medium supplemented with LIF but ES cell might needed membrane-bound or matrix association factors to support the self-renewal process. Therefore, most laboratories rely on feeder layer to provide factors that enhance the proliferation and maintain the undifferentiated state of ES cells (Rathjen et al., 1990). There are several types of cell can provide this feeder function. The most widely used are either STO (Thioguanine and ouabain resistant) cell line or mouse embryonic fibroblasts (MEFs). The advantage of MEF is that they reproducible source of feeder cells but MEFs can not maintain indefinitely in culture, have limited life span and must be replenished continuously from frozen stocks so there is no temptation to use them beyond the time when they have lost their growth-enhancing ability. Actually, any strain of mouse can be used for producing MEFs as feeder layers but the largest litters are more satisfied. The big litter can be obtained from either random bred mice or F1 hybrid such as CD-1 strain. However, supplementation of the medium with recombinant leukemia inhibitory growth factor (LIF) is now an alternative to use as feeder.

LIF is also known as differentiation inhibitory factor (DIA). It is a secreted cytokine that inhibits the spontaneous differentiation of ES cell (Smith et al., 1988; Willium et al., 1988). LIF functions by binding to LIFR at the cell surface, which causes it to heterodimerize with another transmembrane protein, glycoprotein-130 (gp130). LIF bind to a two-part receptor complex that consists of the LIF receptor and the gp130 receptor. The binding of LIF triggers the activation of the latent transcription factor STAT3, a necessary event *in vitro* for the continued proliferation of mouse ES cells (Niwa et al., 1998; Burdon et al., 1999; Matsuda et al., 1999). Then the dimerisation of STAT3 proteins moves into nucleus and activate the DNA for self-renewal. The self-renewal of mouse ES cells also appears to be influenced by SHP-2 and ERK activity. SHP-2 modulates ES cell self-renewal and differentiation via bi-directional regulation of the ERK and STAT3 pathways. ERK, Extra-cellular Regulated Kinase, is one of several kinds of enzymes that become activated when the gp130 receptor and other cell-surface receptors are stimulated. Both ERK and SHP-2 are components of a signal-transduction pathway that counteracts the proliferative effects of STAT3 activation (Fig 2.6). Therefore, if ERK and SHP-2 are active, they inhibit ES cell self-renewal (Burdon et al., 1999).

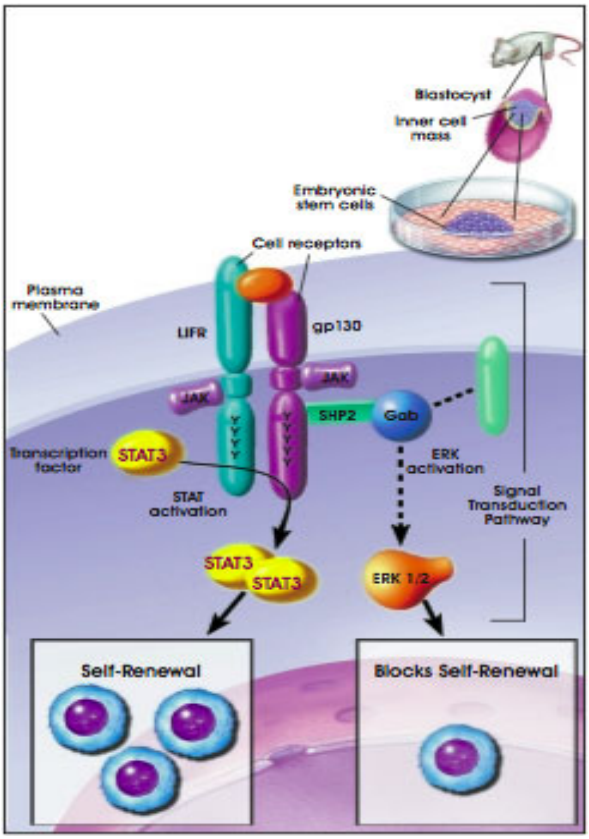


Figure 2.6 LIF – STAT3 signaling pathway control ES cell self renewal, in the mean time, it could inhibit ERK signaling which normally induce the cell differentiation.

Wnt signaling in ES cells

Wnt proteins are important regulators of cell proliferation and differentiation (Cadigan and Nusse, 1997). The Wnt signaling pathways involves proteins that directly participate in both gene transcription and cell adhesion. Wnt molecules are secreted lipid modified signaling proteins that bind to Frizzed receptors in the cell surface (Fig 2.7). Several cytoplasmic components transduce the signal to β-catenin, which enter the nucleus and forms a complex with a high mobility group (HMG) box containing DNA binding protein such as TCF (T cell factor) and LEF (lymphoid enhancer factor). The central player of the canonical Wnt signaling pathway is β-catenin, which is degraded in the absence of Wnt in cytoplasm. Express β-catenin is

phosphorylated by glycogen synthase kinase 3β (GSK-3β) then targeted for proteosome-mediated degradation. In the presence of Wnt signaling, Dishevelled (Dsh) becomes activated which leads to the uncoupling of β-catenin from the degradation pathway by inhibition of GSK-3β activity. This results in the accumulation of β-catenin, which enters the nucleus and interacts with partners such as TCF/LEF. Therefore, stabilization of β-catenin and its accumulation in the cytoplasm is a crucial step in canonical Wnt dependent target gene expression (Eisenmann, 2005; Paratore and Sommer, 2006). Recently, Wnt/β-catenin has been considered to maintain long-term pluripotency state of ES cell (Miyabayashi et al., 2007). The large-scale gene expression profiling studies of undifferentiated mouse and human ES cells found that the main components of the canonical Wnt signaling pathways are expressed (Aubert et al., 2002; Sato et al., 2003) and the activation of Wnt signaling pathway could inhibit ES cells differentiation. Recent studies have been showed that activation of Wnt signaling pathway using either a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK03), 6-bromoindirubin-3-oxime (BIO), (Sato et al., 2005) or a small molecule, IQ-1, (Miyabayashi et al., 2007) or a purine derivative, QS11, (Zhang et al., 2007) is sufficient to maintain undifferentiated state of ES cell by working through different down stream targets of Wnt signaling pathway.

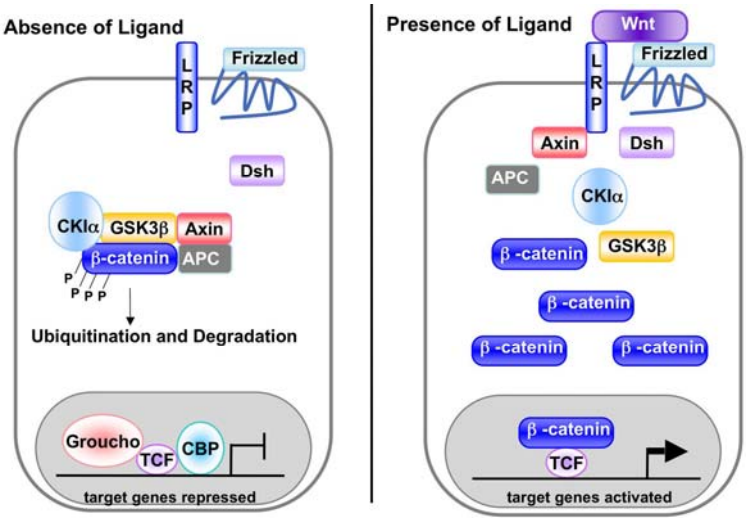


Figure 2.7 A canonical Wnt signaling pathway. In the absence of signal, action of the destruction complex (CKIα, GSK3β, APC, Axin) creates a hyperphosphorylated β-catenin, which is a target for ubiqitination and degradation by the proteosome. Binding of Wnt ligand to a Frizzled receptor complex leads to stabilization of hypophosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription (Eisenmann, 2005).

BMP signaling in ES cells

In addition to Wnt signaling, gene expression profiling studies suggested that BMP4 might support ES cell self-renewal especially in serum-free culture environment. LIF alone is insufficient to prevent mouse ES cells from differentiating into neural cells and also not sufficient to support self-renewal of human ES cell. Ying and colleagues (2003) and Qi and colleagues (2004) reported that the combination of bone morphogenetic proteins (BMPs) and LIF is sufficient to support the self-renewal of mouse ES cells. The effects of BMPs on mouse ES cells involve induction of inhibitor of differentiation (Id) proteins, and inhibition of extra-cellular receptor kinase (ERK) and p38 mitogen-activated protein kinases (MAPK) (Fig 2.8).

In summary, in ES cells the two signaling pathways initiated by LIF and BMP act in combination and are highly controlled in order to sustain self-renewal. Since there are several signaling pathways both defined and undefined involved for maintaining ES cell in pluripotency state, so, the balance of those pathways to remain in a self-renewal state is needed. If the balance shifts, ES cells might begin to differentiate.

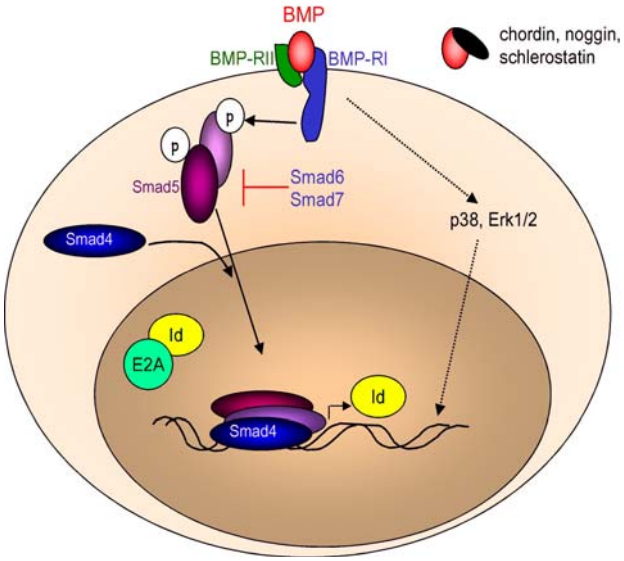


Figure 2.8 BMP binds to BMP receptor type II (BMP-RII) that in turn activates BMP-RI. Activated BMP-RI phosphorylates receptor regulated Smads (Smad1/5/8) which form complexes with Smad4. Activated Smad complexes regulate gene expression of several target genes, including Id1-Id4. Ids act as dominant negative regulators by binding bHLH transcription factors. The inhibitory Smads (Smad6, Smad7) antagonize signaling

Expression of transcription factors indicate pluripotent state of ES cells

One of the hallmarks of an undifferentiated pluripotent cell is the expression of the Pou5f1 gene, which encodes the transcription factor Oct-4. Oct-4 is very important for embryo development throughout blastocyst stage (Nichols et al., 1998). Normally, Oct-4 is express in all blastomeres up to morula stage then Oct-4

expression is more restricted in ICM cells and very low express in TE cells. For ES cells, Oct-4 expression is required for maintain pluripotentcy state of ES cells (Niwa et al., 2000). However, the Oct-4 protein itself is insufficient to maintain ES cells in the undifferentiated state. Few years later, Chamber and colleagues (2003) and Mitsui and colleagues (2003) identified another transcription factor, Nanog, that is essential for the maintenance of the undifferentiated state of mouse ES cells. The expression of Nanog decreased rapidly if mouse ES cells differentiated. If Oct-4 expression is inhibited in cultured ES cells, the cells generate trophectoderm (Niwa et al., 2005). If Oct-4 expression is artificially increased, ES cells would differentiate into primitive endoderm and mesoderm. Therefore, the level of Oct-4 expression dictates a significant aspect of the developmental program of ES cell. Taken all together, Oct-4 transcription factor has been used as one of the pluripotency markers in ES cells (Hay et al., 2004). Cooperative interaction between Oct-4 and Sox2 forms a heterodimer. This synergistic interaction between the two drives transcription of target genes such as Nanog which is a transcription factor that functions in maintaining the pluripotent cells of the inner cell mass and in deriving embryonic stem cells from these cells (Rodda et al., 2005). So, the set of Oct-4, Sox2 and Nanog expression is considered as transcription factors involved in maintaining the pluripotent ES cell phenotype. Even thought, recent knowledge about mechanism of Oct-4 to maintain pluripotent state is not yet been clearly identified but it is possible that some of the components of signaling pathways in cultured mouse ES cells such as adaptor protein, Gab1, may suppress interactions of specific receptors to the Ras-ERK signaling pathway. Further, the expression of this altered form of Gab1 may be promoted by the transcription factor Oct-4. In mouse ES cells, Oct-4 expression and increased synthesis of Gab1 may help suppress induction of differentiation by suppress ERK down stream

signaling pathway result in the inhibition of differentiate. (http://stemcells.nih.gov/info/scireport/appendixB.asp).

2.6 Blastomere of early stage embryo has difference fate of commitment

The differentiation competence of early embryonic sister blastomeres is one of the fundamental questions in developmental biology that has not been fully elucidated (Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989; Takowski and Wroblewska, 1967; Rossant, 1976; Chan et al., 2000; Piotrowska et al., 2001; Piotrowska-Nitsche et al., 2005). The successful implantation of a mouse four-cell blastomere has been reported, but, no full term development resulted (Rossant, 1976). Later studies on two-cell mouse embryos have demonstrated that only one blastomere of a two-cell mouse embryo was capable of normal development resulting in a live birth (Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989). Recent studies on embryo splitting have further demonstrated the pluripotency of embryonic blastomere. Unfortunately, not all blastomeres were able to develop to term (Heyman et al., 1998; Chan et al., 2000; Mitalipov et al., 2002; Schramm and Paprocki, 2004). Although the production of monozygotic twins by separating two-cell embryos has been achieved in some species (Ozil et al., 1982; Papaioannou et al., 1989; Matsumoto et al., 1989; Tsunoda et al., 1984; Allen and Pashen, 1984; Willadsen et al.,1981; Willadsen, 1981), there was only one report showing the pluripotency of four-cell blastomeres which produced quadruplets (four identical calves) (Johnson et al., 1995). The switching of embryonic blastomeres at the two- or four-cell stage (blastomere transfer) further demonstrates the competency of blastomeres without affecting later development. This study suggests the sister

blastomeres are capable of complementing the lost or damaged blastomeres in an embryo. However, whether lineage fate is committed in an early embryo, and if there is a time limit during early development when lineage fate could be reverted, remains unknown (Piotrowska et al., 2001). Based on the studies of embryo splitting and blastomere transfer, the lineage commitment may only be reversible prior to a specific lineage checkpoint in early embryos. Because early embryonic blastomeres are considered pluripotent, a mechanism would be capable of reverting the differentiation commitment established at both the zygotic stage and the two-cell stage. The mechanism could be triggered when the sister blastomeres are lost or damaged, resulting in normal development. However, it should be noted, embryonic arrest is expected, and lineage fate can not be reverted when damages occur beyond the developmental stage.

For years, individual blastomeres in early embryos, prior to polarization at the morula stage, were thought to be identical and pluripotent, if not totipotent. The question now is whether the development of a single blastomere can be seen as a linear clonal process whereby a particular stem cell gives rise to the inner cell mass (ICM) or trophectoderm (TE). The results of lineage-tracing experiments on labeled two- and four-cell blastomeres support the idea of a developmental bias. It has also been found that although each two-cell stage blastomere gives rise to both the ICM and the TE lineages, one cell tends to contribute more to the embryonic part of the blastocyst and the other cell contributes to the abembryonic part of the blastocyst (Gardner, 2001; Piotrowska-Nitsche et al., 2001; Fujimori et al., 2003; Piotrowska-Nitsche and Zernicka, 2005). Additionally, at the four-cell stage, when embryos are cleaving in a common but specific way, cells have predictable fates (Piotrowska-Nitsche et al., 2005). This bias in the allocation of a two- and four-cell blastomere

progeny suggests the differential developmental competence of the sister blastomeres, and their lineage fate, perhaps are committed at even earlier embryonic stage before the polarization at the morula stage. Recently, few reports have been shown that ES cell could be established from single biopsied blastomere from both mouse and human early stage embryo but the success rate of those two reports were low. This might because of the biopsied blastomere committed to be TE not ICM. The efficiency of ES cell establishment from each blastomere based on stem cell markers expression will be evaluated in this study.

2.7 The mitogen-activated protein kinase (MAPK)

The MAPK family is consisted of four groups of kinases: extracellular signal-regulated kinases (ERKs) 1 and 2; ERK5; c-Jun amino terminal kinases (JNKs) 1,2 and 3; and P38MAPK α, β, δ and γ, where each isoform is encoded by its own gene (Binetruy et al., 2007). Among the MAPK family, the ERK1/2, JNK and P38MAPK pathway were the most studied in stem cell research (Binetruy et al., 2007). These kinases are important regulators of the proliferation and differentiation of the ES cells, they are also involved in regulating apoptotic process (Fig 2.9).

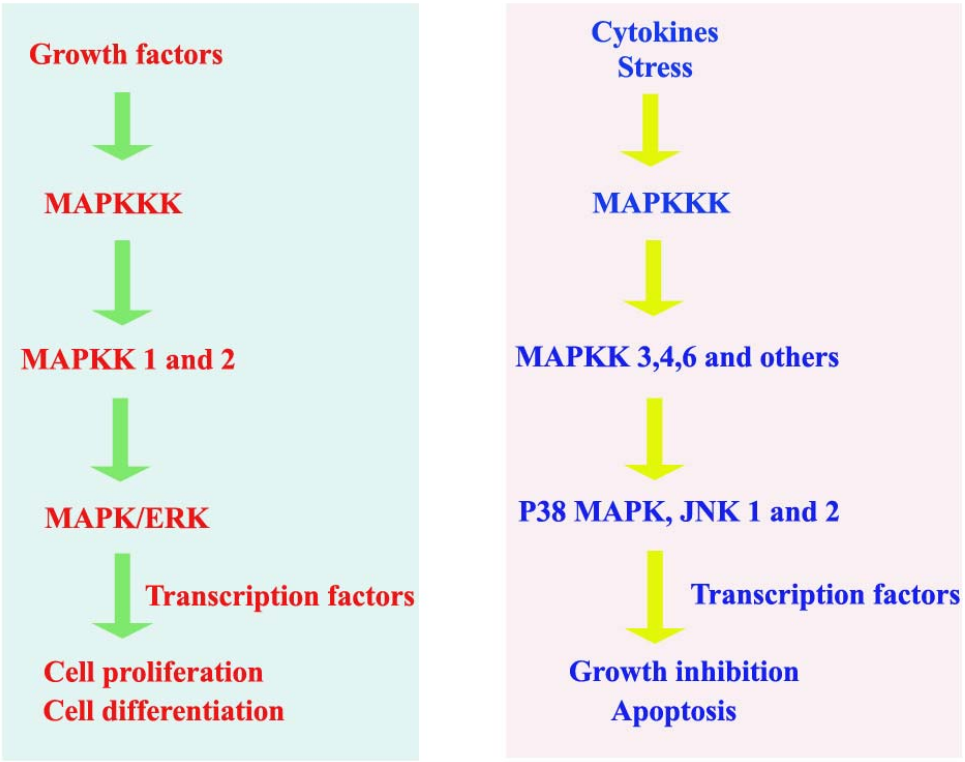


Figure 2.9 MAPK signaling pathway. Growth factors signals are channeled in the MAPKKK/MAPKKs pathway, resulting in cell proliferation and differentiation whereas the cytokine signals are directed via MAPKKK and MAPKKs to P38 MAPK. They causes in growth inhibition and apoptosis.

Several MAPK inhibitors have been investigated for their roles in early embryo development, these include MEK-1 (I) (Chung et al., 2006; Paliga et al., 2005) and P38MAPK (I) (Natale et al., 2004; Maekawa et al., 2005; Paliga et al., 2005; Madan et al., 2006). The MEK-1 (I) has been used for deriving mouse ES cells from embryonic blastomere, which was co-cultured with established mouse ES cell (Chung et al., 2006). Although ES cell lines have been successfully established, the role of MEK-1 (I) and ES cells co-culture on single blastomere development has not yet been determined. P38MAPK (I) has shown to have inhibiting effect on the development of TE cells in mouse morula stage embryos (Maekawa et al., 2005), which may enhance the development toward the ICM fate while TE is being

suppressed. Thus the ICM enhancement effect of P38MAPK (I) merit further investigation. Therefore, one of the objectives of this study will study the effects of MEK-1 (I) and P38MAPK (I) on whole and single blastomere derived embryo development and determine if they will have any benefit on ES cell establishment from single blastomere?.

2.8 Adrenocorticotropic hormone (ACTH)

Adrenocorticotropic hormone (ACTH or corticotropin) is a polypeptide hormone produced and secreted by the pituitary gland. Within the pituitary gland, ACTH is produced in a process that also generates several other hormones. A large precursor protein named proopiomelanocortin (POMC, "Big Mama") is synthesized and proteolytically chopped into several fragments as depicted below (Fig 2.10) (Bowen, 1998). The synthesized hormones will be secreted from the endocrine organ and transported to the target cells by the way of bloodstream.

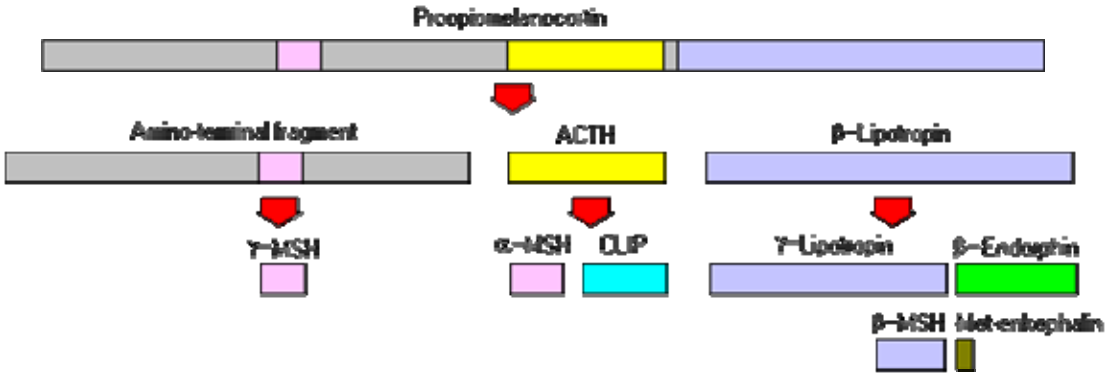


Figure 2.10 The process of proopiomelanocortin synthesized and proteolytically choppe into several fragment including ACTH (Bowen, 1998).

In target cell, ACTH binds a trans-membrane receptor that coupled with G-proteins, called G-protein coupled receptor. Dhanasekaran and colleagues (1998) indicated that the α-subunits as well as the βγ-subunits of G-proteins regulate several

critical signaling pathways involved in cell proliferation, differentiation and apoptosis (Dhanasekaran et al., 1998). Once the G-protein coupled receptor is activated by ACTH, the α-subunit will be released to further activate adenylyl cyclase (AC). Then the active AC will synthesis 3'-5'-cAMP (cAMP) from ATP (Fig 2.11). However, active cAMP will be inactivated by phosphodiesterase enzyme to produce 5'-AMP.

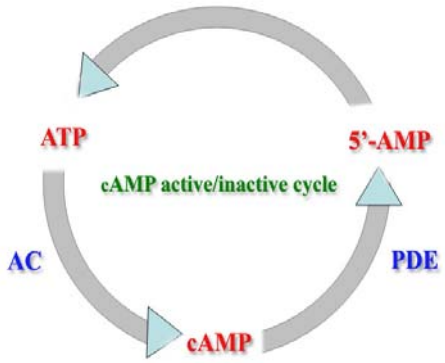


Figure 2.11 AC enzyme produce active cAMP whereas PDE catalyse the in active cAMP called 5'-AMP

The cAMP is central intracellular messengers that influence many cellular functions such as gluconeogenesis, glycolysis, muscle contraction, membrane secretion, learning process, ion transport, differentiation, growth control and apoptosis. The active cAMP functions mainly in two ways, regulation of ion channels by binding to the cytoplasmic structural elements of channels and regulates their open state, or activation of protein kinases by directly activating Protein Kinase A (PKA) and the activation of PKA leads directly to the phosphorylation of many proteins and gene transcription (Fig 2.12).

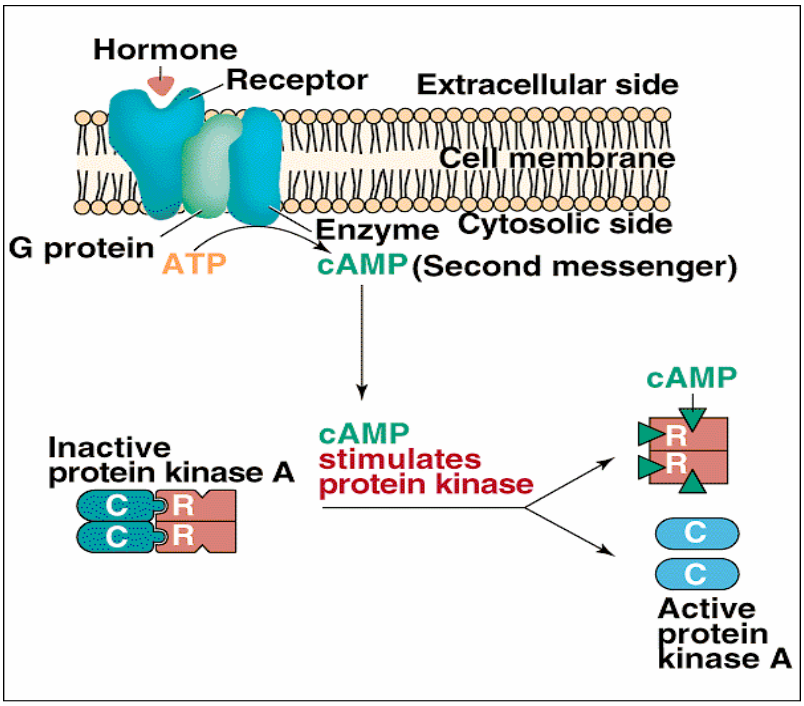


Figure 2.12 Hormone work through the G-protein couple receptor and then activate the plasma membrane receptor, adenylyl cyclase (AC) to produce cAMP secondary messenger.

ACTH on mammalian embryo development

ACTH had been used with several animal species such as chicken, sheep, pig and mouse to evaluate the effect of adrenal stimulation (Jenkins et al., 2007; Carter et al., 2002; Razdan et al., 2000; O'Shaughnessy et al., 2003**).** There were several studies have been evaluated the effect of egg and embryo development after ACTH administration to the animals. The trend of those results shows that ACTH had effects on mammalian egg/embryo quality, quantity and development rate. Moreover, in sow, administrated with ACTH had significantly reduce number of spermatozoa bind to the zona pellucida of oocytes when compare to control embryo and also found that ACTH has negative effect on sow embryo development and possibly induce faster embryo transportation through the oviduct, shortened the duration of standing oestrus and

changed the hormonal pattern of progesterone. (Razdan et al., 2000; Brendt, 2006; Brendt et al., 2007).

ACTH on ES cell establishment

Dhanasekaran and colleagues (1998) has been reported that ACTH could induce the AC activity. Once the AC activity is high, the cAMP level will also high since AC converse ATP to cAMP. Recently, Faherty and colleagues (2007) studied about the role of cAMP/PKA pathway on mouse ES cell self renewal. They suggest that using the forskolin, the adenylate cyclase agonist, could increase cAMP level. The high cAMP level could induce the ES cell self renewal but only if the culture medium did not supplemented with LIF and FBS.

2.9 References

Allen, W.R., and Pashen, R.L. (1984). Production of monozygotic (identical) horse twins by embryo micromanipulation**. J. Reprod. Fertil.** 71: 607-613.

Aubert, J., Dunstan, H., Chamber, I., and Smith, A. (2002). Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. **Nat. Biot.** 20: 1240 – 1245.

Biggers, J.D., Moore, B.D., and Whittingham, D.G. (1965). Development of mouse embryos *in vivo* after cultivation from two cell ova to blastocysts *in vitro*. **Nature** 15: 734 – 735.

Binetruy, B., Heasley, L., Bost, F., Caron, L., and Aouadi, M. (2007). Regulation of embryonic stem cell lineage commitment by mitogen activated protein kinases. **Stem cells** 25: 1090-1095.

Bongso, A., and Lee, E.H. (2005). Stem Cells: Their definition, classification and sources: In A. Bongso, and E.H., Lee (Eds). **Stem cells from bench to bedside** (pp. 1-13). Singapore, World scientific publishing Co. Pte.

Bowen, R. (1998). **Adrenocorticotropic hormone.** [On-line]. Available: http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/hypopit/acth.html

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. **Nature** 309: 255-256.

Brandt, Y., Medej, A., Rodriguez-Martinez, H., and Einarsson, S. (2007). Effects of exogenous ACTH during oestrus on early embryo development and oviductal transport in the sow. Reprod. Dom. Amim. 42: 118 – 125.

Brandt, Y.C.B. (2006). The effect of ACTH during oestrus on the reproduction in the sow. [On-line]. Available: http://diss-epsilon.slu.se/archive/00001078/01/ Acta200614 .pdf.

Brinster, R L. (1965). Studies on the development of mouse embryos in vitro: The interaction of energy source. **J. Reprcst. Fend.** 10: 227 - 240.

Browning, J.Y., and Wolf, R.C. (1981). Maternal recognition of pregnancy in the rabbit: effect of conceptus removal. **Biol. Reprod.** 24: 293 – 297.

Burdon, T., Chambers, I., Stracey, C., Niwa, H., and Smith, A. (1999). Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. **Cells Tissues Organs.** 16: 131 – 143.

Cai, L., Hayer, N.L., and Nowakowski, R.S. (2000). Synchrony of clonal cell proliferation and contiguity of clonally related cells: production of mosaicismin the ventricular zone of developing mouse neocortex. **J. Neurosci.** 17: 2088 – 2100.

Carter, A.M., Petersen, Y.M., Towstoless, M., Andreasen, D., and Jensen, B.L. (2002). Adrenocorticotrophic hormone (ACTH) stimulation of sheep fetal adrenal cortex can occur without increased expression of ACTH receptor (ACTH-R) mRNA. **Reprod. Fert. Dev.** 14: 1-6.

Cavaleri, F., and Schöler, H. (2004). Molecular Facets of pluripotency: In R. Lanza, J. Gearhart, B. Hogen, D. Melton, R. Pedersen, J. Thomson, and M. West, (Eds). **Hand book of stem cell** (pp. 27 – 44). London: Elsevier Academic Press.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. **Cell.** 113: 643-655.

Chan, A.W.S., Dominko, T., Luetjens, C., Neuber, E., Martinovich, C., Hewitson, L., Simerly, C.R., and Schatten, G.P. (2000). Clonal propagation of primate offspring by embryo splitting. **Science** 287:317-319.

Chang, M.C. (1959). Fertilization of rabbit ova *in vitro*. **Nature** 193: 466-467.

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L., and Lanza, R. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. **Nature** 439: 216-219.

Eistetter, H. R. (1989). Pluripotent embryonal stem cells can be established from disaggregated mouse morulae. **Dev. Growth Differ.** 31: 275-282.

Eisenmann, D.M. (2005). Wnt signaling [On-line]. Available: http://www.wormbook.org /chapters/www_wntsignaling/wntsignaling.pdf.

Eliot, T.S. (2003). Chapter 6: Blastomere cleavage: In A.A., Kiessling, and S.C., Anderson (Eds). **Human embryonic stem cells: An introduction to the science and therapeutic potencial** (pp. 65-80). London: Jones and Bartlett Publishers International.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. **Nature** 292: 154-156.

Faherty, S., Fitzgerald, A., Keohan, M., and Quinlan, L.R. (2007). Self-renewal and differentiation of mouse embryonic stem cells as measured by Oct-4 expression: the role of the cAMP/PKA pathway. ***In Vitro*** **Cell Dev Biol Anim.** 43: 37-47.

Fujimori, T., Kurotaki, J., Miyazaki, J.L., and Nabeshima, Y.I. (2003). Analysis of cell lineage in two- and four-cell mouse embryos. **Development** 130: 5113-22.

Gardner, R. (2004). Pluripotential stem cells from vertebrate embryos: present perspective and future challenges: In R., Lanza, J., Gearhart, B., Hogen, D., Melton, R., Pedersen, J. Thomson, and M. West, (Eds). **Hand book of stem cell** (pp. 15 – 26). London: Elsevier Academic Press.

Gardner, R.L. (2001). Specification of embryonic axes begins before cleavage in normal mouse development. **Development** 128:839–847.

Genbacev, O., Krtolica, A., Zdravkovic, T., Brunette, E., Powell, S., Nath, A., Caceres, E., McMaster, M., McDonagh, S., and Li, Y. (2005). Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. **Fertil. Steril. 83**:1517–1529.

Gulyas, B. J. (1975). A re-examination of the cleavage patterns in eutherian mammalian eggs: rotation of the blastomere pairs during second cleavage in the rabbit. **J. Exp. Zool.** 193: 235–248.

Hammond, L. (1949). Recovery end culture of tubal mouse ova. **Nature** 163: 28-29.

Hay, D.C., Sutherland, L., Clark, J., and Bordon, T. (2004). Oct-4 knockdown induces similar patterns of endoderm and trophoectoderm differentiation markers in human and mouse embryonic stem cell. **Stem Cells** 22: 225 – 235.

Heyman, Y., Vignon, X., Chesne, P., Le Bourhis, D., Marchal, J., and Renard, J.P. (1998). Cloning in cattle: from embryo splitting to somatic nuclear transfer. **Reprod Nutr Dev** 38: 595-603.

Heape, W. (1890). Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. **Proc. R. Soc. London** 48: 457-458.

Johnson, W.H., Loskutoff, N.M., Plante, Y., and Betteridge, K.L. (1995). Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo. **Vet. Rec.** 137:15-16.

James, J. (2006). **Blastocyst culture** [On-line]. Available: http://www.canberra fertilitycenter.com.au/treatment_blastocyst.htm

Jenkins, S.A., Muchow, M., Richards, M.P., McMurtry, J.P., and Porter, T.E. (2007). Administration of adrenocorticotropic hormone during chicken embryonic development prematurely induces pituitary growth hormone cells. [On-line]. Available: http://endo.endojournals.org/cgi/rapidpdf/en.2006-1102v1.

Kiessling, A.A., and Anderson, S.C. (2003). Chapter 8: The blastocyst and inner cell mass cells. In A.A., Kiessling and S.C., Anderson (Eds). S**tem cells: an introduction to the science and therapeutic potential** (pp. 98 – 108). London: Jones and Bartlett Publishers International.

Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. **Nature** 444: 481-485.

Lu, B., Jan, L., and Jan, Y.N. (2000). Control of cell divisions in the newvous system: symmetry and asymmetry. **Annu. Rev. Neurosci.** 23: 531 – 556.

Madan, P., Calder, M.D., and Watson, A.J. (2006). Mitogen activated protein kinase (MAPK) blocked of bovine preimplantation embryogenesis requires

inhibition of both P38MAPK and extracellular signal-regulated kinase (ERK) pathways. **Reproduction** 130: 41-51

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. **Proc. Natl. Acad. Sci. U. S. A.** 78: 7634-7638.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. **EMBO J.** 18: 4261 – 4269.

Matsumoto, K., Miyake, M., Utsumi, K., and Iritani, A. (1989). Production of identical twins by separating two-cell rat embryos. **Gamete Res** 22:257-263.

Maekawa, M., Yamamoto, T., Tanoue, T., Yuasa, Y., Chisaka, O., and Nishida, E. (2005). Requirement of the MAP kinase signaling pathways for mouse pre-implantation development. **Development** 132: 1773-1783.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Muruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. **Cell.** 113:631-642.

Mitalipov, M.M., Yeoman, R.R., Kuo, H.C., and Wolf, D.P. (2002). Monozygotic twinning in rhesus monkeys by manipulation of in vitro derived embryos. **Biol Reprod** 66: 1449-1455.

Mateizel, I., Temmerman, N.D., Ullmann, U., Cauffman, G., Sermon, K., Velde, V.D., Rycke, M.D., Degreef, E., Devroey, P., Liebaers, I., and Steirteghem, A.V. (2006). Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorder. **Hum. Reprod**. 21: 503 – 511.

Miyabayashi, T., Teo, J.L., Yumamoto, M., McMillan, M., Nguyen, C., and Kahn, M. (2007). Wnt/β-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. **Proc. Natl. Acad. Sci. U. S. A.** 104: 5668 – 5673.

Nagy, A. (2005). **Derivation of new ES cell lines** (Andras Nagy Lab) [On-line]. Available: http://www.mshri.on.ca/cores/ESfacility/EScDer2005.pdf

Nagy, A. (1994). Developmental genetics and embryology of the mouse: part, present and future: In A. Nagy (Ed). **Manipulating the mouse embryo** (pp. 1-96). USA: Cold Spring Harbor Laboratory Press.

Natale, D.R., Paliga, A,J.M., Beier, F., D'Souza, S.J.A., and Watson, A.J. (2004). p38 MAPK signalling during murine preimplantation development. **Dev. Biol.** 268 : 76-88

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. **Cell** 95: 379–391.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. **Genes Dev.** 12: 2048 – 2060.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. **Nat. Genet.** 24: 372 – 376.

Niwa, H., Toyooka, D., Shimosato, D., Strumpf, K., Takahashi, R., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation. **Cell** 123: 917 – 929.

O'Shaughnessy, P.J., Fleming, L.M., Jackson, G., Hochgeschwender, U., Reed, P., and Baker, P.J. (2003). Adrenocorticotropic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. **Endocrinology** 144: 3279 – 3284.

Ozil, J.P. (1983). Production of identical twins by bisection of blastocysts in the cow. **J. Reprod Fertil**. 69: 463-468.

Paliga, A.J.M., Natale, D.R., and Andrew J. (2005). p38 mitogen-activated protein kinase (MAPK) first regulates filamentous actin at the 8-16 cell stage during preimplantation development. **Biol. Cell**. 97: 629-640.

Papaioannou, V.E., Mkandawire, J., and Biggers, J.D. (1989). Development and phenotypic variability of genetically identical half mouse embryos. **Development** 106: 817-827.

Paratore, C., and Sommer, L. (2006). Stem cells. In K. Unsicker and K. Krieglstein (Eds). **Cell signaling and growth factors in development** (pp. 1-37). Darmstadt: Wiley-vch verlag GmbH and Co. KGaA, Weinheim.

Piotrowska, K., Wianny, F., Pedersen, R.A., and Zernicka-Goetz, M. (2001). Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. **Development** 128:3739–3748.

Piotrowska-Nitsche, K., Gomez, A.P., Haraguchi, S., and Goetz, M.Z. (2005). Four-cell stage mouse blastomeres have different developmental properties. **Development** 132:479-490.

Qi, X., Li, T.G., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y., and Zhao, G.Q. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. **Proc. Natl. Acad. Sci. U.S.A.** 101:6027-6032.

Razdan, P., Mwanza, A.M., Kindahl, H., Rodriguez-Martinez, H., Hulten, F., and Einarsson, S. (2002). Effect of repeated ACTH-stimulation on early embryonic development and hormonal profiles in sows. **Anim. Reprod. Sci.** 70: 127 – 137.

Rathjen, P.D., Toth, S., Willis, A., Heath, J.K., and Smith, A.G. (1990). Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. **Cell**. 62, 1105–1114.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. **Nature Biotechnol.** 18: 399 – 404.

Rodda, D. J., Chew, J. L., Lim, L. H., Loh, Y. H., Wang, B., Ng, H. H., and Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. **J Biol Chem** 280: 24731-24737.

Rossant, J. (1976). Postimplantation development of blastomeres isolated from 4- and 8-cell mouse eggs. **J. Embryol Exp Morphol** 36: 283-290.

Sato, N., Sanjuan, I.M., Heke, M., Uchida, M., Naef, F., and Brivanlou, A.H. (2003). Molecular signature of human embryonic stem cells and its comparison with the mouse. **Dev. Biol.** 260: 404 – 413.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. **Nat. Med.** 10: 55 – 63.

Schramm, R.D., and Paproki, A.M. (2004). In vitro development and cell allocation following aggregation of split embryos with tetraploid or developmentally

asynchronous blastomeres in rhesus mmonkeys. **Cloning Stem Cells** 6:302-314.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Morean, J., Stahl, M., and Roger, D.D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. **Nature** 336: 688 – 690.

Solter, D., and Knowles, B.B. (1975). Immunosurgery of mouse blastocyst. **Proc. Natl. Acad. Sci. U.S.A**. 72: 5099 - 5102.

Strelchenko, N., Verlinsky, O., Kukharenko, V., and Verlinsky, V. (2004). Morula derived human embryonic stem cells. . **Reprod. Biomed.** 9: 623-629.

Strumpf, D.C., Mao, A., Yamanaka, Y., Ralstom, A., Chewengsaksophak, K., Beck, F., and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. **Development** 132: 2093 – 2102.

Sunoda, Y., Uasui, T., and Sugie, T. (1984). Production of monozygotic twins following transfer of separated half embryos in the goat. **Jpn J. Zootech Sci** 55: 643-647.

Tabar, N.H. (2005). Isolation and differentiation of mouse embryonic stem cells . **J. Reprod. Med**. 3: 42 – 45.

Tanaka, N., Takeuchi, T., Neri, Q.V., Sills, E.S., and Palermo, G.D. (2005). Effect of immunosurgery on ICM attachment and embryonic stem cell harvesting. **Fert. Steril.** 84: 401.

Tanaka, N., Takeuchi, T., Neri, Q.V., Sills, E.S., and Palermo, G.D. (2006). Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results

in a murine model. **J. Transl. Med.** 4: 20 [On-line]. Available: http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=1479373&blobtype.

Tarkowski, A.K., and Wroblewska, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. **J. Embryol. Exp. Morphol.** 18: 155-180.

Tarkowski, A.K. (1959). Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. **Ada. Theriologica.** 3: 191-267.

Tesar, P. (2005). Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. **Proc. Natl. Acad. Sci. U. S. A.** 102: 8239-8244.

The national Institutes of Health. (2001). Mouse embryonic stem cell cultures [On-line]. Available: http://stemcells.nih.gov/info/scireport/appendixB.asp.

Thomson, J.A. Kalishman, J, Golos,T. G. Durning, M., Harris, C.P., Becker, R.A., and Hearn J.P. (1995). Isolation of a primate embryonic stem cell line. **Proc. Natl. Acad. Sci. U. S. A.** 92: 7844-7848.

Tsunoda, Y., and Mclaren, A. (1983). Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. **J. Reprod. Fert.** 69: 315-322.

Wang, Q., Fang, Z.F., Jin, F., Lu, Y., Gai, H., and Sheng, H.Z. (2005). Derivation and growing human embryonic stem cells on feeders derived from themselves. **Stem Cells** 23: 1221 – 1227.

Wakayama, S., Hikichi, T., Suetsugu, R., Sakaide, Y., Bui, H.T., Mizutani, E., and Wakayama, T. (2007). Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. **Stem Cells** 25:986-993.

Whitten, W.K. (1956). Culture of tubal mouse ova. **Nature** 176: 96.

Whitten, W.K. (1957). Culture of tubal mouse ova. **Nature** 179: 1081-1082.

Willadsen, S.M., Lehn-Jensen, H., Fehily, C.B., and Newcomb, R. (1981). The production of monozygotic twins of preselected parentage by micromanipulation of nonsurgically collected cow embryos. **Theriogenology**; 15:23-29.

Willadsen, S.M., and Polge, C. (1981). Attempts to produce monozygotic quadruplets in cattle by blastomere separation. **Vet Rec**; 10:211-213.

Williums, R.L., Hilton, D.J., Pease, S., Wilson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1988). Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. **Nature** 336: 684 – 687.

Ying, Q.L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. **Cell.** 115: 281-292.

Zhang, Q., Major, M.B., Takanashi, S., Camp, N.D., Nishiya, N., Peters, E.C., Ginsberg, M.H., Jian, X., Randazzo, P.A., Schultz, P.G., Moon, R.T., and Ding, S. (2007). Small molecule synergist of the Wnt/β-catenin signaling pathway. **Proc. Natl. Acad. Sci. U. S. A.** 104: 7444 – 7448.

CHAPTER III

SUCCESSFUL OF ES CELL LINES ESTABLISHMENT FROM SINGLE BLASTOMERES OF AN EARLY MOUSE EMBRYO

3.1 Abstract

The recent development of establishing embryonic stem (ES) cell lines from single blastomeres of an early mouse and human embryo has created a strong interest in the idea of personalized ES cells. However, the question about if every blastomere of the preimplantation embryo has equal ES cell derivation capability still need more evidences to prove it. Single blastomeres could be cultured individually and formed blastocyst and ICM outgrowth. Based on the expression patterns of ES cell markers (Sox-2, Oct-4), and a trophectoderm marker (Cdx2), only some, but not all sister blastomeres derived from embryos and embryonic outgrowth expressed stem cell markers when two- and four-cell stage embryonic blastomeres were individually cultured. Our findings support the concept that sister blastomeres of two- and four-cell mouse embryos also have a differential differentiation competence, and the random retrieval of blastomeres from an early embryo (two-to-four cell stage) may effect the later development and successful establishment of ES cells in mice. This experiment thus demonstrated the differential competence of sister blastomeres of early mouse embryo based on the expression patterns of stem cell markers, the capability of forming ICM, and the establishment of ES cells. This study

successfully established the drug and ES supporting cells-free culture condition for single blastomere culture. There were four ES cell lines (5.6%; 4/72) which could be established from single blastomere derived from two cell stage embryo.

3.2 Introduction

The differentiation competence of early embryonic sister blastomeres is one of the fundamental questions in developmental biology that has not been fully elucidated (Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989; Takowski and Wroblewska, 1967; Rossant, 1976; Chan et al., 2000; Piotrowska et al., 2001; Piotrowska-Nitsche et al., 2005). The successful implantation of a mouse four-cell blastomere has been reported, but, no full term development resulted (Rossant, 1976). Later studies on two-cell mouse embryos have demonstrated that only one blastomere of a two-cell mouse embryo was capable of normal development resulting in a live birth (Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989). Recent studies on embryo splitting have further demonstrated the pluripotency of embryonic blastomere. Unfortunately, not all blastomeres were able to develop to term (Heyman et al., 1998; Chan et al., 2000; Mitalipov et al., 2002; Schramm and Paprocki, 2004). Although the production of monozygotic twins by separating two-cell embryos has been achieved in some species (Ozil et al., 1982; Papaioannou et a., 1989; Matsumoto et al., 1989; Tsunoda et al., 1984; Allen and Rashen, 1984; Willadsen et al.,1981; Willadsen, 1981), there was only one report showing the pluripotency of four-cell blastomeres which produced quadruplets (four identical calves) (Johnson et al., 1995). The switching of embryonic blastomeres at the two- or four-cell stage (blastomere transfer) further demonstrates the competency

of blastomeres without affecting later development. This study suggests the sister blastomeres are capable of complementing the lost or damaged blastomeres in an embryo. However, whether lineage fate is committed in an early embryo, and if there is a time limit during early development when lineage fate could be reverted, remains unknown (Piotrowska et al., 2001). Based on the studies of embryo splitting and blastomere transfer, the lineage commitment may only be reversible prior to a specific lineage checkpoint in early embryos. Because early embryonic blastomeres are considered pluripotent, a mechanism would be capable of reverting the differentiation commitment established at both the zygotic stage and the two-cell stage, The mechanism could be triggered when the sister blastomeres are lost or damaged, resulting in normal development. However, it should be noted, embryonic arrest is expected, and lineage fate can not be reverted when damages occur beyond the developmental stage.

For years, individual blastomeres in early embryos, prior to polarization at the morula stage, were thought to be identical and pluripotent, if not totipotent. The question now is whether the development of a single blastomere can be seen as a linear clonal process whereby a particular stem cell gives rise to the inner cell mass (ICM) or trophectoderm (TE). The results of lineage-tracing experiments on labeled two- and four-cell blastomeres support the idea of a developmental bias. It has also been found that although each two-cell stage blastomere gives rise to both the ICM and the TE lineages, one cell tends to contribute more to the embryonic part of the blastocyst and the other cell contributes to the abembryonic part of the blastocyst (Gardner, 2001; Piotrowska-Nitsche et al., 2001; Fujimori et al., 2003; Piotrowska-Nitsche and Zernicka-Goetz, 2005). Additionally, at the four-cell stage, when

embryos are cleaving in a common but specific way, cells have predictable fates (Piotrowska-Nitsche et al., 2005). This bias in the allocation of a two- and four-cell blastomere progeny suggests the differential developmental competence of the sister blastomeres, and their lineage fate, perhaps are committed at even earlier embryonic stage before the polarization at the morula stage.

Embryonic stem (ES) cells have been successfully established from early embryos instead of using the inner cell mass (ICM) of a blastocyst. (Eistetter, 1989; Strelchenko et al., 2004; Tesar, 2005). It is noted that only a small number of blastomeres from eight-cell (Delhaise et al, 1996) and 16-cell (Eistetter et al., 1989) mouse embryos were viable for deriving ES cells. In 1989, Wilton and Trounson attempted to establish ES cells from the biopsy blastomere of a four-cell mouse embryo and the attempt was unsuccessful that only trophoblast-like cells resulted. Recently, Wakayama and colleagues (2007) demonstrated the successful derivation of ES cell lines from the blastomeres of two–cell, early four-cell, late four-cell, and eight-cell mouse embryos with a derivation rate of 50-69%, 28%-40%, 22%, and 14%-16% respectively. The establishment of an ES cell from a two-cell blastomere had the highest success rate. It was also noted that the success rate was reduced approximately 50% at each additional cell division. This study suggests that only one or two blastomeres of an embryo retain stem cell properties or are capable of deriving ES cells up to the eight-cell stage.

The latest reports support the idea of establishing personalized ES cell lines from the single biopsy blastomeres of eight-cell stage mouse embryos and the establishment of an extra-embryonic stem cell line from a single blastomere recovered from eight- to ten-cell stage human embryos (Chung et al., 2006; Klimanskaya et al.,

2006). Because of a low success rate establishing ES cells from a single biopsy blastomere (Chung et al., 2006; Klimanskaya et al., 2006), and that only a few of the blastomeres retain their stem cell properties and are capable of deriving ES cells, the question becomes: Can an ES cell line be established from the sister blastomeres with a distinct lineage fate? It is important to determine if the random retrieval of early embryonic blastomeres for deriving ES cells would jeopardize the integrity of an embryo and its later development. We aim to investigate if the sister blastomeres of two- and four-cell mouse embryos have equal developmental capacity, a distinct lineage fate based on the expression pattern of the ES cell markers in the single blastomere derived embryos and their outgrowth, and if they are equally competent in deriving ES cell lines.

3.3 Materials and Methods

3.3.1 Animals

Female CD-1 mice (four-six weeks) were superovulated with 10 IU of pregnant mares serum gondotrophin (PMSG, Sigma; IP). This was followed by 10 IU of human chorionic gonadotrophin (hCG, Sigma; IP) 48 hours later, and then natural mating with CD-1 male mice. Two-cell embryos were collected 43-45 hours after hCG injection from the oviducts and then cultured in 20 μl drop of KSOM + AA media (Specialty Media) under mineral oil with 5% CO_2 in air at 37°C.

3.3.2 Nomenclature

Embryos derived from the blastomere break-down of 2C and 4C stage embryos were named 2CBD and 4CBD, respectively. The zona-pellucida free 2C and 4C

stages embryos were named as 2C whole (2CW) and 4C whole (4CW) embryos.

3.3.3 Blastomeres break down

The zona pellucida of 2C and 4C stage mouse embryos was removed by 0.5% Protease (Sigma). The blastomeres were then separated by incubating the zona-free embryo in PBS without calcium and magnesium [PBS (-)] followed by gentle pipeting. The blastomeres of the same embryo were cultured individually alongside with their sister blastomeres.

3.3.4 Feeder cell preparation.

Mouse fetal fibroblasts (MFF) were prepared from 13.5 dpc mouse fetuses. The MFFs were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 200mM L-glutamine (Invitrogen) and 1x Penicillin/Steptomycin (Invitrogen). The MFFs were inactivated with 5 μg/ml mitomycin C (Sigma) for two hrs followed by a thorough wash before plating.

3.3.5 Single blastomere culture.

The blastomeres of the 2CBD and 4CBD embryos were cultured individually in a 72-well plate pre-coated with 0.1% gelatin (Sigma). The MFFs were plated at 1,000 cell/well in 10 μl of culture medium 24 hours prior use. The blastomeres and embryos were cultured in two different media, mouse ES (mES) and KSOM media. The mES medium contains DMEM supplemented with 10% FBS, 200mM L-glutamine, 0.1mM β-mercaptoethanol (Sigma), 1x Minimum essential amino acid (Invitrogen), 1x Penicillin/Steptomycin and 1000 IU/ml hLIF (Chemicon). Once the embryos were

attached, the mES medium was replaced for the continued culture of outgrowth. Blastomeres and embryos were cultured in air at 37°C with 5% CO_2.

3.3.6 Immunocytochemistry on pre-implantation embryos and embryo outgrowth.

The embryos were washed twice in PBS (-), fixed in 4% paraformaldehyde (PFA; Polyscience) for 30 minutes and incubated with a blocking buffer consisting of 0.2% triton-x 100 (Sigma), 3 mM sodium azide (sigma), 0.1% saponin (Sigma), 2% BSA (Sigma), and 5% house serum (Hyclone) in PBS (-) for one hour. Then the embryos were incubated at room temperature using the following primary antibodies: either mouse monoclonal antibody against human Oct-4 (1:250; Santa Cruz Biotechnolgy), rabbit polyclonal antibody against mouse and human Sox-2 (1:100; Stem Cell Technologies), or mouse monoclonal antibody against a full-length Cdx2 recombinant protein (1: 1000; BioGenex) for another two hours. The samples were then washed several times with PBS(-) before being incubated at room temperature with the following secondary antibodies: Alexa fluor® 488 donkey anti mouse IgG (1:1000; Molecular probes, Inc) and/or Rhodamine Red™- goat anti rabbit IgG (1:1000; Molecular probes, Inc) for one hour. Finally, the embryos were incubated with 5µg/mL Hoechst 33342 (Sigma) for five minutes before a thorough last wash with PBS(-). They were then mounted on slides with 10 µl of vectashield (Vector Lab) and examined with an Olympus BX51 epifluorescent microscope containing high numerical aperture objectives. The images were captured by an Orca ER G digital CCD camera (Hamamatsu, Inc) and analyzed using the MetaMorph software (Universal Imaging, Inc). An AXIOVERT 200M equipped with LSM 510 META

confocal unit (Zeiss, Inc) was used to perform the high resolution laser scanning confocal microscopy.

The same immunocytochemistry procedures noted above were performed on the embryo outgrowths with a minor change in the last step. After the embryo outgrowths were stained with 5μg/mL Hoechst 33342, fresh PBS(-) was added to cover the outgrowths before microscopic examination.

3.3.7 Blastocyst cell count

Embryos were counterstained with specific antibodies to distinguish the ICM and TE cells (Sox-2 and Cdx2, respectively). Immunocytochemistry was performed, as described in the previous section, except the rabbit polyclonal antibody against Sox-2 (1:100) and the mouse monoclonal antibody against a full-length Cdx2 recombinant protein (1: 1000) were co-labeled, and then detected by incubation with the Alexa fluor® 488 donkey anti mouse IgG (1:1000) and Rhodamine Red™ - goat anti rabbit IgG (1:1000). The Hoechst (nucleus, blue), Sox-2 positive (ICM cells, red), Cdx-2 positive (TE cells, green) cells were visualized and counted under fluorescence microscopy at different focal planes across the embryos.

3.3.8 Establishment of embryonic stem cells from single blastomere

The 2CBD and 4CBD blastomeres were cultured in KSOM until attaching onto the feeder cells when mES medium was replaced. Ten days after blastomere separation, the embryo outgrowth with prominent ICM was manually selected and subcultured onto freshly prepared MFF. Visible ES colonies were then selected for subculture based on cell morphology and maintained by standard methodology (Nagy

et al., 2003). Fresh mES medium was replaced every other day and passaged at two - three day intervals. Besides morphology, ES cell lines were characterized by the expression of stem cell markers, which composed of Oct-4 (1:250; Santa Cruz Biotechnology), Sox-2 (1:100; StemCell Technologies), Nanog (1:50; Santa Cruz Biotechnology), SSEA-1 (1:50; Chemicon), TRA-1-60 (1:100; Chemicon), TRA-1-81 (1:100; Chemicon) and Alkaline phosphatase activity (Vector lab).

3.3.9 *In vitro* differentiation of embryonic stem cells derived from single blastomeres

ES cells derived from the 2CBD embryo were cultured in suspension for 7 days for the formation of EBs. EBs were then allowed to attach on gelatin coated plate and cultured in N1 medium for 7 days, N2 medium for 14 days and N3 medium for 7 days. The N1 medium composed of DMEM/F12 (Invitrogen) supplemented with minimum essential amino acid, 200mM of L-glutamine and N2 supplement (Invitrogen). The N2 medium composed of N1 medium supplemented with 20 μg/mL basic fibroblast growth factor (bFGF). The N3 medium composed of DMEM/F12 supplemented with 1% FBS and B27 supplement (Invitrogen). EBs were stained with alpha-fetoprotein (AFP) and vimentin. Neuroprogenitor cells were stained with nestin, whereas successful differentiation of neuronal cell types was confirmed by the expression of neuron specific β-III tubulin (TuJ1), tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT)

3.3.10 Statistical analysis

Data analyses for differences in the embryonic development were carried out by

ANOVA in Statistical Analysis Systems (SAS, version 9.0, SAS Inc., Cary, NC, USA). For embryo development, the percentages of cleavage to blastocyst development were calculated by dividing those numbers with the total number of the cultured embryo and then analyzed by ANOVA in Statistical Analysis Systems.

3.4 Results

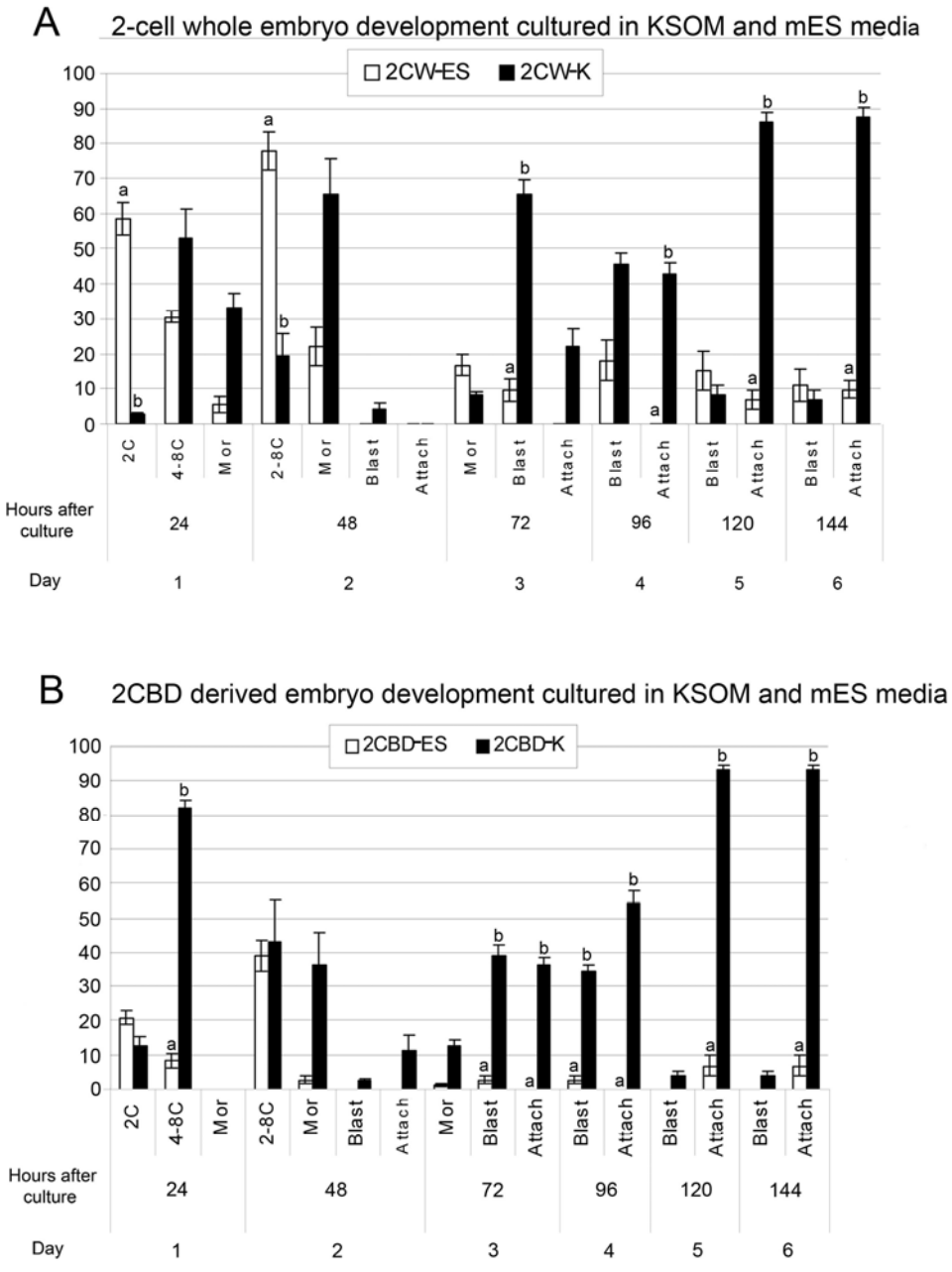
3.4.1 Development of two- and four-cell mouse embryo and 2/4CBD mouse embryo in KSOM and mES media

The dynamic change of nutrient requirement as embryo develops is one of the determining factors for the success of *in vitro* embryo culture in various species. The sequential culture of human embryos has resulted in optimal embryonic development *in vitro* and the birth of thousands of babies. Due to the developmental block of early embryos, it is important to provide an optimal nutrient supplement to advance through each embryonic stage. mES medium is optimized for ES cell cultures, whereas KSOM is one of the most commonly used medium for mouse embryo cultures with high developmental rate. However, mES medium has been reported that it can be used for culture the single blastomere and those blastomere could develop to be ES cell lines (Wakayama et al., 2007). In order to increase the chances for success in isolating ES cells from a single blastomere, an optimal culture scheme is essential for embryo development with enhanced ES cell population.

We first compared the developmental rate of the zona free 2CW and 4CW mouse embryos and the individual culture of the single blastomeres derived from a 2CBD and 4CBD mouse embryos in both the KSOM and mES media. The 2CW embryos had a normal developmental rate in the KSOM medium (2CW-K) with

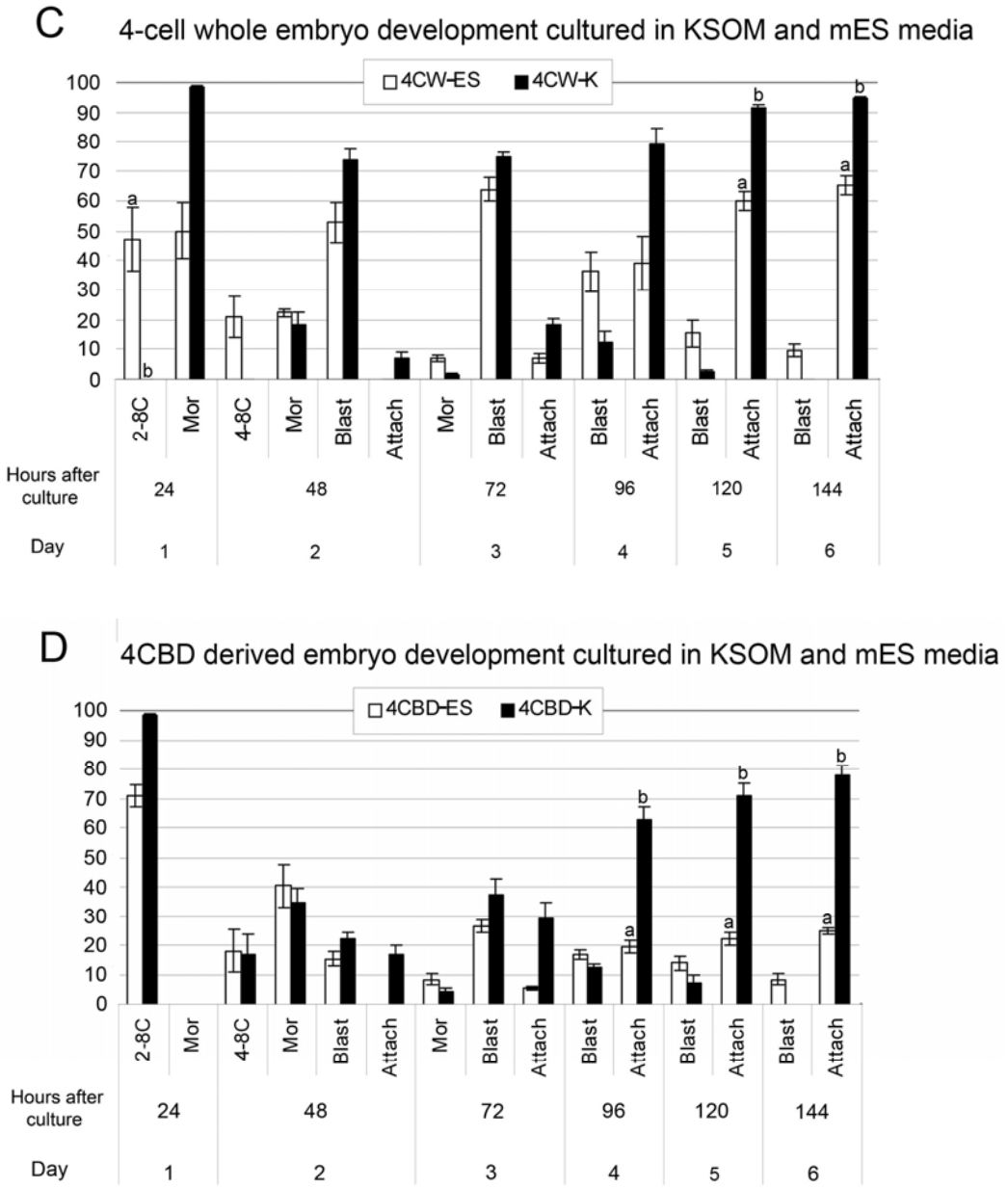
65.3% (47/72) of the 2CW-K embryos reaching the blastocyst stage on day 3.5 (Figure 3.1A), which was comparable with previously published data (Nagy et al., 2003). 2CW-K embryos began to attach onto the feeder 72 hours after *in vitro* culture and over 87.5% (63/72) were attached by day 6 (Fig 3.1A). Compared to KSOM, 58.3% (42/72) of the 2CW embryo cultured in the mES (2CW-ES) medium were arrested at the two-cell stage after 24 hours of culture (Figure 3.1A), only 22.2% (16/72) and 9.7% (7/72) developed to morula (day 2) and blastocyst stage (day 3) respectively, which was much lower than that of KSOM results (Fig 3.1A; $P<0.05$). These results suggest KSOM is better suited to support the development of zona-free two-cell mouse embryo through the two-cell block.

When 2CBD KSOM (2CBD-K) embryos were cultured individually, more than 81.9% (59/72) of the 2CBD-K blastomeres passing the two-cell block (Fig 3.1B). 2CBD-K blastomeres developed to morula and blastocyst stage at high rate, and over 90% (67/72) of the blastocyst attached onto feeder by day 5 (Fig 3.1B). On the other hand, only 2.8% (2/72) of the 2CBD-ES blastomere cultures reached the morula stage and 6.9% (5/72) of the blastocyst attached onto the feeder cells on day 5. This was much lower than that of the 2CBD-K (Fig 3.1B; $P<0.05$).



K = KSOM; ES = mouse ES medium; Number of blastomere in culture = 72 blastomeres

Figure 3.1 Development of 2CW (3.1A) and 2CBD embryos (3.1B) after cultured in ES and KSOM medium for 6 days.



K = KSOM; ES = mouse ES medium; Number of blastomere in culture = 72 blastomeres

Figure 3.1 Development of 4CW (3.1C) and 4CBD embryos (3.1D) after cultured in ES and KSOM medium for 6 days.

Although both of the 2CBD blastomere derived embryos formed blastocysts and outgrowths when cultured in KSOM, 25.0% (9/36) of those blastocysts and outgrowths had one with a prominent and visible ICM clump (Fig 3.2A; Table 3.1), and 61.1% (22/36) had a visible ICM in both the blastomere derived blastocysts and outgrowths (Fig 3.3). However, the 2CBD-ES derived embryos were not able to survive and form outgrowth. Immunocytochemistry demonstrated that more than 88% (16/18; Fig 3.10) of the 2CBD derived blastocysts had at least one Sox-2 positive cell; however, most of the blastocysts had a small number of positive cells with relatively low intensity. Our results suggested that the development of the 2CW and 2CBD embryos was affected by culture media, especially when the development was advancing through the maternal embryonic transition at the two-cell stage.

In addition to two-cell embryos, 4CW and 4CBD mouse embryos were also used for determining the effect of culture media on early embryonic development (Fig 3.1C). The overall developmental rate in 4CW-K embryos was much higher than those cultured in the mES medium (Fig 3.1C). Besides having a low development rate, it was found that 4CW-ES derived embryos had a low attachment rate compared to that of 4CW-K derived embryos (Fig 3.1C; $P<0.05$). In the 4CBD blastomere culture, individually cultured blastomeres were able to divide at least one-to-three times within a 24 hour period in the KSOM and mES media. It was also found that the 4CBD-K had a much higher attachment rate compared to the 4CBD-ES on day four, five, and six (Fig 3.1D; $P<0.05$).

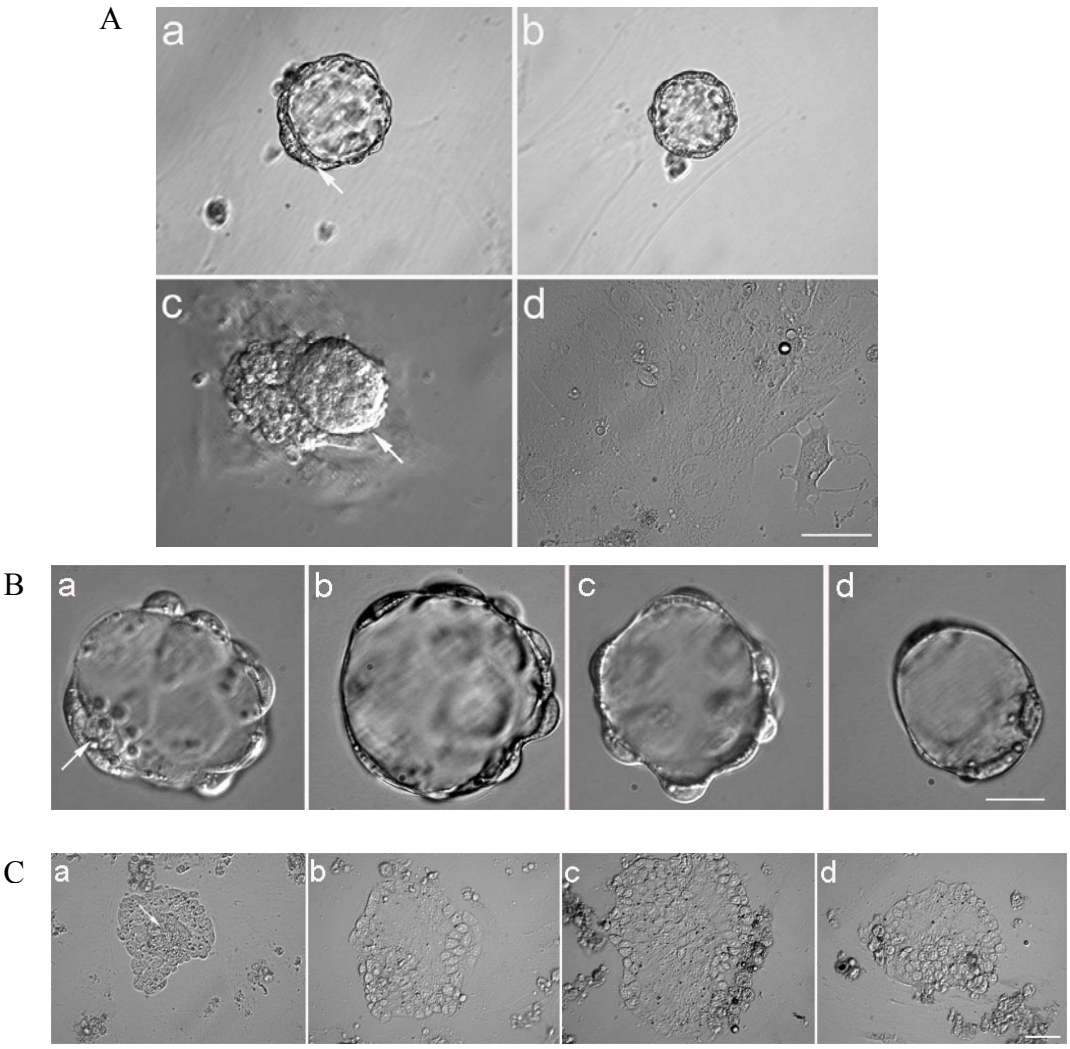


Figure 3.2 Blastocysts and outgrowths derived from 2CBD and 4CBD blastomeres. **(3.2A):** 2CBD blastomere derived blastocysts (a and b) and embryonic outgrowths c and d). Visible ICM could be observed in one of the embryonic outgrowth after attached onto feeder cells, whereas the other outgrowth developed to TE. Arrow: ICM; Bar = 50 µm. **(3.2B)** 4CBD blastomere derived blastocysts in which only one blastomere (a) has visible ICM, and no visible ICM was observed in sister blastomere (b-d) derived blastocysts. Arrow: ICM; Bar = 30 µm. **(3.2C)** 4CBD derived embryo outgrowths. There is only one outgrowth that could form a visible ICM clump (a; arrow) while the others could attach onto the feeder but do not have ICM clump (b-d). Bar = 50 µm.

This result indicated the culture medium may have had less effect on the 4CW or 4CBD derived embryos compared to the 2C embryos. Although the developmental rate was not affected when cultured in either media, the attachment rate was greatly enhanced in the 4CBD-K derived blastocysts (Fig 3.1D). Even most of the 4CBD derived blastocysts were able to attach and form outgrowths, only 44.4% (8/18), 22.2% (4/18) and 5.5% (1/18) of the 4CBD derived embryos had one, two or three of the four sister outgrowths form visible ICM respectively (Figs. 3.2B and 3.2C; Table 3.2).

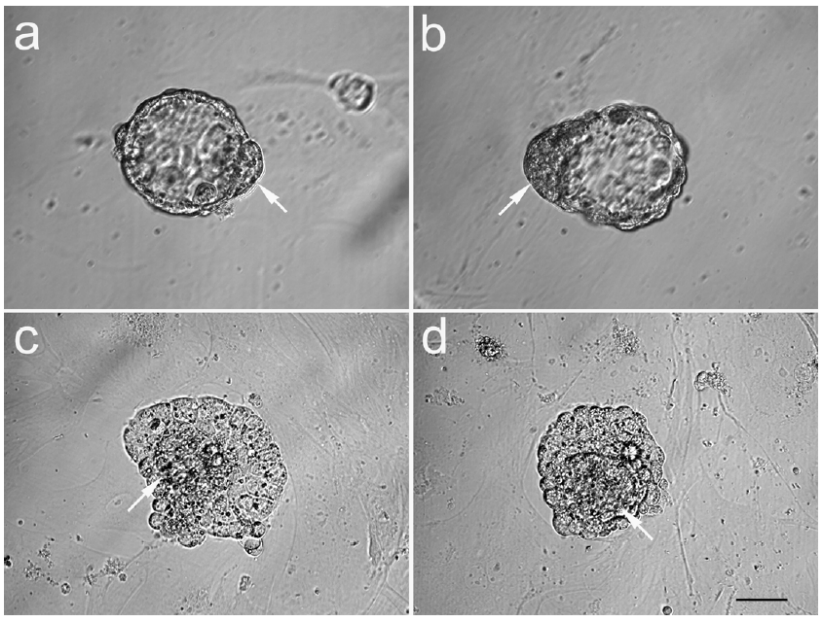


Figure 3.3 2CBD blastomere derived blastocysts (a and b) and embryonic outgrowths (c and d). Visible ICM could be observed in embryonic outgrowth after attached onto feeder cells. Arrow: ICM; Bar = 50 μm.

Table 3.1 Colony formation rate of 2CBD derived embryos cultured in two different media for 6 days

Btm* derived from	Media	Rep	No. emb	ICM appearance (D6)		No colony on D6 (%)	One/two btm arrest (%)
				One btm* (%)	Both btm* (%)		
2C stage embryo	mES	3	36	0 (0.0)	0 (0.0)	0 (0.0)	36 (100.0)
	KSOM	3	36	9 (25.0)	22 (61.1)	3 (8.3)	2 (5.5)

*btm = blastomere ; mES = mouse ES media

Table 3.2 Colony formation rate of 4CBD derived embryo cultured in two different media for 6 days

Btm* derived from	**Media**	**Rep**	**No. emb**	**ICM appearance (D6)**				**1-4 btm* arrest**
				1 btm* (%)	**2 btm* (%)**	**3 btm* (%)**	**4 btm* (%)**	
4C stage embryo	mES	3	18	3 (16.7)	1 (5.5)	0 (0.0)	0 (0.0)	14 (77.7)
	KSOM	3	18	8 (44.4)	4 (22.2)	1 (5.5)	1 (5.5)	4 (22.2)

*btm = blastomere; mES = mouse ES media

In addition to the effect of a culture media on the developmental rate, the size of embryos was also affected. Blastocysts derived from the 2/4CW-K embryos were bigger with more prominent and distinctive ICM and TE cells than those 2/4CW-ES (Fig. 3.4). In the blastomere break-down study, the sizes of the 2CBD and 4CBD blastomere derived blastocysts are about half and one quarter of a normal embryo (Fig. 3.5). Although the sizes of the bisected embryos were reduced, the timing of compaction and the formation of the blastocoel cavity remained tightly regulated by the biological clock of an early embryo.

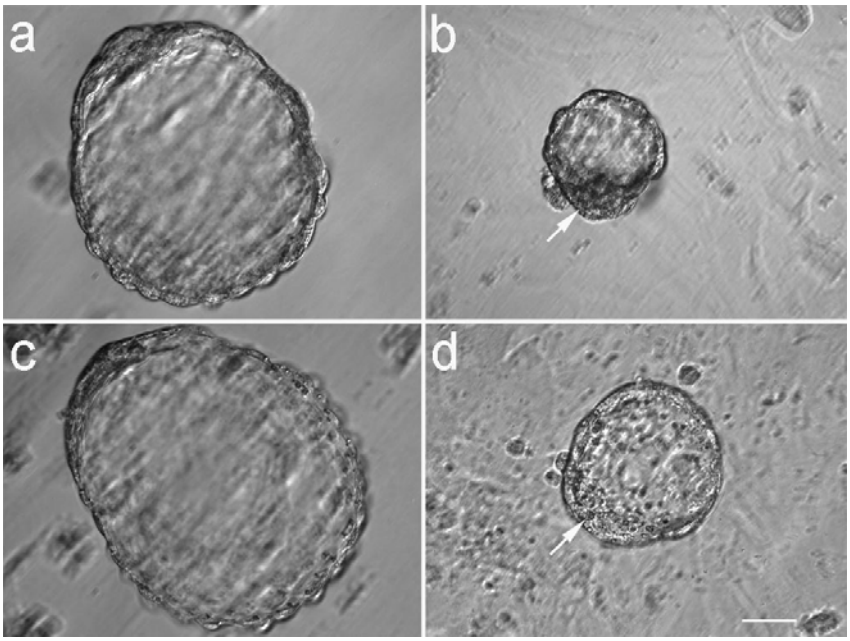


Figure 3.4 Blastocysts of 2CW embryos cultured in KSOM (a) and mES (b) medium, and 4CW embryos cultured in KSOM (c) and mES (d) media. Arrow: ICM of embryo cultured in mES. Bar = 50μm.

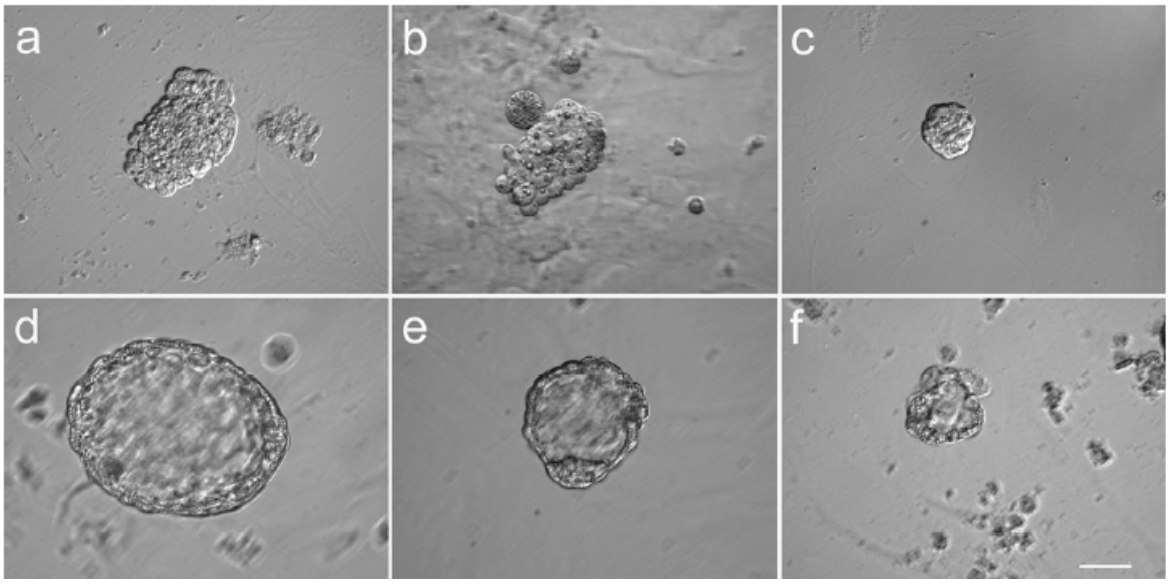


Figure 3.5 Morula (Day 2) and blastocyst (Day 3) derived from zona–free whole embryos (a and d), 2CBD (b and e) and 4CBD (c and f) derived embryos. a-c: morulae; d-f: blastocyst. Bar = 100 µm.

3.4.2 Expression of stem cell markers in embryos cultured in the KSOM and mES media

The effect of culture media on embryo development and morphology was clearly demonstrated in the previous studies. However, the effect of culture media on the expression of stem cell markers such as Oct-4 and Sox-2 has not yet been determined. The fact that early embryos cultured in a mES medium were smaller in size and had a less prominent blastocoel cavity, compared to those cultured in KSOM, these suggest the changes in gene expression pattern resulting in a different developmental rate. Although reduced number of TE cells was found in blastocyst derived in mES medium (Fig 3.4), the expression pattern of Oct-4 and Sox-2 was not different from those cultured in KSOM and was primarily expressed in the ICM cells with very low levels in the TE cells (Fig. 3.6). Our results indicated that mES medium

affects early embryonic development with no effect on the expression of Oct-4 and Sox-2.

Based on these studies of the effect of embryo development, morphology and pluripotent gene expression patterns in different culture media, instead of enhancing the expression of pluripotent genes, the mES medium delayed and reduced the development rate of those embryos with a less prominent morphology in both whole and blastomere break down embryos. Thus, a sequential culture scheme was used in the rest of the studies by having the embryo cultured in the KSOM medium until the attachment onto feeder cells and then the mES medium was replaced.

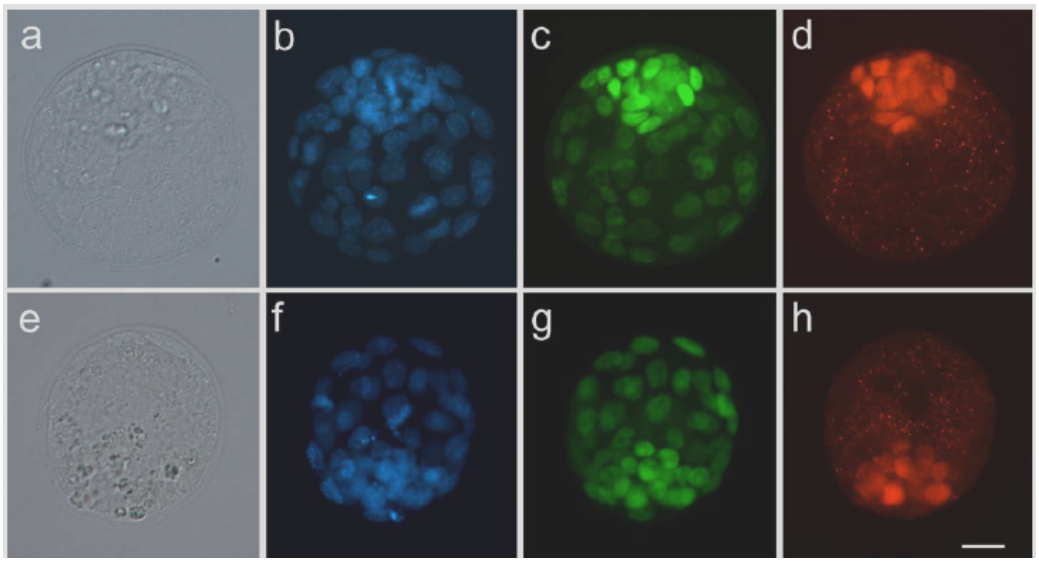


Figure 3.6 Expression of stem cell markers in blastocysts cultured in KSOM and mES media. Blastocysts derived from the whole embryos cultured in KSOM (a-d) and mES (e-h) media have similar expression pattern of Oct-4 (c,g; green) and Sox-2 (d,h; red), Expression of Oct-4 and Sox-2 is co-localized at the ICM cells, whereas low level expression of Oct-4 was also observed in trophoblasts.

It has been demonstrated that the expression of pluripotent genes such as Oct-4 and Sox-2 is crucial for the development and maintenance of pluripotency in ICM

and for the development of embryo proper. It has also been demonstrated that Cdx2 is essential for TE function and the establishment of trophoblast lineage. A balanced reciprocal expression between these two sets of markers is important for normal embryo development. We have demonstrated that the expression of Oct-4 and Sox-2 were not affected by culture media, and it should also be noted that the spatial expression pattern of the pluripotent genes and Cdx2 in blastomere break down derived embryos has not yet been determined. We had chosen Sox-2 to represent the pluripotent genes in the following experiments because it provided a more specific expression pattern and it was tightly restricted in ICM cells; whereas the expression of Oct-4 was primarily in the ICM with a low level expression in the TE cells (Fig. 3.6).

The 2CBD and 4CBD derived embryos at four days after culture were used for determining the ratio of TE cells *vs* ICM cells. Immunocytochemistry was performed using Sox-2 and Cdx2 specific antibodies and the number of positive cells were counted under a fluorescent microscope (Figs. 3.7A and 3.7B). The normal embryos were used as a control. A total of 18, 20 and 20 embryos derived from blastomeres of the 2CBD (9 x 2 blastomeres = 18), 4CBD (5 x 4 blastomeres = 20), and control (20 embryos) were used respectively. The total number of cells in the 2CBD (28.1 ± 2.9 cells) and 4CBD (13.9 ± 1 cells) derived blastocysts were reduced at approximately 55% and 78% of a normal embryo (62.8 ± 2.5 cells) (Fig. 3.9; Table 3.3). A similar ratio was also observed in the Cdx2 positive cells (TE) and the Sox-2 positive cells (ICM) while the overall ratio of the Cdx2 and Sox-2 remained the same in the 2CBD, 4CBD and normal embryos (Fig. 3.9; Table 3.3). This finding was consistent with our previous studies on embryo morphology showing that the reduced size of the 2CBD and 4CBD derived embryos was the result of the reduced cell numbers. Although the

ratio of the total cell numbers between the groups and the ratio of Sox-2 vs Cdx2 remain unchanged, there were only 88.1% (16/18) of the 2CBD and 80% (16/20) of the 4CBD blastomeres having a visible ICM or being positive for Sox-2; whereas all blastocysts from the normal embryos had a visible ICM (Fig. 3.10). Based on our findings in the immunocytochemistry studies, it was not surprising that some of the 2CBD and 4CBD derived blastocysts had a visible ICM. Although some blastocysts contained a small number of Sox-2 positive cells, the low cell numbers may not be sufficient to form a visible ICM or be competent for establishing ES cells.

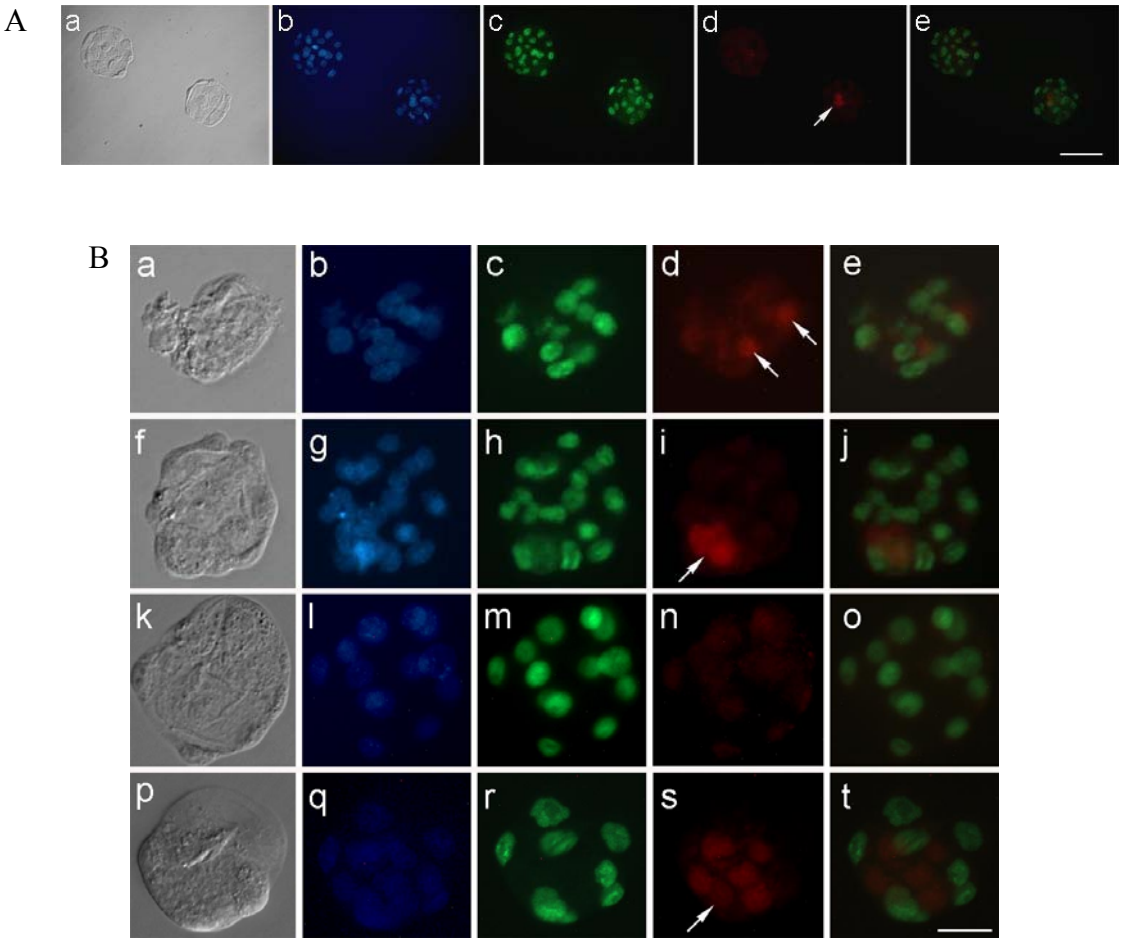


Figure 3.7 The expression of stem cell (Sox-2) and trophectoderm (TE; Cdx2) markers in blastocysts and outgrowths derived from 2CBD and 4CBD blastomere derived embryos. **(3.7A)** Blastocysts derived from two sister blastomeres of a 2CBD embryo. Transmission light image (a); Hoechst DNA staining (b; blue); Cdx2 (c; green); Sox-2 (d; red. Arrow: Sox-2 positive cells. Bar = 100 µm. **(3.7B)** Blastocysts derived from four sister blastomers of a 4CBD embryo. (a, f, k, p); Hoechst (b, g, l, q; blue); Cdx2 (c,h,m,r; green); Sox-2 (d, i, n, s; red) and overlay images of Cdx2 and Sox-2 (e, j, o, t). Arrow: Sox-2 positive cells. Bar = 30 µm.

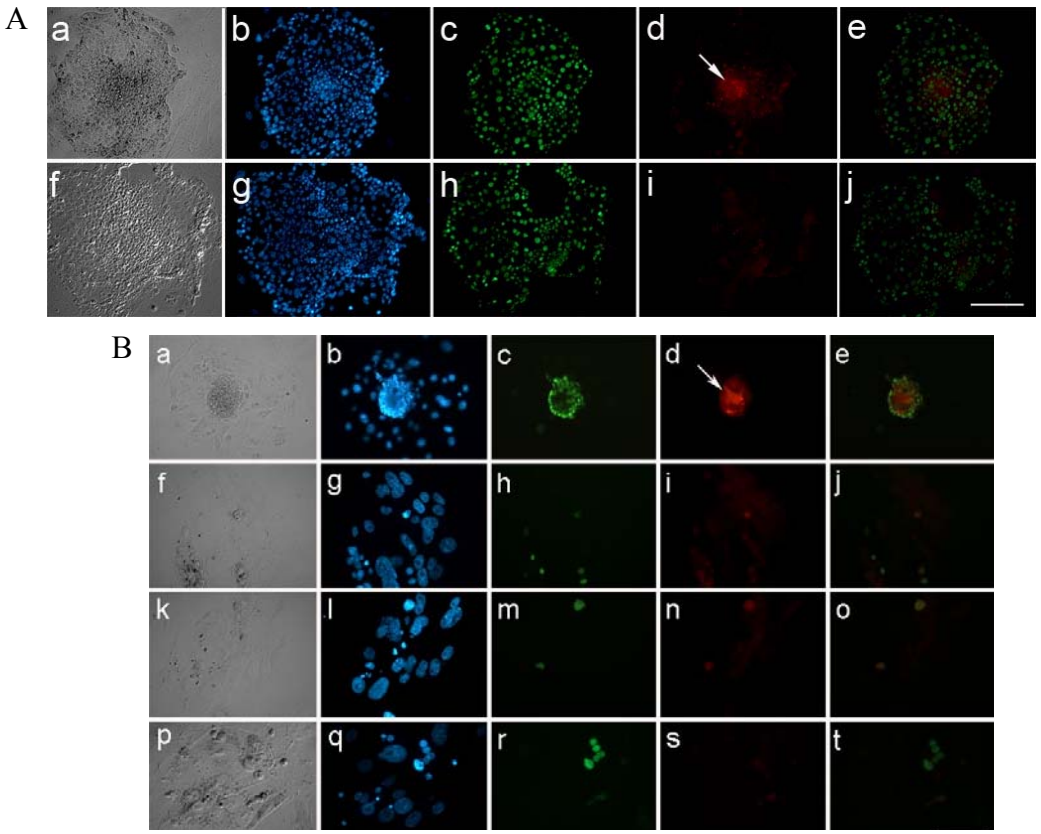


Figure 3.8 Embryonic outgrowths derived from sister blastomeres of a 2/4CBD embryo. **(3.8A)** The first outgrowth (a-e) was derived from 2CBD blastocyst with visible ICM, whereas the second outgrowth (f-j) without visible ICM. Transmission light image (a, f); Hoechst DNA staining (b,g; blue); Cdx2 (c,h; green); Sox-2 (d,i; red), and overlay image of Cdx2 and Sox-2 (e, j). **(3.8B)** Embryonic outgrowths derived from four sister blastomeres of a 4CBD embryo. The first (a-e), second (f-j), third (k-o) and fourth outgrowth (p-t) were cultured individually and had only one outgrowth with visible ICM and Sox-2 positive cells with intense signal. There are cell negative for both Cdx2 and Sox-2 suggesting spontaneous differentiation beyond the TE lineage. Transmission light image (a, f, k, p); Hoechst DNA staining (b, g, l, q); Cdx2 (c, h, m, r); Sox-2 (d, i, n, s), and overlay of Cdx2 and Sox-2 (e, j, o, t). Arrow: ICM. Bar = 100 µm.

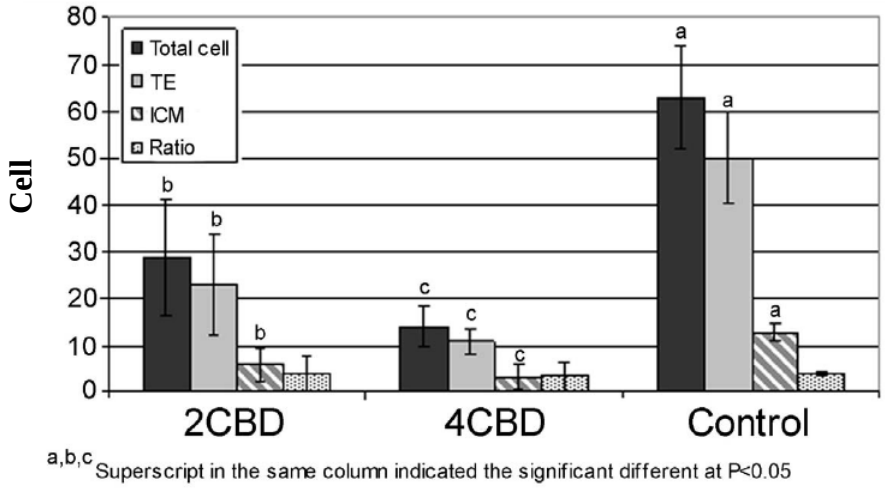


Figure 3.9 Comparison of total cell number, Cdx2 (TE) and Sox-2 (ICM) positive cells between the 2/4CBD derived embryo and normal embryo. Total cell number, Cdx2 and Sox-2 positive cells and Cdx2 : Sox-2 ratio of the 2/4CBD derived embryos and normal embryos represented by a histogram.

In addition to immunocytochemistry on the 2CBD and 4CBD derived blastocysts, we determined the expression patterns of stem cells (Sox-2) and TE (Cdx2) markers in the embryo outgrowth (Figs 3.8A and 3.8B). We aimed to determine whether each blastomere had equally competent for deriving ES cells based on the expression of stem cell markers. Our results in the 2CBD and 4CBD blastomere derived embryonic outgrowth were consistent to the blastocysts. The blastocysts derived from the 2CBD blastomeres attached and developed to the embryonic outgrowth regardless of the presence of ICM.

Table 3.3 The Cdx-2 and Sox-2 positive cells of the blastomere break down derived embryos.

Embryo	**No.**	**Total cell no.**	**Cdx-2 positive (Mean ± SEM)**	**Sox-2 positive (Mean ± SEM)**	**Ratio (Mean ± SEM)**
2CBD	18	28.1 ± 2.9[b]	23 ± 2.5[b]	5.8 ± 3.5 [b]	3.9 ± 0.8 : 1
4CBD	20	13.9 ± 1.0[c]	10.7 ± 0.6[c]	3.2 ± 0.6 [c]	3.0 ± 0.7 : 1
Normal embryo	20	62.8 ± 2.5[a]	50.0 ± 2.2[a]	12.7 ± 0.4 [a]	3.9 ± 0.1 : 1

[a,b,c] superscript in the same column indicated the significant different at $P<0.05$

However, only those with a visible ICM in a blastocyst formed ICM in an outgrowth. It was found that there were two types of embryonic outgrowth. The first type contained both ICM and differentiated cell types such as TE cells. The second type contained only the differentiated cell types from the trophoblastic cell lineage. A slight difference in the differentiation patterns was found in the 4CBD embryonic outgrowth. Besides the Sox-2 and Cdx2 positive cells, there were cell types with a distinctive morphology that were not recognized by the antibodies against Sox-2 and Cdx2. This suggested a spontaneous differentiation toward more advanced cell types besides the TE lineage occurring in the outgrowth (Fig 3.8B). In general, the ICM clump expressed Sox-2 distinctively, whereas the region of differentiated cells contained some positive Cdx2 cells and some other cell types with a distinctive morphology, but negative for Cdx2.

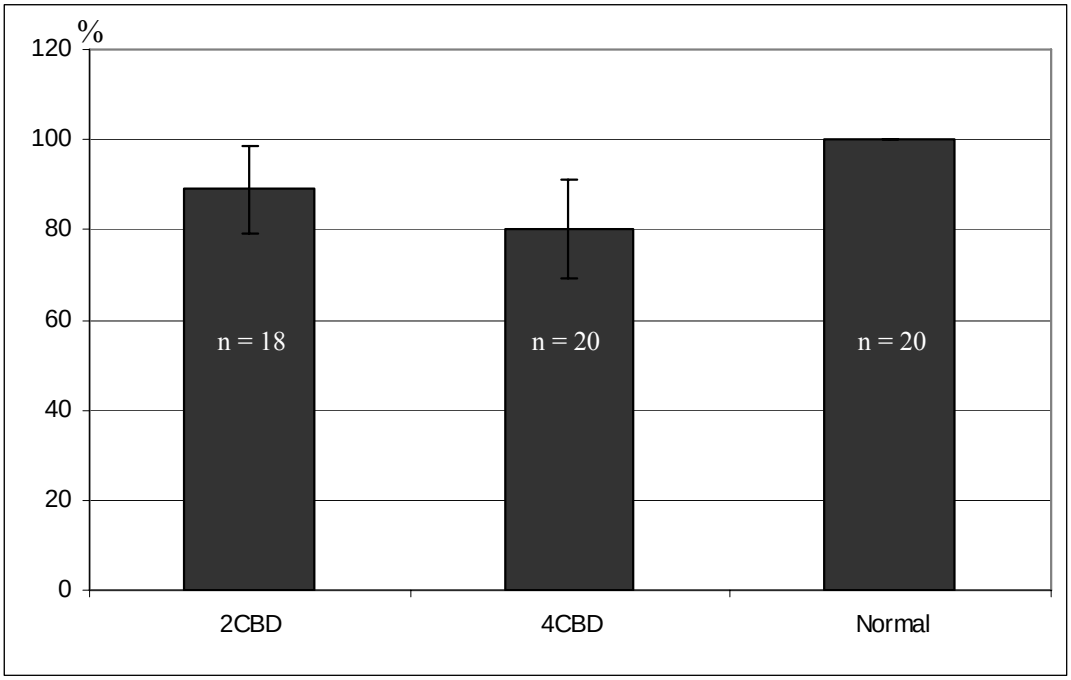


Figure 3.10 The percentage of ICM appearance (Sox-2 positive cells) in the blastomere break down derived

3.4.4 ES cell derivation from single blastomere

We have demonstrated that a single blastomere can be continually cultured and developed *in vitro*. Although an immunocytochemistry study on embryonic outgrowths derived from 2CBD and 4CBD blastomeres suggested the unequal formation of ICM, the biased expressions of stem cell determinant genes in embryonic outgrowth derived from sister blastomeres and the competence of the ICM of sister embryonic outgrowths, whether they share the same pluripotency as those derived from normal blastocyst has not yet been determined. Moreover, the validation of our single blastomere culture system by

establishing ES cell lines is an important step towards affirming our findings on the differential competence of early sister embryonic blastomeres.

The 2CBD and 4CBD blastomeres were individually cultured in the KSOM medium until the resulting blastocysts attached onto the feeder cells. At that point the KSOM was replaced by the mES medium. Ten days after the *in vitro* culture, any visible ICM was mechanically isolated and trypsinized. The ES cell colonies were then observed for about two-three days after being sub-cultured and were considered as passage one (P1). The established ES cell lines were then sub-cultured every two-three days for further characterization and analyses. As a result, we established four ES cell lines from the 2CBD derived blastomere (5.6%, 4/72; Table 3.4), while no ES cell lines could be established from the 4CBD (0%, 0/72; Table 3.4) derived blastomeres. There was no ES cell lines established from the sister outgrowth that derived from the 2CBD derived embryo.

Table 3.4 Efficiency of ES cell derivation from 2CBD and 4CBD derived embryos.

Embryo stage	**No. Embryo**	**No. Blastomere**	**No. ICM Sub-cultured (%)***	**No. ES cell line Day 2 (%)***	**No. ES cell line P.6 (%)***
2CBD	36	72	30 (41.7)	8 (11.1)	4 (5.6)
4CBD	18	72	8 (11.1)	0 (0.0)	0 (0.0)

* The percentage was calculated from number of blastomere.

These results suggested that only one blastomere of the 2CBD derived embryos was capable of retaining its stem cell properties and then deriving ES cells,

while the sister outgrowth was differentiated toward the TE lineage or other cell types. The stem cell properties of the resulting ES cell lines were confirmed by the expression of stem cell markers commonly used in mES cells, which included AP, Oct-4, Sox-2, SSEA-1, Nanog, TRA-1-60 and TRA-1-81 (Fig 3.11). In order to determine the pluripotency of the ES cell lines derived from 2CBD blastomeres, *in vitro* differentiation was performed followed by immunostaining using endoderm (α-fetoprotein) and ectoderm (vimentin) specific antibodies on embryoid bodies (EBs), and antibodies specifically recognized neuroprogenitor cells (nestin), and neuronal cell types (β-III tubulin, TuJ1; tyrosine hydroxylase, TH; choline acetyltransferase, ChAT). The differentiation results have been demonstrated that ES cell derived from single blastomere could differentiate after *in vitro* differentiation to be neuron and show positive to several neuron markers (Figure 3.12). Here we demonstrated the successful establishment of the ES cells from a single blastomere derived embryo, outgrowth and ICM. This validated our single blastomere culture system and confirmed that the pluripotency of ES cell lines derived from the 2CBD of outbred CD-1 mice could be maintained.

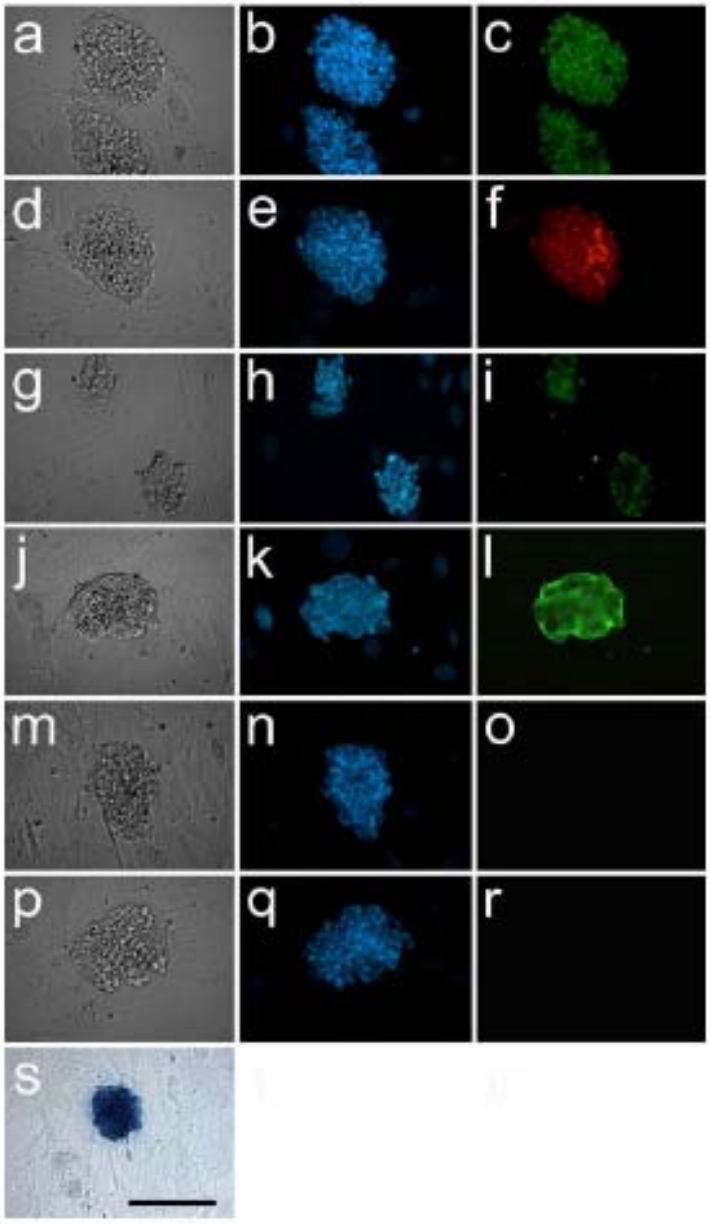


Figure 3.11 ES cell markers expression of the 2CBD derived ES cell line. The ES cell properties was confirmed by the expression of mouse specific stem cell markers (Oct-4, Sox-2, Nanog, SSEA-1) and human ES cell specific marker (TRA-1-60 and TRA-1-81) as negative control. Oct-4 (c), Sox-2 (f), Nanog (i), SSEA-1(l), TRA-1-60 (o) and TRA-1-81 (r) and Alkaline phosphatase (s). Transmission light images (a, d, g, j, m, p, s). Hoechst DNA staining (b, e, h, k, n, q; Blue). Bar = 100 μm.

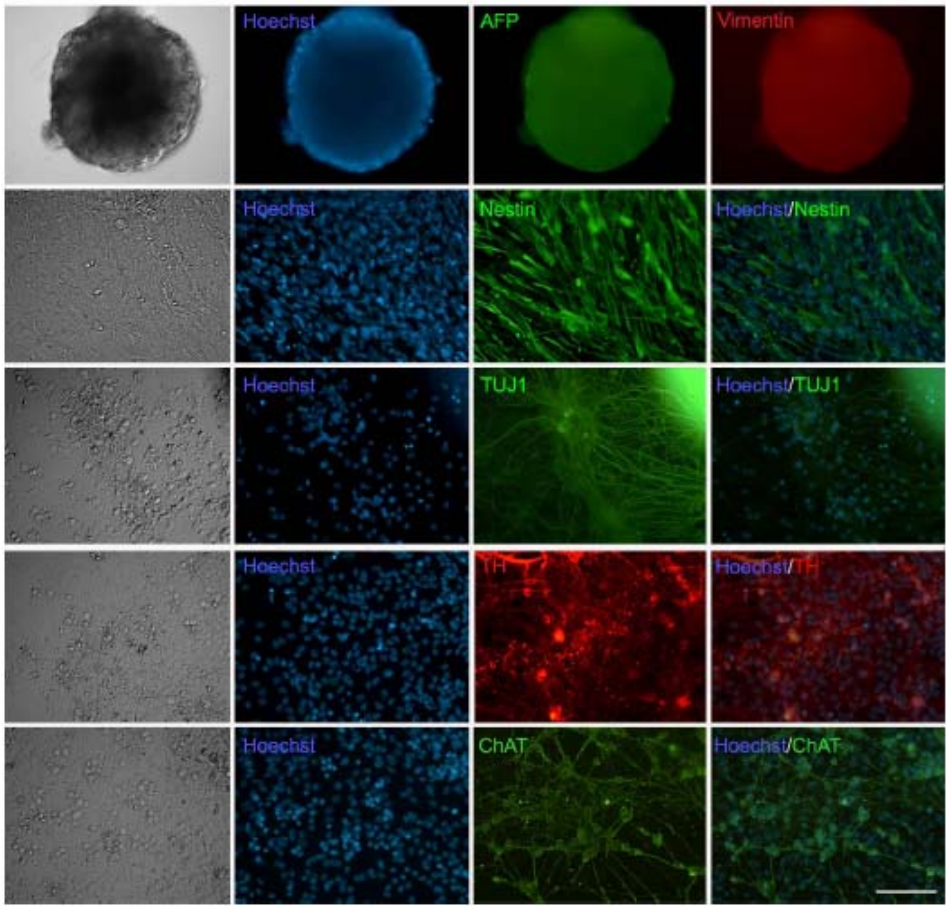


Figure 3.12 *In vitro* differentiation of ES cells derived from 2CBD derived mouse embryos. Each row represents the same sample. The first row is embryoid body (EB; endoderm: α-fetaprotein; ectoderm: vimentin), the second row is ES cells derived progenitor cells (PGC; Nestin) in N1medium and the 3rd-5th rows are ES cells derived neuronal cell types (β-III tubulin, TuJ1; tyrosine hydroxylase, TH; choline acetyltransferase, ChAT) in N3 medium. The first column from the left is the transmission light image, the second column is DNA (blue) stained with Hoechst, the third column is immunostaining using specific antibodies (green and red) and the fourth column is the overlay images of the columns 2 and 3 except for EB, which was stained with vimentin. Bar = 100 μm.

3.5 Discussion

The objective of this study was to determine if the blastomeres of a two- and four-cell mouse embryo are equally competent in developing to blastocyst and the derivation of ES cells. The expression of stem cell (Sox-2) and TE (Cdx2) markers in blastomere breakdown derived embryos and outgrowths were compared with normal mouse embryos in order to determine their lineage preference. We first developed an optimal culture method that could enhance single blastomere development and the establishment of embryonic outgrowth. We had adopted the co-culture system of mouse ES cells and modified with sequential medium. A much higher blastocyst rate ($P<0.05$) resulted when 2CBD and 4CBD blastomeres were cultured individually with MFF in KSOM medium than those cultured in mES medium (Fig 3.1B and 3.1D). This suggested that KSOM was sufficient to support the development of a single blastomere derived from a two-cell mouse embryo, overcome the two-cell block, and developed to blastocyst at a comparable rate to that of whole embryo culture (Fig 3.1A). A similar result was also observed in 4CBD blastomere culture (Fig 3.1C and 3.1D). When single blastomere derived embryos attached onto the MFF feeder cells, KSOM medium was replaced by mES medium in order to enhance ICM formation for later derivation of ES cells. While most of the embryos were developed to blastocyst and attached onto feeder cell in KSOM medium, nearly all of the blastocyst established embryonic outgrowth. Continuous culture of the embryonic outgrowth in mES medium has resulted in the establishment of four mES cell lines, which were derived from a single blastomere of four two-cell embryos. All mES cell lines expressed common stem cell markers for mES cells but not those specifically for human ES cells (Fig 3.11). We have developed an efficient method for culturing a

single blastomere, the establishment of embryonic outgrowth, and the derivation of ES cells. Our results clearly showed that KSOM was superior to mES medium in supporting single blastomere development to blastocyst although a previous study suggested that mES medium was sufficient for continue culture of single blastomere and the isolation of ES cells (Wakayama et al., 2007). The discrepancy between the two studies could be explained by the mouse strains that were used for preparing embryos. Several reports have demonstrated the importance of mouse genetic background on early embryonic development (Bagis et al., 2003) and the establishment of ES cells (Kawase et al., 1994). Most ES cell lines including the single blastomere derived ES cells described by Wakayama and colleagues were derived from the 129/SV strain (Sukoyan et al., 2002; Tesar, 2005; Wakayama et al., 2007) which has been shown to be the best mouse strain for establishing an ES cell line.

In this study we used an outbred CD-1 stock instead of commonly used 129/SV, which may have resulted in variation responding to the culture medium of choice and later establishment of ES cells (Figure 1; Table 3). Although CD-1 stock has been used for mES cell establishment in several attempts (Suzuki et al., 1999; Brook et al., 2003), no mES cell line has yet been reported in the CD-1 strain. Wakayama and colleagues (2005) had attempted to use somatic cells from a male and female CD -1 mouse as a donor cell for nuclear transfer (NT) and the establishment of ES cell from the reconstructed blastocyst (Wakayama et al., 2005). A much lower morulae/blastocyst rate was occurred, compared to B6D2 mice (10.4% for male donor cell and 4.4% for female donor cell *vs* 47.4%, respectively). Although no live offspring resulted from the cloned embryos, they were successful in establishing CD-

1 ES cells from cloned embryos at a relatively low rate, 9% for the male donor cell and 23% for the female donor cell. These studies suggested that ES cell could be established from an outbred strain at a much lower efficiency compared to that of an inbred strain, which explains our low establishment rate when deriving ES cells from 2CBD blastomere in CD-1. Furthermore, despite the influence of genetic background, the epigenetic differences between genders may also affect ES cell establishment rate in an outbred strain such as CD-1 (Wakayama et al., 2005).

We have also demonstrated that not all sister blastomeres from 2CBD and 4CBD embryos formed the visible ICM in embryonic outgrowth (Figures 3.2A-3.2C). The immunocytochemistry study revealed a unique expression pattern of stem cell and TE markers in embryos and outgrowths derived from a single blastomere. Only some but not all 2CBD and 4CBD blastomere derived embryos expressed Sox-2 (Figures 3.7A and 3.7B). It is not surprising because there were only a few reports of live offspring derived from a set of bisect/split embryos, which was even more unusual in an advanced stage embryo. Although the production of monozygotic twins by the separation of a two-cell embryos has been reported by several groups in various species (Ozil et al., 1982; Papaioannou et al., 1989; Matsumoto et al., 1989; Tsunoda et al., 1984; Allen and Rashen, 1984; Willadsen and Polge, 1981; Willadsen et al., 1981), there was only one report on the production of a complete set of quadruplet calves (Johnson et al., 1995). In most cases, live offspring from a full set of bisected embryos have not resulted (Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Chan et al., 2000). Instead of embryo splitting, Kwon and Kono (1996) demonstrated that a full set of offspring could be produced from a four-cell mouse embryo by nuclear transfer (Kwon and Kono, 1996). This study suggested that four-

cell blastomeres are competent for reprogramming and capable to regain pluripotency through nuclear transplantation. This study also supported the notion that sister blastomeres may be capable of complementing the loss or damaged blastomeres of an embryo. Nonetheless, a very limited number of successful cases in producing a full set of identical offspring by either embryo splitting or nuclear transplantation were reported (Johnson et al., 1995; Kwon and Kono 1996). Our results supported the idea that not all sister blastomeres resulted in live offspring, because only some of the blastomere derived embryos and outgrowths homogenously expressed a high level of stem cell markers capable of forming ICM.

We have observed that the total cell number of 2CBD and 4CBD derived embryos were reduced by approximately 50% and 75% when compared to the whole embryo (Table 3.3), however, the ICM:TE ratio remained unchange. Our finding is consistent with previous studies in bisected mouse embryo (Wang et al., 1990) and non human primate embryo (Chan et al., 2000; Mitalipov et al., 2002). Even the cell numbers of a blastomere break down derived embryo were less than the whole embryo, yet the timing when morula, compaction and the formation of blastocyst occurred was comparable between break-down and whole embryos. This result suggested that whole embryos shared the same biological clock with blastomere break-down derived embryos regardless of the cell number, thus the developmental sequence of embryonic blastomeres could be committed prior to blastomere separation. In addition to the decrease of cell numbers in blastomere derived embryos, Tsunoda and McLaren (1983) reported that 18 d.p.c. mouse fetal weight was significantly lower than those derived from normal embryos (Tsunoda and McLaren, 1983). Altogether these studies demonstrated the profound effect of developmental

commitment which is established in early embryos on subsequent embryonic and fetal development.

The development of a personalized ES cell line for cell therapy has been considered as a potential cure for various diseases including neurodegenerative diseases and diabetes. The idea of establishing personalized ES cells was aggressively pursued after the birth of Dolly, a sheep created by nuclear transplantation (Wilmut, et al., 1997). Rideout III and colleagues (2002) then demonstrated the possibility of producing cloned mouse embryos using somatic cells and the creation of personalized mouse ES cells followed by genetic modification which were transplanted into the genetically defected somatic cell donor. This resulted in significant improvement to its clinical symptoms (Rideout III et al., 2002). In theory, somatic cell cloning (or therapeutic cloning) and the creation of personalized ES cells from a cloned embryo is an ideal approach for the production of unlimited matched cell types for cell therapy; however, the advancement in clinical application has been limited by the low efficiency in nuclear transplantation and the ethical concern of therapeutic cloning.

Recent success in establishing mouse and human ES cell lines from a single blastomere of preimplantation embryos has led to a new development in stem cell technology (Wakayama et al., 2007; Chung et al., 2006; Klimanskaya et al., 2006). This breakthrough demonstrates the possibility of personalized ES cells that would eliminate the ethical concerns surrounding therapeutic cloning. Although it was only applied to *in vitro* derived embryos, ES cells derived from single blastomeres were first demonstrated in blastomere biopsies of mouse embryos as the embryos continued to develop to term (Wakayama et al., 2007). The feasibility of deriving ES cells from human blastomeres was also demonstrated using the similar isolation method

(Klimanskaya et al., 2006). Even though the embryo from a blastomere biopsy for deriving ES cells has not been used for embryo transfer and live offspring (Klimanskaya et al., 2006), the procedure is commonly used in fertility clinics for preimplantation genetic diagnosis (PGD) and is considered a safe and practical method for blastomere retrieval. As a result, there was a strong interest in single blastomere derived ES cells for clinical application of deriving personalized human ES cells (Wakayama et al., 2007; Chung et al., 2006; Klimanskaya et al., 2006). However, a relatively low success rate in establishing ES cells from a single biopsy blastomere and the unsuccessful derivation of ES cell lines from all sister blastomeres has raised the question of whether all sister blastomeres are equally competent in deriving ES cells, or if they have distinct fate. In mice, the ES cell establishment rate was inversely related to the number of divisions or cell numbers of an embryo. The efficiency in establishing ES cell lines from blastomeres of two-cell, early four-cell, late four-cell, and eight-cell embryos were 50-69%, 28-40%, 22% and 14-16% respectively (Wakayama et al., 2007). This result demonstrated an interesting pattern that only one or two blastomeres from each embryonic stage were capable of deriving an ES cell, but not all blastomeres. This is consistent with our findings that the expression pattern of stem cell markers and the development of ICM were not uniform among sister blastomere derived embryos and outgrowths (Figures 3.7A-3.7B and 3.8A-3.8B). Our result shares similar phenomenon with studies on the generation of identical animals (Heyman et al., 1998; Chan et al., 2000; Mitalipov et al., 2002; Schramm and Paprocki, 2004) and the generation of an ES cell from a single blastomere (Wakayama et al., 2007; Chung et al., 2006; Klimanskaya et al., 2006) in that sister blastomeres were in general not equally competent in establishing

ES cell lines and the generation of identical offspring. However, the capability of sister blastomeres in complementing the lost or damaged blastomere of an embryo could not be determined. One of the fundamental questions is whether lineage fate is committed in an early embryo, and if this lineage fate can be reverted, or if it is limited to a particular blastomere(s) but not all sister blastomeres that retains pluripotency. Our findings demonstrated that sister blastomeres were not identical based on the expression pattern of stem cells markers, the capability of forming ICM, and the derivation of ES cells.

Although sister blastomere may be capable of complementing the lost or damaged sister blastomere, limitation is expected based on previous studies and our recent findings. Therefore, it is very important to determine if the random retrieval of a single blastomere for deriving an ES cell would jeopardize an embryo's integrity and its later development, because blastomeres with ICM lineage preference can be retrieved and result in reducing the developmental capability of the donor embryo. Therefore, an in-depth investigation should be performed before attempting the retrieval of single blastomeres from human embryos for the establishment of ES cells as a clinical practice. Moreover, one of the major drawbacks on the approaches previously described was the co-culture of mouse and human ES cell during the derivation process (Chung et al., 2006; Wakayama et al., 2007). In addition to ES cell co-culture, mitogen-activated protein kinase inhibitor (MAPK inhibitor), MEK-1 (I), was also supplemented (Chung et al., 2006). The peptide hormone, ACTH, has also been supplemented in mES medium to improve the success rate of ES cell establishment from single blastomere (Wakayama et al., 2007). However, it is still unclear whether ES cell or the MEK-1 (I) or both have played an important role for

the establishment of ES cell from single blastomere despite the relatively low efficiency. In this study, we have demonstrated that the ES cell could be established from single blastomere of two cell stage embryo without using any supporting cells or drugs. there was four ES cell lines could be established and these lines were free from the cross contaminated with the ES supporting cell or free from some unclear effects of the drugs.

In conclusion, we have established an efficient sequential culture method for single blastomere culture with successful *in vitro* development, establishment of embryonic outgrowth and the derivation of ES cell lines. We also demonstrated that sister blastomeres of two- and four-cell embryos were not identical and are not equally competent based on the expression pattern of stem cell markers, the formation of ICM and the derivation of ES cells. We hypothesize that the low efficiency in establishing ES cells from a single blastomere of mouse and human embryos was due to the differentiation commitment in early embryos that resulted in unequal pluripotence of sister blastomeres. Hence, there is a potential risk in retrieving a single human blastomere from an early embryo for the establishment of an ES cell, because it may jeopardize the integrity of the embryo for continued development.

3.6 References

Allen, W.R., and Pashen, R.L. (1984). Production of monozygotic (identical) horse twins by embryo micromanipulation. **J. Reprod Fertil** 71:607-613.

Bagis, H., Mercan, H.O., Sauirkaya, H., Turgut, G., and Dinnyes A. (2001). The effect of genetic background on the *in vitro* development of mouse embryos in

potassium simplex optimized medium supplemented with amino acid ($KSOM^{AA}$). **Turk J. Vet Anima Sci** 27: 409-415.

Brook, F.A., Evans, E.P., Lord, C.J., Lyons, P.A., Rainbow, D.B., Howlett, S.K., Wicker, L.S., Todd, J.A., and Gardner, R.L. (2003). The derivation of highly germline competent embryonic stem cells containing NOD-derived genome. **Diabetes** 52:205-208.

Chan, A.W.S., Dominko, T., Luetjens, C., Neuber, E., Martinovich, C., Hewitson, L., Simerly, C.R., and Schatten, G.P. (2000). Clonal propagation of primate offspring by embryo splitting. **Science** 287:317-319.

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L., and Lanza, R. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. **Nature** 439:216-219.

Delhaise, F., Bralion, V., Schuurbiers, N., and Dessy, F. (1989). Establishment of an embryonic stem cell line from 8-cell stage mouse embryos. **Eur. J Morphol.** 34:237-243.

Eistetter, H.R. (1989). Pluripotent embryonal stem cells can be established from disaggregated mouse morulae. **Dev. Growth Differ.** 31:275-282.

Fujimori, T., Kurotaki, J., Miyazaki, J.L., and Nabeshima, Y.I. (2003). Analysis of cell lineage in two- and four-cell mouse embryos. **Development** 130:5113-22.

Gardner, R.L. (2001). Specification of embryonic axes begins before cleavage in normal mouse development. **Development** 128:839–847.

Heyman, Y., Vignon, X., Chesne, P., Le Bourhis, D., Marchal, J., and Renard, J.P. (1998). Cloning in cattle: from embryo splitting to somatic nuclear transfer. **Reprod. Nutr. Dev.** 38:595-603.

Johnson, W.H., Loskutoff, N.M., Plante, Y., and Betteridge, K.L. (1995). Production of four identical calves by the separation of blastomeres from an *in vitro* derived four-cell embryo. **Vet. Rec.** 137:15-16.

Kawase, E., Suemori, H., Takahashi, N., Hashimoto, K., and Nakatsuji, N. (1994). Strain difference in establishment of mouse embryonic stem (ES) cell lines. **Int. J. Dev. Biol.** 38:385-90.

Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. **Nature** 444:481-485.

Kwon, O.Y., and Kono, T. (1996). Production of identical sextruplet mice by transfeering metaphase nuclei from four cell embryos. **Proc. Natl. Acad. Sci. U. S. A.** 93:13010-13013.

Matsumoto, K., Miyake, M., Utsumi, K., and Iritani, A. (1989). Production of identical twins by separating two-cell rat embryos. **Gamete Res.** 22:257-263.

Mitalipov, M.M., Yeoman, R.R., Kuo, H.C., and Wolf, D.P. (2002). Monozygotic twinning in rhesus monkeys by manipulation of *in vitro* derived embryos. **Biol. Reprod.** 66:1449-1455.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003). Manipulating the mouse embryo. In A. Nagy, M. Gertsenstein, K. Vintersten, and R. Behringer. (Eds). **A laboratory manual (3rd ed).** Cold Spring Harbor: Cold Spring Harbor Press.

Ozil, J.P. (1983). Production of identical twins by bisection of blastocysts in the cow. **J. Reprod. Fertil.** 69:463-468.

Papaioannou, V.E., Mkandawire, J., and Biggers, J.D. (1989). Development and phenotypic variability of genetically identical half mouse embryos. **Development** 106:817-827.

Piotrowska, K., Wianny. F., Pedersen., R.A., and Zernicka-Goetz, M. (2001). Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. **Development** 128:3739–3748.

Piotrowska-Nitsche, K., Gomez, A.P., Haraguchi, S., and Goetz, M.Z. (2005). Four-cell stage mouse blastomeres have different developmental properties. **Development** 132:479-490.

Piotrowska-Nitsche, K., and Zernicka-Goetz, M. (2005). Spatial arrangement of individual 4-cell stage blastomeres and the order in which they are generated correlate with blastocyst pattern in the mouse embryo. **Mech. Dev.** 122:487-500.

Rideout III, W.M., Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. **Cells** 109:17-27.

Rossant, J. (1976). Postimplantation development of blastomeres isolated from 4- and 8-cell mouse eggs. **J. Embryol. Exp. Morphol.** 36:283-290.

Schramm, R.D., and Paproki, A.M. (2004). *In vitro* development and cell allocation following aggregation of split embryos with tetraploid or developmentally asynchronous blastomeres in rhesus mmonkeys. **Cloning Stem Cells** 6:302-314.

Strelchenko, N., Verlinsky, O., Kukharenko, V., and Verlinsky, V. (2004). Morula derived human embryonic stem cells. **Reproductive Biomedicine** 9:623-629.

Sukoyan, M.A., Kerkis, A.Y., Mello, M.R.B., Kerkis, I.E., Visintin, J.A., and Pereira, L.A. (2002). Establishment of new murine embryonic stem cell lines for the generation of mouse models of human genetic diseases. **Brazillian J. of Medical and Biological Research** 35:535-542.

Sunoda, Y., Uasui, T., and Sugie, T. (1984). Production of monozygotic twins following transfer of separated half embryos in the goat. **Jpn. J. Zootech. Sci.** 55:643-647.

Suzuki, O., Matsuda, J., Takano, K., Yamamoto, Y., Asano, T., Naiki, M., and Kusanagi, M. (1999). Effect of genetic background on establishment of mouse embryonic stem cells. **Exp. Anim.** 48:213-216.

Tarkowski, A.K. (1959). Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. **Ada. Theriologica.** 3:191-267.

Tarkowski, A.K., and Wroblewska, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. **J. Embryol. Exp. Morphol.** 18:155-180.

Tesar, P.J. (2005). Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. **Proc. Natl. Acad. Sci. U. S. A.** 102:8239-8244.

Tsunoda, Y., and Mclaren, A. (1983). Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. **J. Reprod. Fert.** 69:315-322.

Wakayama, S., Kishigami, S., Thuan, N.V., Ohta, H., Hikichi, T., Mizutani, E., Yanagimachi, R., and Wakayama, T. (2005). Propagation of an infertile

hermaphrodite mouse lacking germ cells by using nuclear transfer and embryonic stem cell technology. **Proc. Natl. Acad. Sci. U. S. A.** 102:29-33.

Wakayama, S., Hikichi, T., Suetsugu, R., Sakaide, Y., Bui, H.T., Mizutani, E., and Wakayama, T. (2007). Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. **Stem Cells** 25:986-993.

Wang, Z.J., Trounson, A., and Dziadek, M. (1990). Developmental capacity of mechanically bisected mouse morulae and blastocysts. **Reprod. Fert. Dev.** 2:683-691.

Willadsen, S.M., Lehn-Jensen, H., Fehily, C.B., and Newcomb, R. (1981). The production of monozygotic twins of preselected parentage by micromanipulation of nonsurgically collected cow embryos. **Theriogenology** 15:23-29.

Willadsen, S.M., and Polge, C. (1981). Attempts to produce monozygotic quadruplets in cattle by blastomere separation. **Vet. Rec.** 10:211-213.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable

offspring derived from fetal and adult mammalian cells. **Nature** 385:810-813.

Wilton, L.J., and Trounson, A.O. (1989). Biopsy of preimplantation mouse embryos: development of micromanipulated embryos and proliferation of single blastomeres *in vitro*. **Biol. Repord.** 40:145-152.

CHAPTER IV

CHEMICALS ENHANCEMENT IN ES CELL DERIVATION FROM SINGLE BLASTOMERE

4.1 Abstract

Personal ES cells is one of the most anticipated area of interests in biomedical research because of the promise of stem cell therapy and immune-compatibility. Although culture of a single blastomere recovered from early pre-implantation embryo has resulted in the successful derivation of ES cell, optimal conditions has not yet developed. Several chemicals target the MAPK/ERK signaling pathway, which play an important role in regulating cell growth and differentiation, have shown enhancing effect on the development of inner cell mass (ICM) and the establishment of ES cells from single blastomere. However, in-depth investigation of such chemicals on early embryonic development and the derivation of ES cell lines have not determined. This study was aimed to determine if adrenocorticotropic hormone (ACTH), MEK-1 inhibitor [MEK-1 (I)] and P38MAPK inhibitor [P38MAPK (I)], SB203580, could enhance the development of ICM and the derivation of ES cell lines from single blastomere of mouse two- and four-cell stage embryos. Our results demonstrated that among both MAPK inhibitors, P38MAPK (I) has stronger delayed early embryonic development and inhibited the development of single blastomere derived embryos than MEK-1 (I). As a result, a total of seventeen ES cell lines were established. Among these ES cell lines, nine (12.5%; 9/72) and five (6.9%; 5/72) of

such ES cell lines were established from single blastomere derived from two-cell embryo with and without the supplement of ACTH, respectively. In addition to 2CBD, three (4.2%; 3/72) ES cell lines were established from blastomere of four-cell stage embryo supplemented with ACTH. However, ES cell line was not able to establish from those supplemented with either MEK-1 (I) or P38MAPK (I). The results of this study demonstrated that ACTH could enhance the derivation of ES cells from single blastomere, whereas MEK-1 (I) and P38MAPK (I) were not able to enhance the derivation of ES cells.

4.2 Introduction

ES cell is one of the most promising stem cell sources for cell therapy and tissue engineering. ES cells have been successfully established from several species in the past decades including mouse (Evans and Kaufman, 1981; Martin, 1981), monkey (Thomson et al., 1995; Suemori et al., 2001) and human (Thomson et al., 1998; Baharvand et al., 2006; Heins et al., 2006). Although ES cell lines could be established by different methods (Evans and Kaufman, 1981; Munsie et al., 2000; Gallagher et al., 2003; Tavares et al., 2005), most of the currently available ES cell lines were derived from the ICM cells of the blastocyst stage embryos. Blastomeres of eight-cell and morula stage embryos have shown to be an alternative source for establishing ES cell lines despite the low efficiency compared to traditional method of deriving from ICM (Eistetter, 1989; Delhaise et al., 1996; Strelchenko et al., 2004; Sills et al., 2005; Tesar, 2005). One of the major barriers of clinical application of ES cells is to identify immune compatible ES cells for patients. Therefore, the success in establishing ES cell lines from eight-cell blastomeres instead of using ICM or morula

stage embryo has led to a new era of personalized ES cells (Strelchenko et al., 2004). Besides providing an alternative source of immune compatible ES cell, blastomeres recovered from early embryos do not require the destruction of embryos, which has been raising great ethic concerns. Recent reports have further demonstrated the feasibility of establishing ES cell lines from single blastomeres of mouse (Chung et al., 2006) and human (Klimanskaya et al., 2006) early embryos. Blastomere collected by biopsy of early eight-cell stage embryos was capable of deriving ES cells, whereas the embryonic counterpart was capable of developing to blastocyst stage. Although embryo transfer and full term development of the biopsied blastocyst was not demonstrated, similar blastomere biopsy procedure is commonly used in fertility clinic for preimplantation genetics diagnosis, thus viable blastocyst for later development is expected. One of the major drawbacks on the approaches described is the co-culture of mouse and human ES cell during the derivation process (Chung et al., 2006; Klimanskaya et al., 2006). In addition to ES cell co-culture, mitogen-activated protein kinase inhibitor (MAPK inhibitor), MEK-1 (I), was also supplemented (Chung et al., 2006). It is unclear whether MEK-1 (I) played an important role for the establishment of ES cell from single blastomere despite the relatively low efficiency.

The MAPK family is consisted of four groups of kinases: extracellular signal-regulated kinases (ERKs) 1 and 2; ERK5; c-Jun amino terminal kinases (JNKs) 1,2 and 3; and P38MAPK α, β, δ and γ, where each isoform is encoded by its own gene (Binetruy et al., 2007). Among the MAPK family, the ERK1/2, JNK and P38MAPK pathway were the most studied in stem cell research (Binetruy et al., 2007). These kinases are important regulators of the proliferation and differentiation of the ES cells,

they are also involved in regulating apoptotic process. Several MAPK inhibitors have been investigated for their roles in early embryo development, these include MEK-1 (I) (Chung et al., 2006; Paliga et al., 2005) and P38MAPK (I) (Natale et al., 2004; Maekawa et al., 2005; Paliga et al., 2005; Madan et al., 2006). The MEK-1 (I) has been used for deriving mouse ES cells from embryonic blastomere that was co-cultured with mouse ES cell (Chung et al., 2006). Although ES cell lines have been successfully established, the role of MEK-1 (I) and ES cells co-culture on single blastomere development has not yet been determined. On the other hand, P38MAPK (I) has shown to have inhibiting effect on the development of TE cells in mouse morula stage embryos (Maekawa et al., 2005), which may enhance the development toward the ICM fate. Thus the ICM enhancement effect of P38MAPK (I) merit further investigation. Recently, Wakayama and colleagues (2007) reported the establishment of mouse ES cell lines from single blastomere of 2-, 4- and 8 - stage embryos with the supplement of ACTH. The effect of ACTH on ES cells derivation from single blastomere was clearly demonstrated. However, the derivation efficiency shown interesting pattern that embryos at advance stage such as eight-cells was lower than those derived from four-cells stage embryos, which was lower than that of two-cell embryos (Wakayama et al., 2007). Besides demonstrated the feasibility of deriving ES cells from single blastomere and the potential effect of ACTH, these studies suggested that not all sister blastomeres are capable of deriving pluripotent ES cells. This study was evolved based on the recent advancement in blastomere derivation of ES cell lines and the potential enhancing effect of MAPK inhibitors and peptide hormone. There is no report investigating the effect of such inhibitors and peptide hormone on early embryo and later derivation of ES cells. This study was

interested to evaluate and determine the effect of MEK-1 (I), P38MAPK (I) and ACTH on early mouse embryo development and the derivation of ES cells from single blastomeres of 2- and 4-cell embryos.

4.3 Materials and Methods

4.3.1 Animals

Female CD-1 mice (four-six weeks) were superovulated with 10 IU of pregnant mares serum gonadotrophin (PMSG, Sigma; IP). This was followed by 10 IU of human chorionic gonadotrophin (hCG, Sigma; IP) 48 hours later, and then natural mating with CD-1 male mice. Two-cell embryos were collected 43-45 hours after hCG injection from the oviducts and then cultured in 20 μl drop of KSOM + AA media (Specialty Media) under mineral oil with 5% CO_2 in air at 37°C.

4.3.2 Nomenclature

The blastomeres were recovered by the break-down of two- and four-cell (2C and 4C) stage mouse embryos. The blastomeres were named to indicate its origin of embryonic stage from which they were derived. For example, the embryos that were derived from blastomeres break-down of 2C and 4C stage embryos were named as 2CBD and 4CBD embryos, respectively. The K was used as an initial for KSOM. The ACTH, MEK-1 (I) and P38MAPK (I) were used as an initial for adrenocorticotropic hormone (ACTH, fragments 1.24; American Peptide Company, Sunnyvale, CA, USA), MEK-1 inhibitor (Cell Signaling Technology) and P38MAPK inhibitor (SB203580, Calbiochem), respectively. The ES was used for representing mouse embryonic stem cell.

4.3.3 Blastomeres break down

The zona pellucida of two- and four-cell stage mouse embryos was removed by 0.5% Protease (Sigma). The blastomeres were then separated by incubating the zona-free embryo in PBS without calcium and magnesium [PBS (-)] for about 3 minutes followed by gentle pipeting. The blastomeres of the same embryo were cultured individually alongside with their sister blastomeres.

4.3.4 Feeder cell preparation

Mouse fetal fibroblasts (MFF) were prepared from 13.5 dpc mouse fetuses. The MFFs were cultured in DMEM supplemented with 10% FBS, L-glutamine and Penicillin/Steptomycin. The MFFs were inactivated with 5 μg/ml mitomycin C for two hrs followed by a thorough wash before plating.

4.3.5 Single blastomere culture treatments

The blastomeres of 2CBD and 4CBD embryos were cultured individually in a 72-well plate which was pre-coated with 0.1% gelatin. The MFFs were plated onto a 72-well plate at 1,000 cell/well in 10 μl of culture media at 24 hours prior to blastomere and embryo culture. The 2CBD and 4CBD embryos were cultured in four differences medium as described in 4.3.6. The most width and height diameter of the ICM outgrowths from every treatment was measured under the microscope at 200 magnifications (Fig. 4.1) and calculated by the following equation.

The outgrowth area (μm^2) = width (μm) x height (μm)

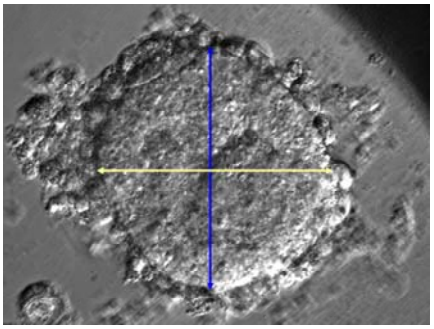


Figure 4.1 The measurement of the outgrowth diameter. The outgrowth area was calculated from the inner area of the primitive endoderm. The lines from two axes were draw across the outgrowth and measure the width (yellow) and height (Blue).

4.3.6 Culture media

As mentioned before, ACTH and MEK-1 (I) have been used for single blastomere culture and ES cell establishment (Wakayama et al., 2007; Chung et al., 2006) but there is no report on P38MAPK (I) culture of single mouse blastomere development and the the effects on ES cell properties. Therefore, in this experiment, we first determine the effect of P38MAPK (I) on mouse early embryos and to determine the optimal culture condition. The resulted culture scheme was then used for the derivation of ES cells from single blastomeres of 2C and 4C embryos, which were alongside with ACTH and MEK-1 (I) treatments.

The blastomeres and embryos were cultured in four different medium. These included: (1) control group: the 2CBD and 4CBD blastomere derived embryos were cultured in 10 µl of KSOM+AA media under mineral oil (Sigma), co-cultured with feeder cells and cultured at 37°C with 5% CO_2 in air. (2) ACTH group: the

blastomeres were cultured in KSOM + 0.1mg/mL ACTH until attached onto the feeder. mES + 20%KSR + 0.1mg/mL ACTH (Wakayama et al., 2006) was then replaced until the ICM outgrowths were sub-cultured. (3) MEK-1 (I) group: the blastomeres were cultured in KSOM + 50μM MEK-1 (I) for 3 days (Modified from Chung et al., 2006) with feeder cells when fresh KSOM was replaced and cultured until the attachment onto the feeder. mES media was then replaced until ICM outgrowths were sub cultured. (4) P38MAPK (I) group: the blastomeres were cultured in KSOM + 20μM P38MAPK (I), SB203580, for 15h (Modified from Maekawa et al., 2005) and fresh KSOM was then replaced until the blastomere attached onto the feeder. Then mES media was used for continue culture until the ICM outgrowths were sub culture. The mES media is consisted of DMEM supplemented with 10% FBS, 200mM L-Glutamine, 0.1mM β-Mercaptoethanol, 1x Minimum essential amino acid, 1x Penicillin /Steptomycin and 1000 IU/ml hLIF. The 2CBD and 4CBD embryos were cultured at 37°C with 5% CO_2 in air until analysis or further treatment.

4.3.7 Blastocyst cell count

To distinguish cells of the ICM and trophectoderm (TE), embryos were counterstained with specific antibodies. The embryos were washed twice in PBS, fixed in 4% paraformaldehyde for 30 minutes, and followed by incubation with blocking buffer which consists of 0.2% Triton-x 100, 3mM sodium azide, 0.1% saponin, 2% BSA, 5% horse serum in PBS (-) for 1 hour. The embryos were then incubated with the primary antibodies, rabbit polyclonal antibody against mouse and human Sox-2 (1:100) and mouse monoclonal antibody against a full-length Cdx2 recombinant protein (1: 1000) for 2 hours at room temperature. The samples were

then washed several times with PBS before incubation with the secondary antibodies, Rhodamine Red™- goat anti rabbit IgG (1:1000) and Alexa fluor® 488 donkey anti mouse IgG (1:1000) for 1 hour at room temperature followed by thorough wash with PBS (-). The embryos were then incubated with 5µg/mL Hoechst 33342 for 5 minutes before the last wash. The embryos were mounted on slide with 10 µl of vectashield. The embryos will then be examined with an Olympus BX51 epifluorescent microscope containing high numerical aperture objectives. The images will be captured by an Orca ER G digital CCD camera and analyzed by MetaMorph software. The Hoechst (nucleus, blue), Sox-2 positive (ICM cells, red), Cdx-2 positive (TE cells, green) cells were visualized and counted under fluorescence microscopy at different focal planes across the embryos.

4.3.8 Stem cell establishment from single blastomere

The 2CBD and 4CBD blastomere were cultured in different culture treatments as described in 4.3.6 until the attachment onto the feeder and form ICM outgrowth. At day 10 after separation, the ICM outgrowth from 2CBD and 4CBD were subcultured and transferred to new feeder with mES media. Visible ES colonies were then selected for subculture based on cell morphology and maintained by standard methodology (Nagy et al., 2003). The ES colony can be found at about 2-3 days after subculture. Fresh mES medium was replaced every other day and passaged at three - four day intervals. Besides morphology, ES cell lines were characterized by the expression of stem cell markers, which include Oct-4 (1:250), Sox-2 (1:100), Nanog (1:50), SSEA-1 (1:50), TRA-1-60 (1:100), TRA-1-81 (1:100) and alkaline phosphatase activity.

4.3.9 *In vitro* differentiation of embryonic stem cells derived from single blastomeres

The resulted ES cells were cultured in suspension for 7 days for the formation of EBs. EBs were then allowed to attach on gelatin coated plate and cultured in N1 medium for 7 days, N2 medium for 14 days and N3 medium for 7 days. The N1 medium composed of DMEM/F12 supplemented with minimum essential amino acid, 200mM of L-glutamine and N2 supplement. The N2 medium composed of N1 medium supplemented with 20 μg/mL basic fibroblast growth factor (bFGF). The N3 medium composed of DMEM/F12 supplemented with 1% FBS and B27 supplement. EBs were stained with alpha-fetoprotein (AFP) and vimentin. Neuroprogenitor cells were stained with nestin, whereas successful differentiation of neuronal cell types was confirmed by the expression of neuron specific β-III tubulin (TuJ1), tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT)

4.3.10 Gene transfer in ES cells

A self-inactivated lentiviral vector (Gift from C. Lois) expressing the GFP gene under the control of ubiquitin promoter was used in this study. VSVG-LVU-GFP was generated by transfection of the 293FT packaging cells (Invitrogen Inc.) with plasmid pLVU-GFP, pΔ8.9 and pVSVG. Supernatant was collected, concentrated by ultracentrifugation, tittered, and stored at -80°C. High titer lentivirus (10^9 cfu/ml) carried the GFP reporting genes were used to infect the ES cells in a 35mm dish supplemented with 8 μg/mL of polybrene.

4.3.11 Blastocyst injection

ICR female mice were natural mated with the ICR male mice. Then collected the two cell stage embryos and cultured in KSOM media until develop to blastocyst (Day 3.5). On the other hand, ICR female mice were mated with the ICR male vasectomized mice to use as recipients. The ES cells with GFP positive injection was performed using a PIEZO device (Primetech, Japan) with a micropipet size of approximately 15 micrometers inner diameter. The 15-20 ES cells with GFP positive were injected into cavity of blastocyst stage embryo and transferred to the uteruses. The fetuses were dissected from the uterus and removed from maternal tissues at day 13.5 to observe the chimera formation. The fetuses were washed thoroughly in cold PBS to remove cellular debris and blood. The DNA of all fetuses were extracted from tail tip for detect the transgenic (GFP) result by using PCR technique. Then the rest of the body were then fixed in 4% fresh PFA at 4°C for over night. The fetuses were then washed and perfused in gradual increase of sucrose solution (5, 10, 15, 30%) at 4°C. Fetuses were then embedded in OCT for cryosection. A section of 8 μm was cut and examined under fluorescent microscope to identify cell types expressing GFP. Fetus sections were examined by Olympus BX51 microscope and images were analyzed by MetaMorph software.

4.3.12 TUNEL assay

A DeadEndTM Fluorometric TUNEL system kit (Promega) was used to detect the apoptotic cells. The blastocyst stage embryos from ACTH, MEK-1 (I), P38MAPK (I) and control groups were fixed in 4% PFA for 30 minutes, wash with PBS for 5 minutes and permeabilize cells by 0.2% Triton® X-100 in PBS for 5 minutes. Then

wash with PBS before equilibrate in Equilibration solution for 5-10 minutes at room temperature. Then incubated in rTDT incubation buffer for 1h at 37°C in dark. Then washed with 2x SSC for 10 minutes and followed by PBS for 5 minutes to remove the unincorporated fluorescein-12-dUTP. Then incubate the embryos in 5µg/mL Hoechst 33342 for 5 minutes before the last wash. The embryos were then mounted on slide with 10 µl of vectashield. The embryos will then be examined with an Olympus BX51 epifluorescent microscope containing high numerical aperture objectives. TUNEL positive cells (green) and hoechst 33342 (blue) were visualized and counted under fluorescence microscopy at different focal planes across the embryos. Dead cell index (DCI) of all treatments was calculated by TUNEL positive cells divided by total cell number (Neuber et al., 2002).

4.3.13 Statistical analysis

Data analyses for differences in the embryonic development were carried out by ANOVA in Statistical Analysis Systems (SAS, version 9.0, SAS Inc., Cary, NC, USA). For embryo development, the percentages of cleavage to blastocyst development were calculated by dividing those numbers with the total number of the cultured embryos and then analyzed by ANOVA (SAS, version 9.0, SAS Inc., Cary, NC, USA).

4.4 Results

4.4.1 Embryo development in differences drugs

Our first experiment was to determine the effect of MEK-1 (I), P38MAPK (I) and ACTH on early mouse embryo development. The toxicity of P38MAPK (I) on mouse

embryo development was determined by supplementing in culture at different embryonic stages including zygote, 2C and 8C to morula (8C/M) stage for four days, three days and two days, respectively. The effect on development to blastocyst stage was determined and compared. We found that P38MAPK (I) has significant impact on embryo development whereas variation was observed while different stages of embryos were being treated. Supplement of P38MAPK (I) at the zygotic stage and 2C embryos result in the lowest blastocyst rate (5%, 1/20 and 0%, 0/20) and if supplementation at 8C/M result only five out of twenty embryos (25%) reached blastocyst while there was about fifteen out of twenty five embryos from control group (75%) reached blastocyst stage. Overall developmental rate of mouse embryos treated with P38MAPK (I) was significantly lower than the control embryos (Table 4.1). The result of this study was clearly demonstrated that P38MAPK (I) has negative effect on zygote and 2C embryo development when the embryos were always being treated during culture. In order to reduce the adverse effect of P38MAPK (I), an alternative strategy should be implemented. Despite the toxicity of P38MAPK (I), its biological effect is a reversible process, therefore, a specific regimen of P38MAPK (I) was developed in order to minimize the toxicity of P38MAPK (I) and sufficient to suppress TE development. The zygote, 2C and 8C/M stage embryos were cultured with the supplement of P38MAPK (I) for 12h, 15h and 24h followed by culture in KSOM medium not supplemented with P38MAPK (I) until blastocyst stage. No significant effect on embryo development was found in all treatment groups. Base on the overall morphology, we have chosen 15 hours treatment time for the rest of the study. Intact 2C stage embryos were separated into four groups and cultured in four different conditions, P38MAPK (I), MEK-1 (I),

ACTH and control. Our results showed that the development rate to 8C and blastocyst stage in MEK-1 (I) and ACTH groups were comparable to the control and were significantly higher than that of the P38MAPK (I) group ($P < 0.05$) (Fig. 4.2). We observed that embryo development was affected as early as 8C stage in P38MAPK (I) when compared to that of ACTH and MEK-1 (I).

Table 4.1 The development of intact embryo at different stages cultured in culture medium supplemented with P38MAPK (I) until develop to blastocyst.

Treatment	Stage	No.	Day 1	Day 2		Day 3		Day 4	
			2C (%)	4C (%)	8C (%)	4C (%)	Mor (%)	Mor (%)	Blast (%)
P38MAPK (I)	Zygote	27	20 (74.1)	12 (60.0)	1 (5.0)	10 (50.0)	6 (30.0)	3 (15.0)	1 (5.0)[a]
	2C	20	0 (0.0)	12 (60.0)	3 (15.0)	7 (35.0)	9 (45.0)	3 (15.0)	0 (0.0)[a]
	8C-Mor	20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	20 (100.0)	6 (30.0)	5 (25.0)[a]
Control	Zygote	25	20 (80.0)	4 (20.0)	15 (75.0)	0 (0.0)	13 (65.0)	5 (25.0)	15 (75.0)[b]

[a,b] superscript in the same column indicate significant different at $P<0.05$

The percentage of the embryo development at day 2 to day 4 were calculated from the number of 2C stage embryo on day 1

105

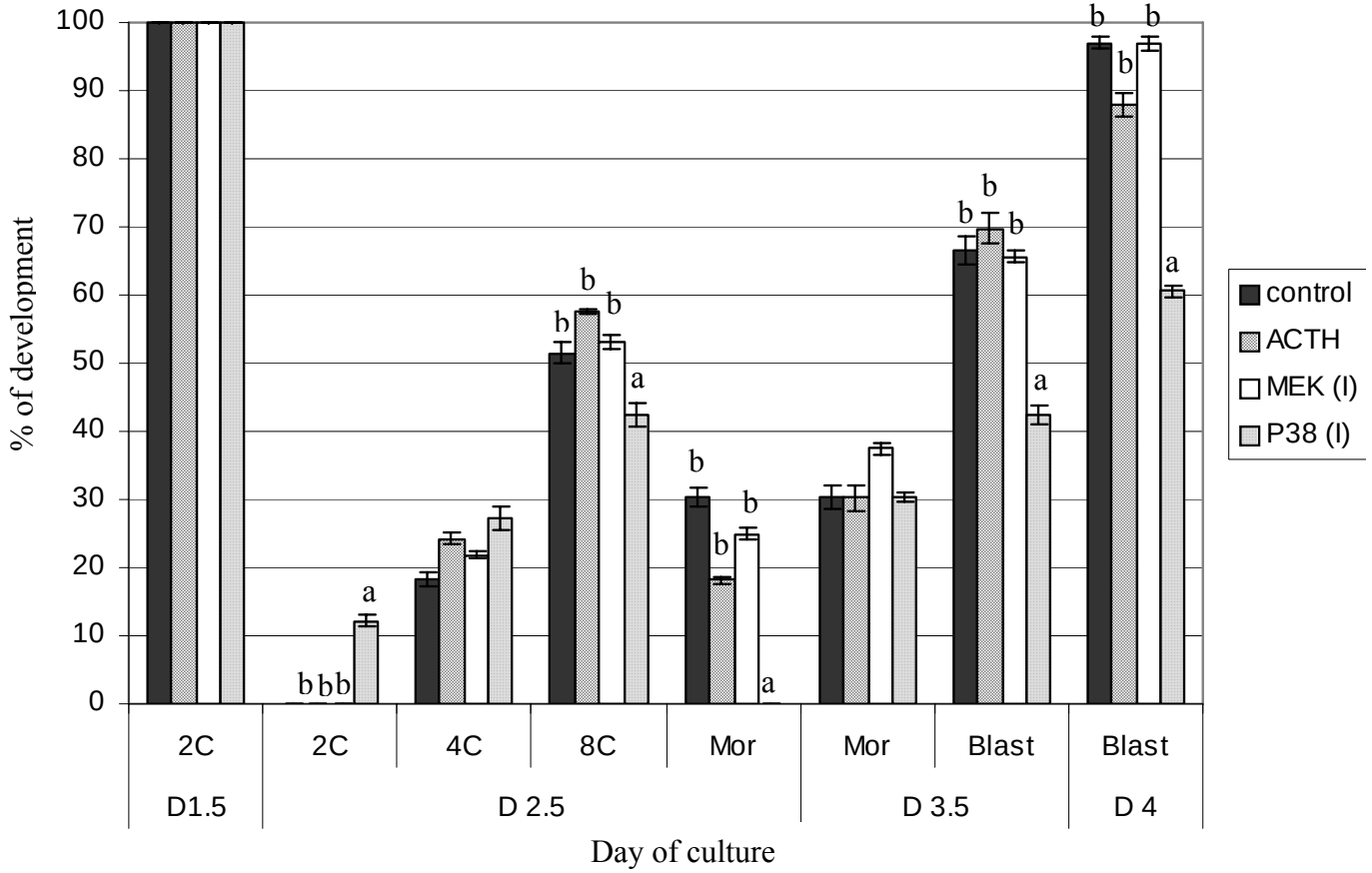


Figure 4.2 Development of intact 2C stage embryo after cultured in ACTH, MEK1 (I), P38 MAPK (I) compared with untreated embryo.

106

In order to determine if the ratio of ICM and TE cells was altered in the resulted blastocysts, we performed double immunostaining using antibodies specifically recognized TE (Cdx2) and ICM or stem cells (Sox-2). Our result on the ratio of ICM *vs* TE was consistent with our expectation that P38MAPK (I) derived blastocysts have the lower number of Cdx2 positive (TE) cells compared to the control, ACTH and MEK-1 (I) derived blastocysts but there were not significant difference (Table 4.2). However, Sox-2 positive cells were significantly reduced in all treatment groups when compared to the control ($P<0.05$). The ratio of Cdx2 and Sox-2 positive cells in all treatment groups was also different from that of the control ($P<0.05$) (Table 4.2). The picture of Cdx2 and Sox-2 positive cells localization of embryo from four different groups were showed in Figure 4.3. Sox-2 positive cells of control embryos have higher expression than those in the other treatments (Fig. 4.3).

Table 4.2 The Cdx2 and Sox-2 positive cells from different drug treatments.

Treatment	**No. Embryo**	**Cdx2 ± S.E.M**	**Sox-2 ± S.E.M**	**Ratio ± S.E.M**
Control	15	46.3 ± 2.5	12.4 ± 0.9[a]	3.7 ± 0.3 : 1[a]
ACTH	15	40.0 ± 3.2	6.0 ± 0.6[b]	6.7 ± 2.1 : 1[b]
MEK-1	15	43.9 ± 2.9	6.7 ± 0.8[b]	6.5 ± 0.6 : 1[b]
P38	15	38.2 ± 4.1	6.1 ± 0.9[b]	6.3 ± 0.9 : 1[b]

[a,b] superscript in the same column indicate significant different at $P<0.05$

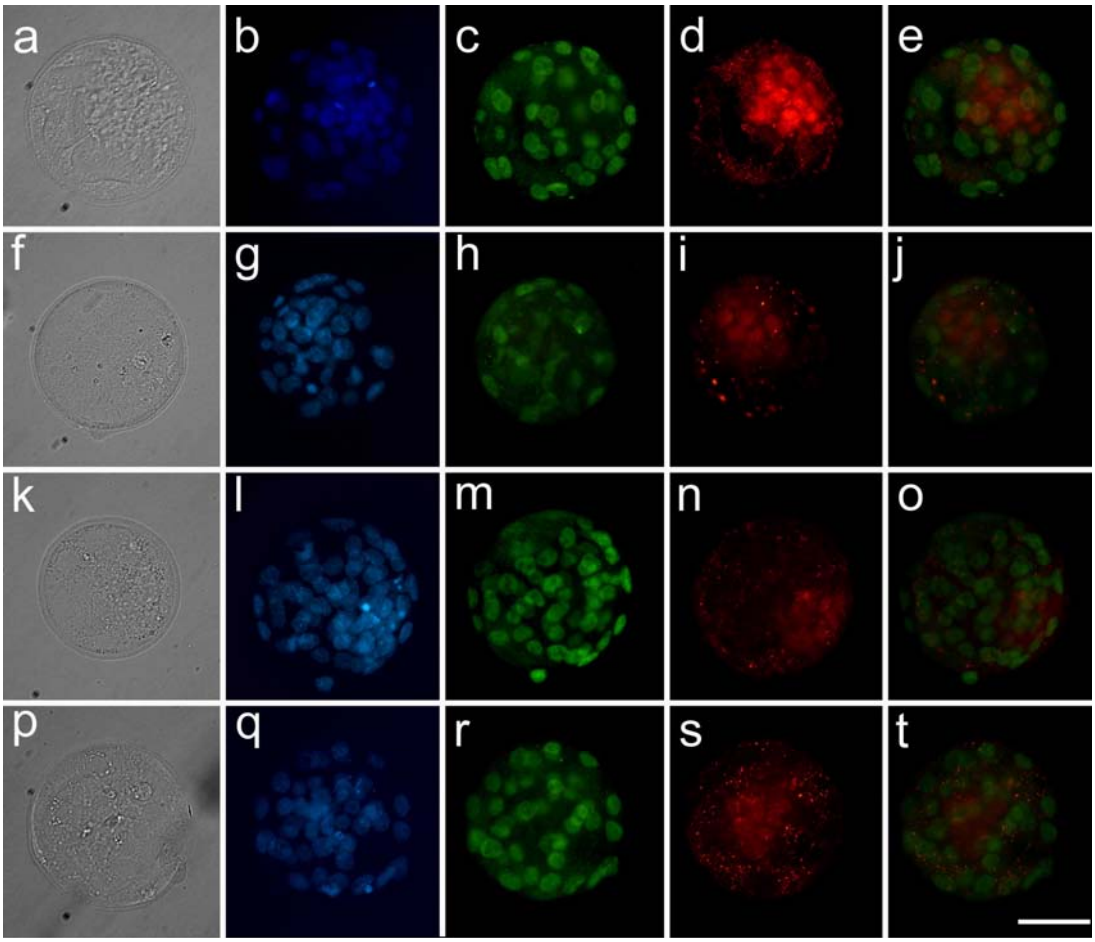


Figure 4.3 The localization of Cdx2-Sox-2 positive cells of the embryos which cultured in KSOM (a-e), ACTH (f-j), MEK1 (I) (k-o) and P38MAPK (I) (p-t). The embryos from each group was stained with Hoechst 33342 (b,g,l and q), Cdx2 (c,h,m and r) and Sox-2 (d,j,n and s), respectively. The Cdx2 and Sox-2 positive cells of the embryos from KSOM, ACTH, MEK-1(I) and P38MAPK (I) were merged (e,j,o and t, respectively) for clearly show the localization of Cdx2 and Sox-2. Bar = 100 μm.

4.4.2 Determining the drugs action on apoptotic cells number by TUNEL assay.

Based on the fact that slowest development was observed when the intact embryos were treated with P38MAPK (I) compared with the control (Fig. 4.2), one may concern whether these supplements increase the apoptotic rate and affect the developmental capability of early embryos as well as the derivation of ES cells. Here we decided to investigate if the supplements have effect on apoptotic rate in the resulted blastocysts. TUNEL assay was used to detect the fragmented DNA in apoptotic cells of the blastocyst (Fig. 4.4). Our result demonstrated that the total cell number of MEK-1 (I) derived blastocysts was not differ from that of the control blastocysts, however, P38MAPK (I) and ACTH derived blastocysts have reduced cell number when compared to the control ($P<0.05$).The highest death cell was found in P38MAPK (I) treatment group as demonstrated by the number of dead cell index (DCI) (Table 4.3). The result demonstrated that P38MAPK (I) has overt adverse effect on cellular integrity and increased cell death compared to the other supplements ($P<0.05$).

From the studies of intact embryo can be summarized that treat the embryo with ACTH, MEK-1 (I) and P38MAPK (I) could reduced the Sox-2 (ICM) positive cell when compare to control but there were no significant effect on Cdx2 (TE) positive cell. The embryo treated with P38MAPK (I) has slowest develop to blastocyst when compare to other treatments groups. Moreover, the result of TUNEL assay also demonstrated that P38MAPK (I) could induce higher death cell when compare to MEK-1 (I), ACTH and control.

Table 4.3 The TUNEL assay result to determine the drugs action on TE cells.

Treatment	**No. Embryo**	**Total cell (Average±S.E.M)**	**TUNEL positive (Average±S.E.M)**	**DCI ± S.E.M**
Control	20	56.1 ± 1.9^{a}	3.4 ± 0.8	0.06 ± 0.01^{a}
ACTH	20	$43.7 \pm 3.0^{b,c}$	2.8 ± 0.7	0.07 ± 0.02^{a}
MEK-1 (I)	20	$50.5 \pm 2.7^{a,b}$	2.6 ± 0.4	0.05 ± 0.01^{a}
P38MAPK (I)	20	42.0 ± 3.5^{c}	4.5 ± 1.4	0.11 ± 0.04^{b}

^{a,b} superscript in the same column indicate significant different at $P<0.05$

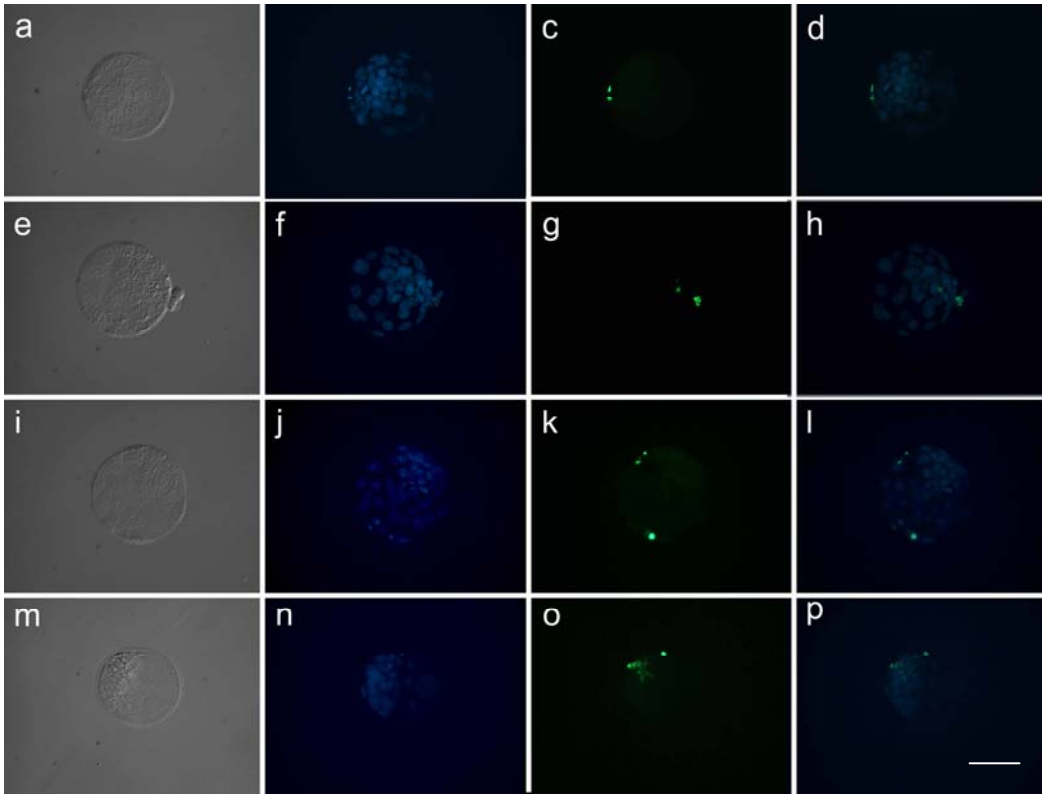


Figure 4.4 The TUNEL positive cells localization from control (a), ACTH (e), MEK1 (I) (i) and P38MAPK (I) (m). The embryonic cell nuclei were stained with Hoechst 33342 (b, f, j and n) and TUNEL positive cells were represented by green spots (c, j, k and o). The merged pictures of Hoechst 33342 with TUNEL positive cell (d, h, l and p), bar = 100 µm.

4.4.3 Development of the single blastomere culture in different drugs.

This study was aimed to develop an optimal culture condition for single blastomere of 2C and 4C embryos, which were then used for the derivation of ES cells. The blastomeres of 2C and 4C embryo were cultured with the medium supplemented with P38MAPK (I), MEK-1 (I), ACTH and control. Among these supplements, ACTH has minimal adverse effect on single blastomere development of 2CBD and 4CBD derived embryos, which was comparable to that of the control (Figs. 4.5 - 4.6). However, both MAPK inhibitors, MEK-1 (I) and P38MAPK (I),

could delay the development on day 1 and 2 after culture the blastomeres derived from 2CBD embryos and reduce the attachment rate when compare to control and ACTH groups ($P<0.05$; Fig. 4.5). Single blastomere from 2CBD and 4CBD culture in P38MAPK (I) has the lowest development rate (Fig. 4.5 - 4.6) and has the lowest overall attachment rate when compare to MEK-1 (I), ACTH and control group ($P<0.05$). As shown in figure 4.7, one of the blastomere outgrowth has a visible ICM clump whereas sister blastomere outgrowths showed small or no ICM clump. The morphology of the outgrowths from different treatments showed different morphologies. Especially, the outgrowth from ACTH group was not spread out as the others with round shaped cells that were not attached to the bottom and look like lysed (Fig. 4.8). The results of single blastomere cultured in different supplement suggested that embryonic stage has profound effect on the effectiveness of the supplements, whereas ACTH was among the less detrimental to single blastomere development compared to that of P38MAPK (I) and MEK-1 (I) (Fig. 4.5 and Fig. 4.6). Among these supplements, P38MAPK (I) was the only one that inhibited the development of both 2CBD and 4CBD derived blastomeres.

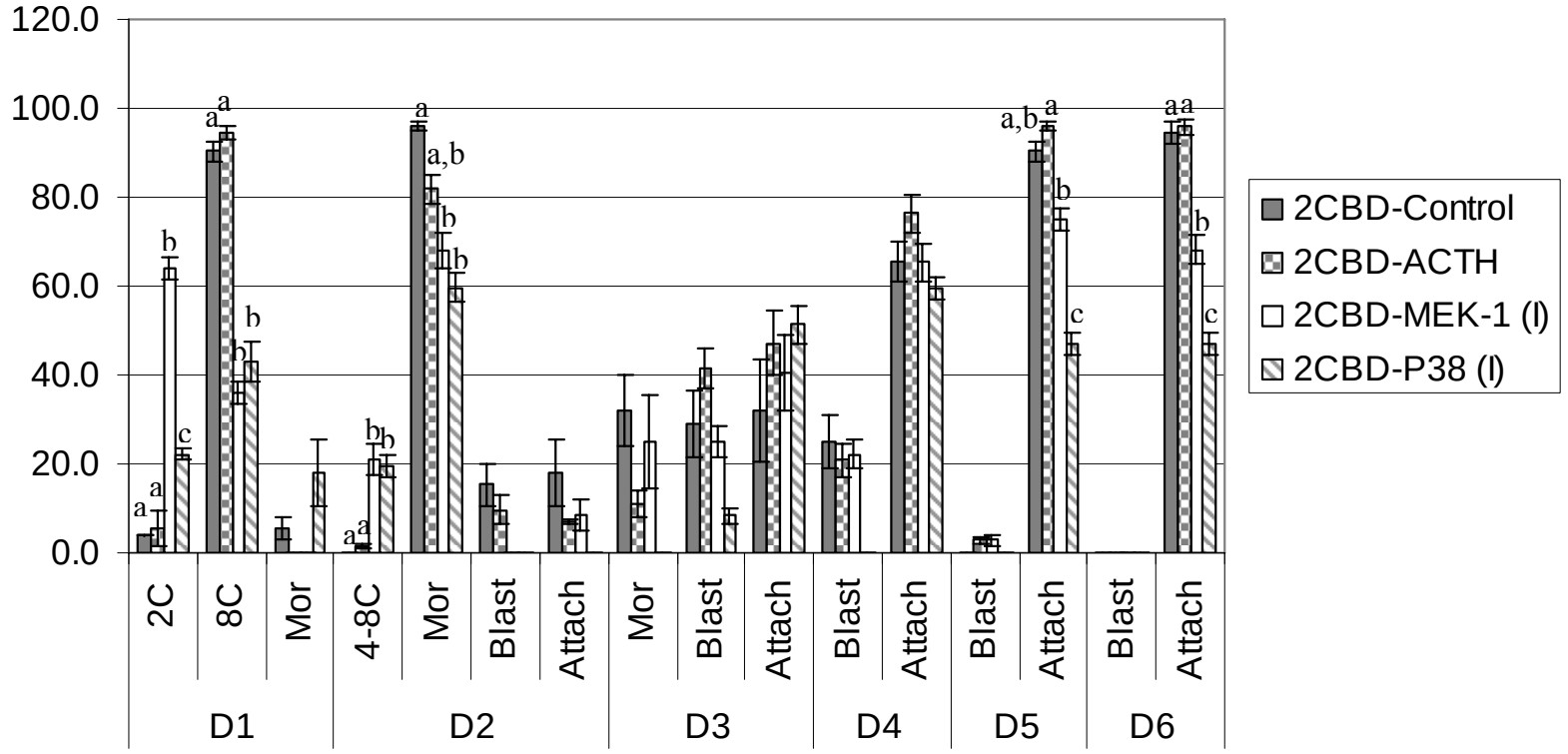


Figure 4.5 The 2CBD derived embryo cultured in ACTH, MEK1 (I), P38MAPK (I) compared with control.

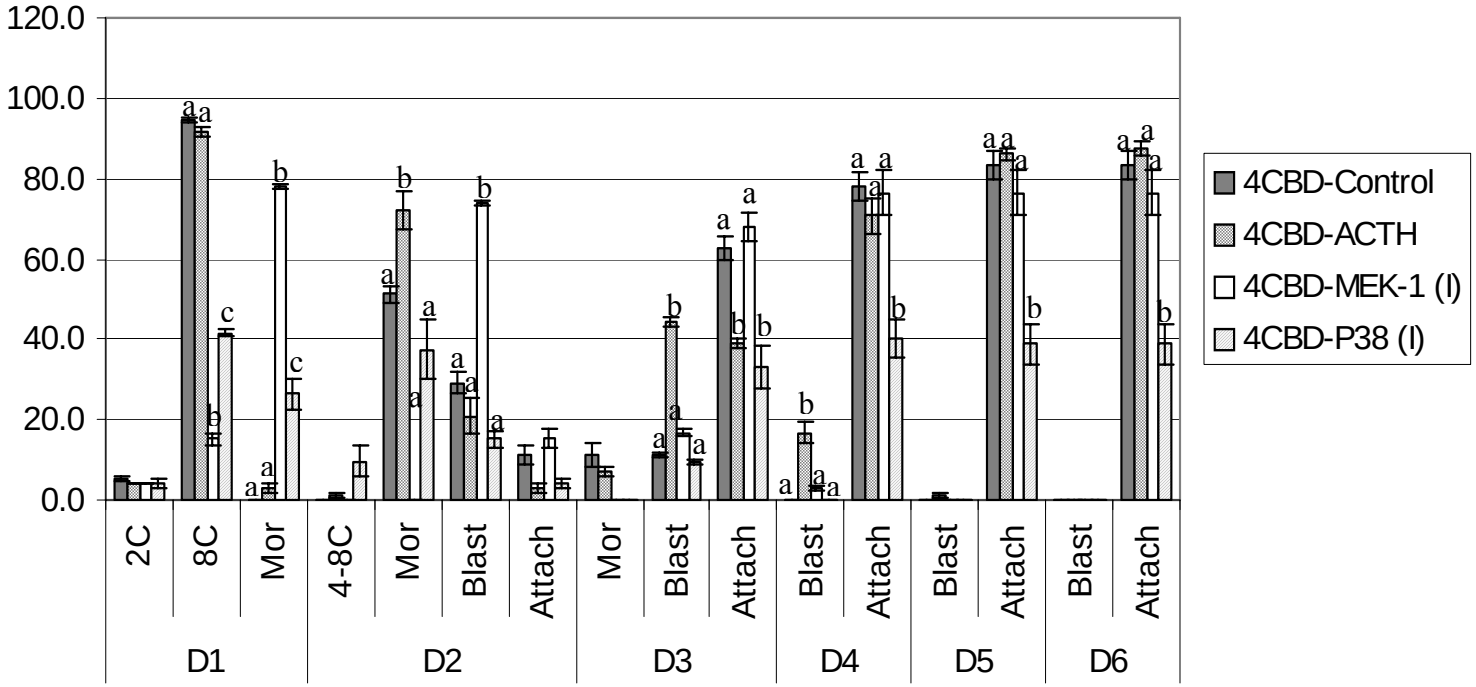


Figure 4.6 The 4CBD derived embryo cultured in ACTH, MEK1 (I), P38MAPK (I) compared with untreated.

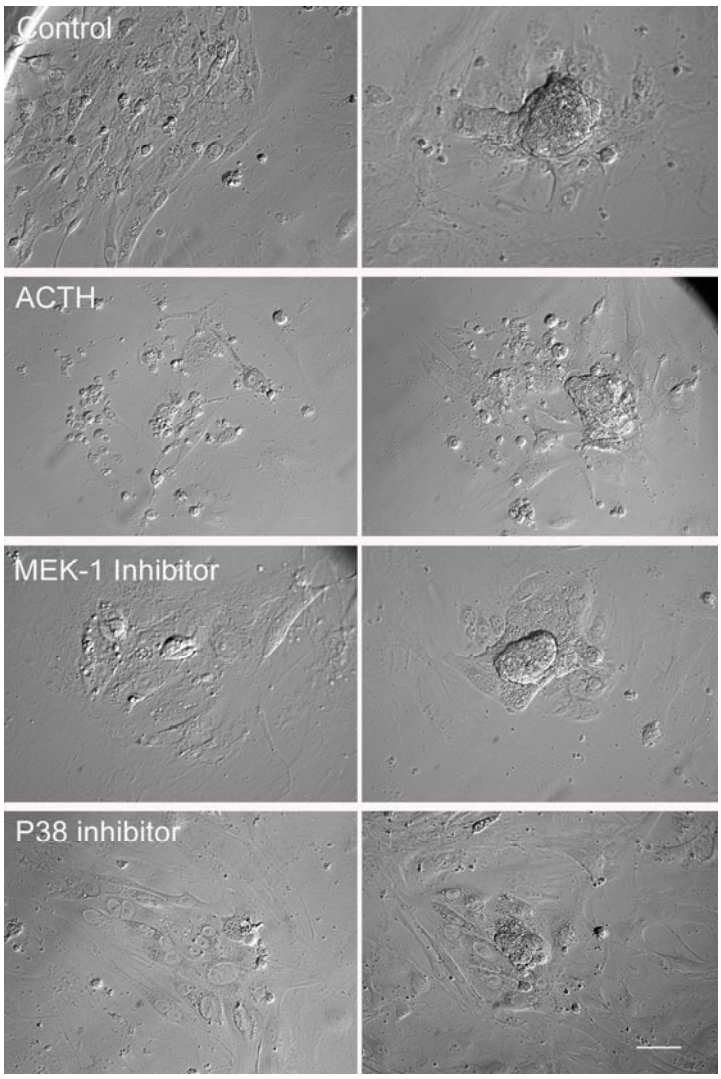


Figure 4.7 The ICM outgrowths of 2CBD derived embryo and it sister blastomere after culture in different drugs. The picture demonstrated that one blastomere had a visible ICM clump whereas another sister showed small or no ICM clump.

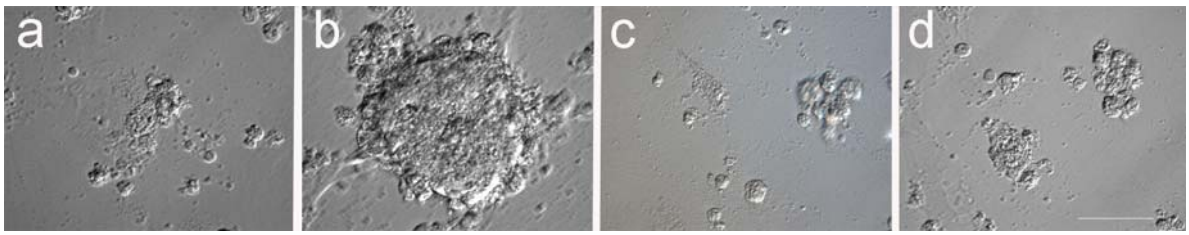


Figure 4.8 The outgrowths of 4CBD derived embryo after cultured in ACTH. The picture demonstrated the three blastomere could not outgrowth while only one blastomere shows a visible ICM clump, bar = 100 μm.

From the studies of 2CBD and 4CBD derived embryo culture in different drug supplement can be summarized that (i) the ACTH has minimal effect on 2CBD and 4CBD derived embryos development and attachment rate when compare with control. (ii) MEK-1 (I) has negative effect on 2CBD derived embryos development than 4CBD derived embryos while (iii) P38MAPK (I) has negative effect to both 2CBD and 4CBD derived embryo development and attachment rate. In conclusion, P38MAPK (I) has strongest negative effect on the development of single blastomere derived from 2CBD and 4CBD while MEK-1 (I) was the second and effect to only 2CBD derived blastomeres. However, the ACTH was the only supplement that has no effect on single blastomere development when compare to control group.

4.4.4 ES cell establishment from single blastomere: effect of supplements and the correlation to ICM size

Besides the variations in developmental competence of single blastomere cultured with different supplements was observed, the size of each blastocyst outgrowth at 10 days after attached onto the feeder cells was also studied. In 2CBD derived embryos, only 9.7% (7/72) of the blastocyst outgrowth from the P38MAPK

(I) group can be sub-cultured, which was significantly lower than that of the control (27.8%; 20/72), ACTH (38.9%; 28/72) and MEK-1 (I) (36.1%; 26/72) (Table 4.4). Besides the formation of blastocyst outgrowth, the development of ICM clump might be correlated to future derivation of ES cells because ICM outgrowth was likely the origin of the ES cells. Based on the average size of the 2CBD derived outgrowths, MEK-1 (I) derived blastocyst outgrowths were the largest (19.3 x 10^4 μm^2), whereas those derived from P38MAPK (I) treatment were the smallest (5.4 x 10^4 μm^2). However, blastocyst outgrowth derived from both MEK-1 (I) and P38MAPK (I) were not capable for the derivation of ES cell lines regardless to the size of the ICM clump. Only the outgrowths of the control and ACTH derived 2CBD blastocyst outgrowth were capable for the establishment of ES cell lines. Even though, the average size of 2CBD derived ICM outgrowth of MEK-1 (I) was the biggest but there was not different from that of control and ACTH (19.3 x 10^4 μm^2, 9.6 x 10^4 μm^2 and 10.8 x 10^4 μm^2, respectively) while it was significantly different from P38MAPK (I) outgrowth (5.4 x 10^4 μm^2) (Table 4.4).

In 4CBD study found that 4CBD from control, ACTH, MEK-1 (I) and P38MAPK (I) could form blastocyst outgrowth at the rate of 11.1% (8/72), 6.9% (5/73), 9.7% (7/72), and 9.7% (7/72), respectively (Table 4.4). MEK-1 (I) has the biggest (8.7 x 10^4 μm^2) ICM size when compare to ACTH (6.6 x 10^4 μm^2), P38MAPK (I) (3.5 x 10^4 μm^2) and control (6.8 x 10^4 μm^2).

In 2CBD blastomeres derived embryos, we have established five ES cell lines from the control group (6.9%; 5/72) and nine ES cell lines from the ACTH group (12.5%; 9/72). However, only three (4.2%; 9/72) ES cell lines were established in ACTH group when blastomeres of 4CBD embryos were used (Table 4.4). One

interesting observation is that no ES cell line could be established from the sister blastocyst outgrowths of both 2CBD and 4CBD embryos. These results suggested that ACTH could improve the ES cell derivation rate from the higher embryonic stage blastomere and also suggested that only some but not all blastomeres of the 2CBD and 4CBD derived embryos retains stem cell properties and capable of deriving ES cells.

4.4.5 ES cell line characterization and *in vitro* differentiation

We have established a total of 17 ES cell lines from embryos derived from single blastomeres of 2CBD and 4CBD embryos. Among these cell lines, 12 were derived with the supplement of ACTH and only five of them were derived without any supplement. Immunostaining of the resulted ES cell lines using stem cells markers commonly used for mouse ES cells have confirmed their stem cell properties, which included alkaline phosphatase, Oct-4, Sox-2, Nanog, and SSEA-1, whereas TRA-1-60 and TRA-1-81 were expected to be negative (Fig. 4.9).

Table 4.4 Single blastomere outgrowth and ES cell derivation from different drug treatments.

Embryo stage	Treatment	No. Embryo	No. Blastomere	No. Subcultred ICM (%)	Average ICM size (μm^2)	ES cell line (%)
2CBD	Control	36	72	20 (27.8)[a]	$9.6 \times 10^{4;\ a,b}$	5 (6.9)[a]
	ACTH	36	72	28 (38.9)[a]	$10.8 \times 10^{4;\ a,b}$	9 (12.5)[a]
	MEK-1 (I)	36	72	26 (36.1)[a]	$19.3 \times 10^{4;\ a}$	0 (0.0)[b]
	P38MAPK (I)	36	72	7 (9.7)[b]	$5.4 \times 10^{4;\ b}$	0 (0.0)[b]
4CBD	Control	18	72	8 (11.1)	6.8×10^4	0 (0.0)[b]
	ACTH	18	72	5 (6.9)	6.6×10^4	3 (4.2)[a]
	MEK-1 (I)	18	72	7 (9.7)	8.7×10^4	0 (0.0)[b]
	P38MAPK (I)	18	72	7 (9.7)	3.5×10^4	0 (0.0)[b]

[a,b] superscript in the same column of the same embryonic stage indicated significant different at $P<0.05$
The percentage were calculated based on the total number of blastomere

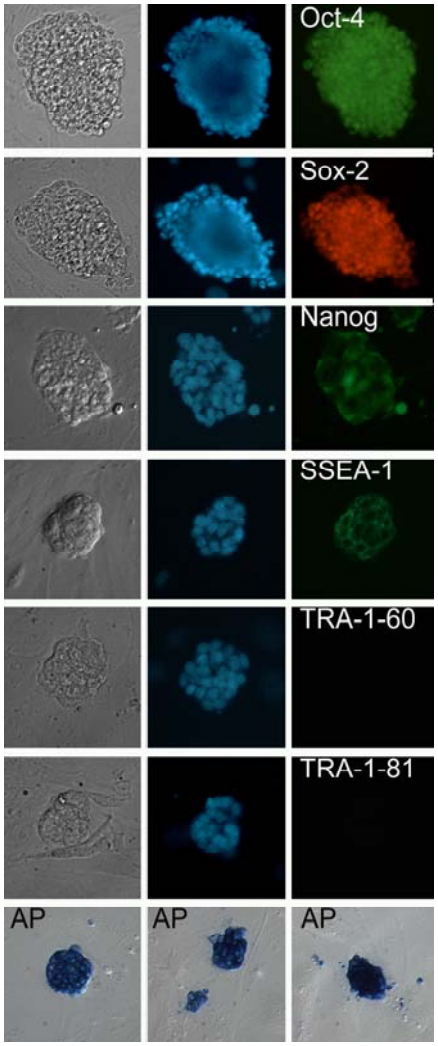


Figure 4.9 ES cell properties of the 4CBD derived ES cell lines was confirmed by the expression of mouse specific ES cell markers (Oct-4, Sox-2, Nanog, SSEA-1) and human ES cell specific marker (TRA-1-60 and TRA-1-81) as negative control. Transmission light images and Hoechst DNA staining were showed in the first and second column. The AP activity was positive and showed in the bottom line of the picture. Bar = 100 μm.

4.4.5 *In vitro* differentiation of the ES cell derived from single blastomere

All ES cell lines were capable of forming EBs, which were positive for endoderm marker alpha-fetaprotein (AFP) and ectoderm marker vimentin (Fig. 4.10). A stepwise differentiation method was used for neuronal differentiation as described by Kuo and colleagues (2003). EBs were allowed to attach onto gelatin coated dish and cultured in N1, N2 and N3 medium, respectively. Neuroprogenitor cells (NPCs) were induced and expanded while culture in N1 and N2 medium, which was confirmed by the expression of Nestin. Furthermore, differentiation of glial cells and mature neuronal cell types were then induced in N3 medium, which were confirmed by the expression of β-tubulin III (TuJ1), tyrosine hydroxylase (TH), and choline acetylase (ChAT). Here we demonstrated the successful establishment of the ES cells from a single blastomere derived embryo and their pluripotent differentiation capability. The success of *in vitro* differentiation was confirmed by the expression of all the above markers as shown in Figure 4.10.

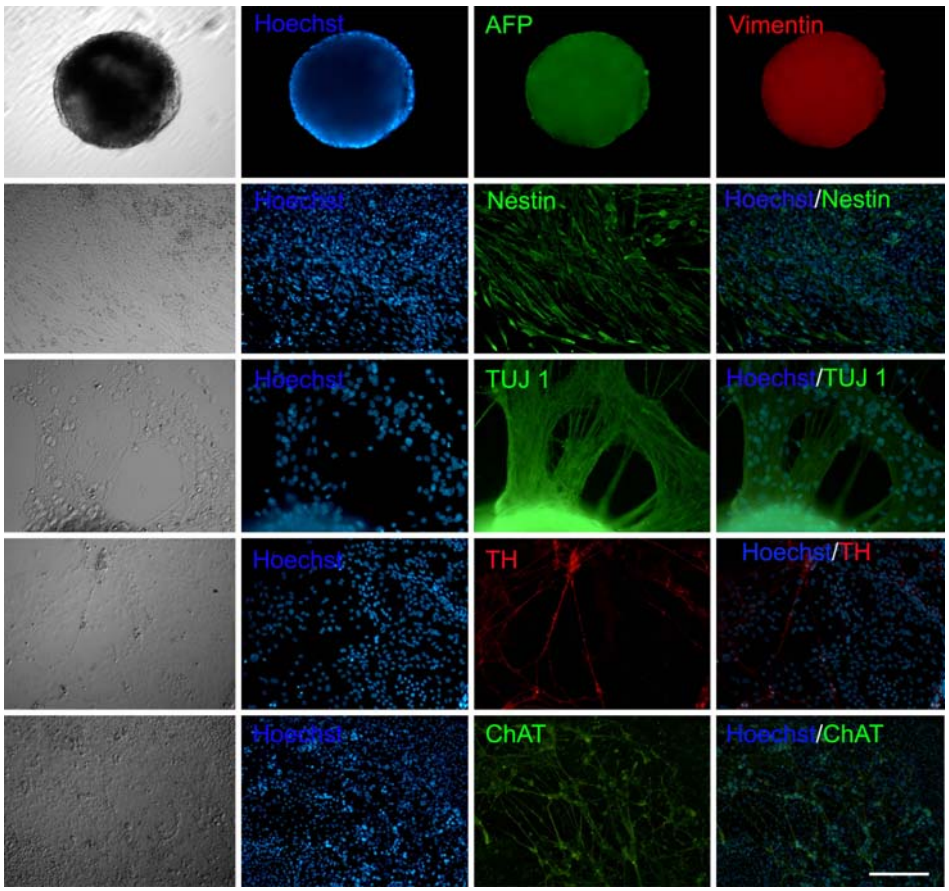


Figure 4.10 *In vitro* differentiation of the ES cell line derived from single blastomere. The EB was stained with Hoechst (Blue), AFP (Green) and vimentin (Red) as the specific marker for EB differentiation. After let the EB attaching to the feeder and cultured in N1, N2 and N3 media, they expressed nestin, TUJ1, TH and ChAT positive. Bar = 100 µm. You probably need the full name of each markers.

4.4.6 Chimera formation rate after injecting the ES cell with GFP into blastocyst cavity

One ES cell lines from each treatment, control-2CBD, ACTH-2CBD and ACTH-4CBD, were selected for transfection with lentivirus expressing the green fluorescent protein (GFP) gene under the control of ubiquitin promoter (FUGW).

Positive colonies were selected based on GFP expression and used for chimera formation test (Fig. 4.11 – Fig. 4.12). Two to three recipient mothers were used for carried the embryo of each cell line. The pregnant mothers were scarified on day 13.5 to collect the fetuses. The result found that mother recipient who carried control ES cell line had 100% pregnancy but ACTH treated ES cell lines had only 50% pregnancy rate (Table 4.5). The mummify fetuses were found in control group higher than the ACTH treated groups. However, there was only one fetus from ACTH-4CBD show GFP positive (10%) (Table 4.5). The DNA was extracted from the tail tip of GFP positive fetus and the rest of the body was subjected for tissue section studied. The PCR result (Fig. 4.13) and tissue section (Fig. 4.14) result confirmed the GFP positive.

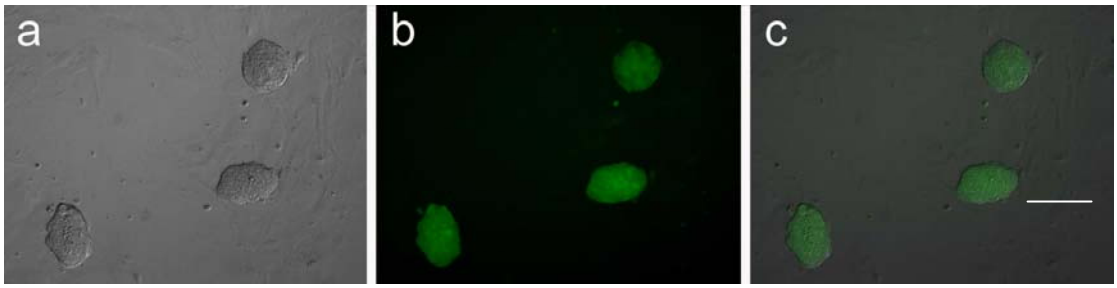


Figure 4.11 The ES colonies (a) derived from ACTH-4CBD embryo were transfected with FUGW-GFP virus showed green color after visualized under fluorescent microscope (b) and all of the cells in colonies showed GFP positive (c), bar = 100 μm.

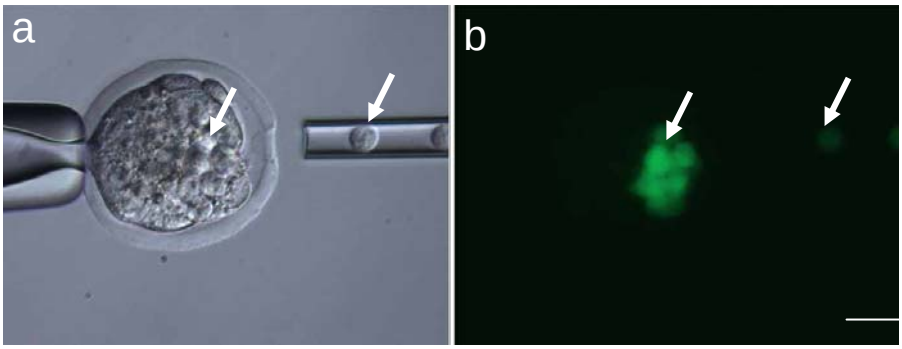


Figure 4.12 The ES - GFP positive cells (arrow) were injected into a cavity of mouse blastocyst, (a) bright field, (b) fluorescent, bar = 100 μm.

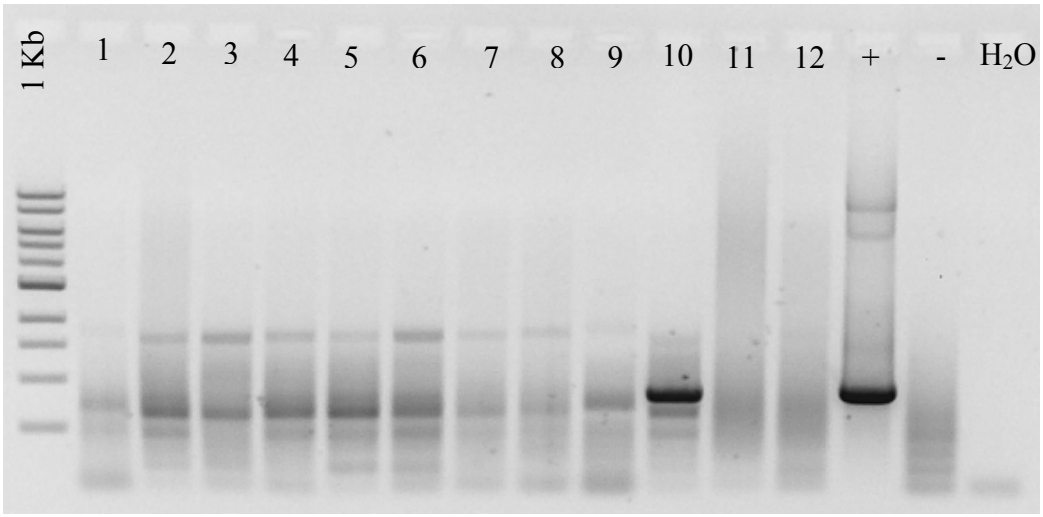


Figure 4.13 PCR analysis showed that a fetus number 10 had positive GFP band. Lane 1 is 1 Kb marker, Number 1-12 stand for number of fetus, + is positive control which is FUGW plasmid, - is negative control which is genomic DNA from wild type mouse and H_2O is water.

Table 4.5 Result of chimera formation from each ES cell line

ES cell line	No. of embryos/ recipient	No. recipient	No. pregnancy (%)	Recipient mother No.	Implanted fetuses (%)	Mummify fetuses (%)	Normal Fetuses (%)	Chimera rate (%)
Control-2CBD	15	3	3 (100)	1	11 (73.3)	7 (63.6)	4 (36.7)	0 (0%)
	15			2	10 (66.7)	2 (20.0)	8 (80.0)	0 (0%)
	15			3	10 (66.7)	4 (40.0)	6 (60.0)	0 (0%)
ACTH-2CBD	13	2	1 (50)	4	13 (100)	3 (23.1)	10 (76.9)	0 (0%)
ACTH-4CBD	14	2	1 (50)	5	13 (92.8)	3 (23.1)	10 (76.9)	1 (10%)

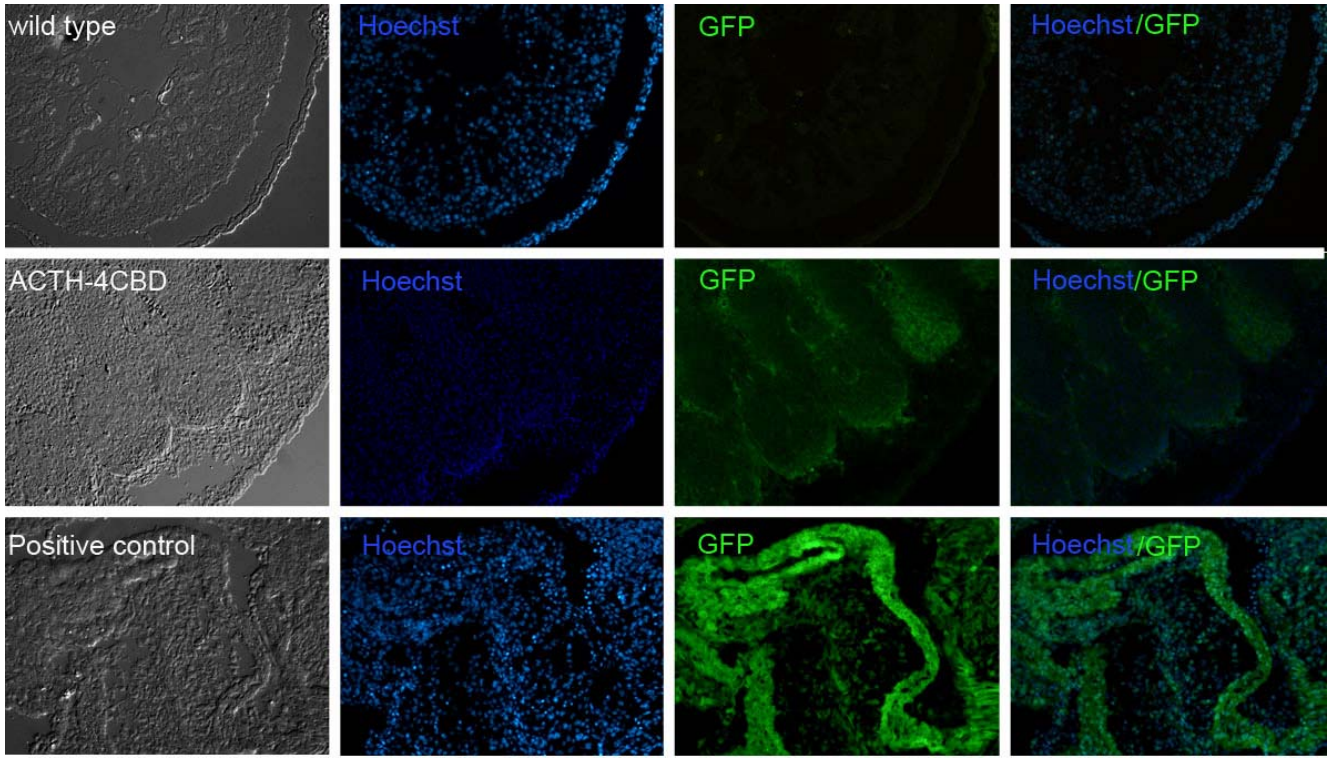


Figure 4.14 Comparison of GFP expressions in tissue of wild type, ACTH-4CBD ES cell, and positive control (FUGW virus infection) E13.5 mouse tissues. Embryos were frozen- sectioned at 8 μm intervals. The GFP expression of chimeric fetuses derived from ACTH-4CBD ES cell was not as strong as the positive control but stronger than a tissue from wild type.

4.5 Discussion

The objective of this study was to determine whether the P38MAPK (I), MEK-1 (I) and ACTH could improve the derivation of ES cell from single blastomeres of early mouse embryos. Direct comparison on the effect of embryo development and the establishment of ES cell lines was performed in parallel with the control group. For embryo development, whole embryo and blastomeres of break down embryos were investigated. ACTH did not pose any adverse effect on either intact or blastomere break down derived embryo development, however, 2CBD derived embryos were inhibited by two MAPK inhibitors, P38MAPK (I) and MEK-1 (I). This was consistent with our findings that no ES cell line was derived from blastomeres cultured in P38MAPK (I) and MEK-1 (I) treatment. Even 2CBD derived embryos were not developed at normal rate in MEK-1 (I) culture (Fig. 4.5) but the formation of blastocyst outgrowths was comparable to that of the ACTH and control group (Table 4.5). However, the negative effect of MEK-1 (I) on 4CBD derived embryos was reduced when compared to that of the 2CBD derived embryos. This finding suggested that ERK1/2 which is a target of MEK-1 (I) might involve in maternal embryonic transition (MET) and the activation of zygotic genome at the two-cell stage, while less detrimental effect was observed in four-cell embryos because embryonic control has been instigated. Another interesting phenomenon is that biological timeline remains in blastomeres derived embryos.

In contrast, blastomeres of two-cell embryos were more competent in deriving ES cells with or without the supplement of ACTH when compared to those derived from the four-cell embryos. Despite of the similar embryo development rate, ES cell lines could be established in 2CBD-ACTH and 2CBD-control groups at 12.5% and

6.9%, respectively (Fig. 4.5; Table 4.4). Furthermore, ES cell lines could also be established from the 4CBD derived embryos only if ACTH was supplemented. These results clearly demonstrated the positive effect of ACTH in supporting ES cells derivation from single blastomere of early mouse embryos (Table 4.4). Our results were consistent with previous study reported by Wakayama and colleagues (2007). The success rate of establishing ES cell line from single blastomere was inversely correlated to the development of the embryos. Higher derivation efficiency was found in less advance embryo such as 2-cell embryos, whereas lower efficiency was found in later stage such as 4-8 cell stage embryos. Although it is unclear on the potency of sister blastomeres, previous studies including our latest results demonstrated that blastomeres of early stage embryo may have committed to a distinct lineage fate as early as the 2C stage embryo. Therefore, only some but not all blastomeres are capable of deriving ES cells.

The G-proteins play an important role in signal transduction pathway. Recent studies have indicated that the α- and βγ-subunits of the G-proteins regulate several critical signaling pathways involved in cell proliferation, differentiation and apoptosis (Dhanasekaran et al., 1998). Different ligands including the neurotransmitter, chemokine, autocrine, paracrine factors and hormones (eg. ACTH) could activate the G-protein coupled receptor. Once the G-protein receptor is activated, the α-subunit will be released, activates the adenylyl cyclase (AC) and the active AC converses ATP to cAMP. An active cAMP involves in different regulatory pathways including the activation of Protein Kinase A (PKA) , which leads to the phosphorylation of downstream proteins and gene regulatory events. Dhanasekaran and colleagues (1998) reported that ACTH could activate AC and increase cAMP that resulted in the

conversion of ATP to cAMP. Recently, Faherty and colleagues (2007) has suggested the role of cAMP/PKA pathway in mouse ES cell self renewal. They suggested that forskolin, an adenylate cyclase agonist, increased cAMP level that enhanced ES cell self renewal. They also suggested that forskolin could work for ES cell renewal only if LIF and FBS were not supplemented. However, the result of our present study show that ACTH could promote the achievement of ES cells derivation from single blastomere even LIF was supplemented. Therefore, it was likely that LIF was not a main factor to inhibit the self-renewal which influenced from cAMP but the FBS might be more important. That was because the FBS contains many unidentified factors that could promote the differentiation (Ogawa et al., 2004; Cheng et al., 2004).

MAPK inhibitors and ACTH have been suggested for their potential effect on enhancing stem cell properties or the suppression of TE development, thus ICM or ES cell development could be enhanced. Among these supplements, P38MAPK (I) and MEK-1 (I) were not capable of improving the derivation of ES cells from single blastomere. In fact, the possible augmentation effect of MEK-1 (I) might require the presence of existing ES cells as it has been used in previous report (Chung et al., 2006). Wakayama and colleagues (2007) reported the establishment of ES cell lines from single blastomeres in mES medium supplemented with ACTH and KSR. However, we are not able to support early development single blastomere in mES medium supplemented with KSR. Thus KSOM medium supplemented with ACTH was used for blastomere culture until blastocyst stage when mES medium was replaced for stem cell enhancement and later derivation of ES cells. As a result, single blastomeres of 2CBD and 4CBD embryos could develop to blastocyst stage, attached and formed blastocyst outgrowth at comparable rate to that of the control groups,

which was superior than the P38MAPK (I) and MEK-1 (I) treatment groups. One interesting observation in ACTH culture was the subsequent cell death of the TE cells in blastocyst outgrowth, whereas ICM clumps were retained in culture (Fig. 4.8). Although the mechanism leading to targeted cell death is not clear, this finding support the notion of enhancing ICM growth by ACTH, thus increases the likelihood of successful derivation of ES cells.

In MEK-1 (I) treatment group, MEK-1 (I) was supplemented in initial culture of single blastomere for three days and then replaced by mES media when outgrowth was attached onto the feeder cells. Although no ES cell line was successfully derived from MEK-1 (I) derived embryos, the size of the ICM clumps in MEK-1 (I) derived 2CBD outgrowth was among the biggest compared to that of the control, P38MAPK (I) and ACTH derived outgrowths while MEK-1 (I) has no different in 4CBD outgrowths derived embryos from the other three treatments (Table 4.4). MEK-1 (I) targets the upstream events of the ERK1/2 pathway and selectively inhibits the ERK1/2 by suppressing the phosphorylation of the ERK1/2 and inhibits the subsequent downstream events (Alessi et al. 1995; Dudley et al.1995). Daoud and colleagues (2006) reported that inhibition of ERK1/2 could delay the TE cell differentiation; so, the ICM outgrowth might be enhanced by MEK-1 (I) instead.

The P38 pathway is in the MAPK family. The P38 includes four genes (P38α, β, γ, and δ) encoding proteins induced by osmotic stress, UV and various cytokines involved in inflammatory responses. The kinases are activated through the phosphorylation at their conserved residues which have different specificities for each P38 protein. The active P38 involved in the cell availability and differentiation processes (Duval et al., 2004). The study of the effect of P38MAPK has been done by

using P38MAPK (I) such as SB203580 which inhibit the P38α and β subunits. The SB203580 works through the P38MAPK pathway and inhibit P38MAPK activity by binding to the ATP site (Young et al. 1997; Davies et al. 2000). The inhibition of P38MAPK by SB203580 could effects to pre-implantation embryo development and placenta cell differentiation as well. Natale and colleagues (2004) was also reported that 2 cell stage embryos treated with SB203580 progressed to the eight cell stage with the same frequency as untreated embryo. However, the treated embryos halted their development at 8-16 cells. In addition, they also reported that P38 pathway is an important regulator of filamentous actin polymerization in many cell types including embryonic actin. Pre-implantation embryos treated with P38MAPK (I) have resulted in a complete loss of filamentous actin. However, the filamentous actin could be resumed after rescued from the P38MAPK (I) supplemented treatment. It is possible that many of these observation on the effects of P38MAPK (I) on pre-implantation development could be mediated via downstream effects to the action skeleton (Natale et al., 2004). Moreover, Duval and colleagues (2004) reported that SB203580 compound does not inhibit the apoptotic process. Therefore, the abnormal of filamentous actin could leading to apoptosis, this reason might describe the high apoptosis rate that found in P38MAPK (I) group. Maekawa and colleagues (2005) reported that SB203580 could inhibit the blastocoel cavitations of mouse blastocysts resulting to the delayed and abnormal blastocyst. Then Daoud and colleagues (2006) found that the inhibition of P38 by SB203580 could delay TE cell differentiation by measuring the level of secreted hCG. Moreover, Lali and colleagues (2000) reported that P38MAPK (I) could inhibit protein kinase B (PKB) which control the cellular survival and cell metabolism, P54MAPK which regulate the cell cycle, and might

involved in the inhibition of Wnt signaling pathway via glycogen synthase kinase 3 (GSK3). According to the above reports can be concluded that P38 pathway involved in embryo developmental processes especially delayed the TE cell differentiation. As a result of this study found that SB203580 could delay the progress to 8-16 cell stage and suppressed TE cell development in early embryos, which was consistent with previous reports (Natale et al., 2004; Paliga et al., 2005; Maekawa et al., 2005; Daoud et al., 2006). Moreover, the study in single blastomeres of the 2CBD and 4CBD embryos cultured in P38MAPK (I) shown the similar result as the intact embryo culture. However, comparison the effect of MEK-1 (I) and P38MAPK (I) on embryo development found that P38MAPK (I) has more robust inhibitory effect on both intact and single blastomere derived embryos development than MEK-1 (I). As this study has been previously hypothesized that suppressing TE differentiation by P38MAPK (I) might enhanced ICM development and increased the likelihood of success in establishing ES cells from single blastomere. But our results clearly demonstrated that by inhibiting the P38 pathway by using 20μM SB203580 was not sufficient to enhance ES cell derivation from single blastomeres derived embryos but the embryo quality and development were effected instead.

In our chimeric study, chimeric fetus could only resulted from ES cell line derived from ACTH-4CBD group with relatively weak green fluorescent signal in tissue sections (Table 4.5). The low chimeric rate could be related to the use of outbred strain of mice and the unknown effects from chemical reagent or the colonies which have low expression level of GFP. Further investigation on chimerism need to be done.

Several reports have showed that the reprogramming of somatic cells by nuclear transfer (Wakayama et al., 1998; Wilmut et al., 1997), cell fusion (Cowan et al., 2005; Teda et al., 2001) and over expression the transcription factors, Oct-4, Sox-2, c-Myc and Klf4, could induce the re-establishment of pluripotency (Hochedlinger and Jaenisch, 2006; Maherali et al., 2007; Yamanaka S, 2007). Those reports were aimed to produce personal stem cell with compatible immunity to that of the patients, who is the donor of the somatic cell. However, this strategy requires further in-depth investigation before clinical application could be implemented. On the other hand, developing personal ES cell from single biopsied blastomere is an alternative approach for deriving personal ES cells for cell therapy.

Here I reported that 17 pluripotent ES cell lines were established from single blastomeres of 2CBD and 4CBD derived embryos. Of these, five (6.9%) and nine (12.5%) ES cell lines were established from 2CBD-Control and 2CBD-ACTH, respectively. Only the supplement of ACTH was capable of supporting the derivation of three (4.2%) ES cells from 4CBD blastomere derived embryos. The MAPK inhibitors, P38MAPK (I) and, MEK-1 (I) were not able to support and enhance the establishment of ES cells from single blastomere. I have performed an in-depth comparative study on three potential supplements, P38MAPK (I), MEK-1 (I) and ACTH, that could enhance the efficiency of ES cell derivation from single blastomere. We have demonstrated that ES cell lines could be efficiently established from single blastomere with the supplement of ACTH. We also demonstrated the feasibility of developing personal ES cell lines, thus headed for the future of personal medicine.

4.6 References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo.* **J. Biol. Chem.** 270:27489–27494.

Baharvand, H., Ashtiani, S.K., Taee, A., Massumi, M., Valojerdi, M.R., Yazdi, P.E., Moradi, S.Z. and Farrokhi, A. (2006). Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. **Develop. Growth Differ.** 48:117-128.

Binetruy, B., Heasley, L., Bost, F., Caron, L. and Aouadi, M. (2007). Regulation of embryonic stem cell lineage commitment by mitogen activated protein kinases. **Stem cells** 25:1090-1095.

Cheng J, Dutra A, Takesono A., Garrett-Beal, L. and Schwartzberg, P.L. (2004). Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. **Genesis** 39:100 - 104.

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L. and Lanza, R. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. **Nature** 439:216-219.

Cowan, C.A., Atienza, J., Melton, D.A. and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. **Science** 309:1369–1373.

Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. **Biochem J** 351:95–105.

Daoud, G., Amyot, M., Rassart, E., Masse, A., Simoneau, L. And Lafond, J. (2006).

ERK1/2 and P38 regulate trophoblasts differentiation in human term placenta. **J. Physiol.** 566: 409-423.

Delhaise, F., Bralion, V., Schuurbiers, N. and Dessy, F. (1989). Establishment of an embryonic stem cell line from 8-cell stage mouse embryos. **Eur. J. Morphol.** 34:237-243.

Dhanasekaran, N., Tsim, S.T., Dermott, J.M. and Onesime, D. (1998). Regulation of cell proliferation by G-proteins. **Oncogene** 17:1383-1394.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. **Proc. Natl. Acad. Sci. U.S.A.** 92**:**7686–7689.

Duval, D., malaise, M., Reinhardt, B., Kedinger, C. and Boeuf, H. (2004). A P38 inhibitor allows to dissociate differentiation and apoptotic precesses triggered upon LIF withdrawal in mouse embryonic stem cells. **Cell. Death. Differ.** 11:331-341.

Eistetter, H.R. (1989). Pluripotent embryonal stem cells can be established from disaggregated mouse morulae. **Develop. Growth Differ.** 31:275-282.

Evans, M.J. and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. **Nature** 292:154-156.

Faherty, S., Fitzgerald, A., Keohan, M. and Quinlan, L.R. (2007). Self renewal and differentiation of mouse embryonic stem cells as measured by Oct-4 expression: the role of the cAMP/PKA pathway. ***In vitro*** **Cell. Dev. Biol.-Animal** 43:37 – 47.

Gallagher, Ed.J., Lodge, P., Ansell, R. and McWhir, J. (2003). Isolation of murine embryonic stem and embryonic germ cells by selective ablation. **Transgenic**

Research 12:451-460.

Heins, N., Lindahl, A., Karlsson, U., Rehnstrom, M., Caisander, G., Emanuelsson, K., Hanson, C., Semb, H., Bjorquist, P., Sartipy, P. and Hyllner, J. (2006). Clonal derivation and characterization of human embryonic stem cell lines. **J. Biotech.** 122:511-520.

Hochedlinger, K. and Jaenisch, R. (2006). Nuclear reprogramming and pluripotency. **Nature** 441:1061–1067.

Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J. and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. **Nature** 444:481-485.

Kuo, H.C., Pau, K.Y.F., Yeoman, R.R., Mitalipov, S.M., Okano, H. and Wolf, D.P. (2003). Differentiation of monkey embryonic stem cells into neural lineages. **Biol. Reprod.** 68:1727-1735.

Lali, F.V., Hunt, A.E., Turner, S.J., and Foxwell, B.M.J. (2000). The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interlwukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. **J. Biol. Chem.** 275: 7395-7402.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K. and Hochedlinger, K. (2007). Direct reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. **Cell Stem Cell** 1:55-70.

Maekawa, M., Yamamoto, T., Tanoue, T., Yuasa, Y., Chisaka, O. and Nishida, E. (2005). Requirement of the MAP kinase signaling pathways for mouse pre-

implantation development. **Development** 132:1773-1783.

Madan, P., Calder, M.D. and Watson, A.J. (2006). Mitogen activated protein kinase (MAPK) blocked of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. **Reproduction**. 130:41-51.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. **Proc. Natl. Acad. Sci. U.S.A**. 78: 7634-7638.

Munsie, M.J., Michalska, A.E., O'Brien, C.M., Trounson, A.O., Pera, M.F. and Mountford, S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. **Curr. Biol.** 10:989-992.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003). Manipulating the mouse embryo. In A. Nagy, M. Gertsenstein, K. Vintersten, and R. Behringer. (Eds). **A laboratory manual (3rd ed).** Cold Spring Harbor: Cold Spring Harbor Press.

Natale, D.R., Paliga, A,J.M., Beier, F., D'Souza, S.J.A. and Watson, A.J. (2004). p38 MAPK signalling during murine preimplantation development. **Dev. Biol**. 268:76-88.

Neuber, E., Luetjens, C.M., Chan, A.W.S. and Schatten, G.P. (2002). Analysis of DNA fragmentation of in vitro cultured bovine blastocysts using TUNEL. **Theriogenology** 57: 2193-2202.

Ogawa, K., Matsui, H., Ohtsuka, S. and Niwa, H. (2004). A novel mechanism for regulating clonal propagation of mouse ES cells. **Genes. Cells.** 9:471 - 477.

Paliga, A.J.M., Natale, D.R. and Andrew, J. (2005). p38 mitogen-activated protein

kinase (MAPK) first regulates filamentous actin at the 8-16 cell stage during preimplantation development. **Biol. Cell.** 97:629-640.

Qi, X., Li, T., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y. and Zhao, G. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. **Proc. Natl. Acad. Sci. U.S.A** 101:6027-6032.

Sills, E.S., Takeuchi, T., Tanaka, N., Neri, Q.V. and Palermo, G.D. (2005). Identification and isolation of embryonic stem cells in reproductive endocrinology: theoretical protocols for conservation of human embryos derived from in vitro fertilization. **Theoretical Biology and Medical Modelling** 2:25.

Strelchenko, N., Verlinsky, O., Kukharenko, V. and Verlinsky, V. (2004). Morula derived human embryonic stem cells. **Reprod. Bio.** 9:623-629.

Suemori, H., Tada, T., Torii, R., Hosoi, Y., Kobayashi, K., Imahie, H., Kondo, Y., Iritani, A. and Nakatsuji N. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. **Dev. Dyn**. 222:273–279.

Tavares, R.L., Freitas, V., Ferreira, A.T., Rosenwaks, Z. and Xu, K. (2005). Primary mouse embryonic fibroblast (PMEF) and embryonic stem cell isolation. **Fertil. Steril.** 84:406.

Tada, M., Takahama, Y., Abe, K., Nakatsuji, N. and Tada, T. (2001). Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells. **Curr. Biol.** 11:1553–1558.

Tesar, P.J. (2005). Derivation of germ-line-competent embryonic stem cell lines from

preblastocyst mouse embryos. **Proc. Natl. Acad. Sci.** 102: 8239-8244.

Thomson, J., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A. and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. **Proc. Natl. Acad. Sci. U.S.A** 92:7844-7848.

Thomson, J.A., Eldor, J.I., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. **Science** 282: 1145–1147.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. **Nature** 394: 369–374.

Wakayama, S., Hikichi, T., Suetsugu, R., Sakaide, Y., Bui, H.T, Mizutani, E. and Wakayama, T. (2007). Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. **Stem Cells** 25:986-993.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. **Nature** 385:810–813.

Yamanaka, S. (2007). Strategies and new developments in the generation of patient specific pluripotent stem cells. **Cell Stem Cell** 1: 39-46.

Young, P.R., McLaughlin, M.M., Kumar, S., Kassis, S., Doyle, M.I., McNulty, D., Gallagher, T.F., Fisher, S., McDonnell, P.C., Carr, S.A., Huddleston, M.J., Seibel, G., Porter, T.G., Livi, G.P., Adams, J.I. and Lee, J.C. (1997). Pyridinyl imidazole inhibitors of p38 mitogenactivated protein kinase bind in the ATP site. **J Biol Chem** 272:12116–12121.

CHAPTER V

A PRERIMINARY STUDY OF ES CELL ESTABLISHMENT FROM MONKEY SINGLE BLASTOMERES DERIVED EMBRYOS

5.1 Abstract

The successful of ES cell line establishment from mouse single blastomeres have previously been demonstrated. Since non human primates (NHPs) are much closer to human than mouse, so, the ES cell produced from NHPs could provide high potential for studies of human ES cell based therapy. Therefore, this experiment has been tried to establish a protocol for monkey ES cell derivation from the intact monkey ICSI derived embryos. Immunosurgery, blastocyst dissection and blastocyst outgrowth were used for ICM isolation. The result found that immunosurgery produced cleaner colonies than other techniques. Then 12 outgrowths from immunosurgery were sub cultured with either trypsin/EDTA or dispase before replated onto new feeder cells. One out of six outgrowths which sub cultured by trypsin/EDTA showed monkey ES-like cell morphology (16.7%). The established colonies showed positive to monkey ES cell markers but the expression level was low. In another experiment, the possibility to establish ES cell of monkey 2CBD and 4CBD derived embryos has been studied. The result found that 4CBD derived embryos could develop after *in vitro* culture but there was only 6 out of 76 blastomeres (7.9%) could develop to blastocyst.

Of those, four blastocysts were used for ES cell establishment. The result found that morphology of the establish colonies were different from standard monkey ES cell, even thought, they showed Oct-4 positive. Our results suggested that immunosurgery was a sufficient method to isolate monkey ICM cells and Trypsin/EDTA was suitable for subculture. Moreover, from the study of monkey single blastomere break down demonstrated that 4CBD derived embryos could develop *in vitro* to blastocyst stage.

5.2 Introduction

NHPs have similar genetic and physiology to humans (VandeBerg and Blangero, 1996). They provide the ultimately experimental animal model for investigating several human diseases including vaccine development, drug and alcohol addiction, brain function and gene therapy (Mitalipov et al., 2002). The NHPs are also more suitable for neurodegenerative study than rodent species because rodents do not have a true neurodegenerative (Chan et al., 2004). Because of these similarity, NHPs are not only ideally suited for research of the above diseases, but also offer a unique system for developing and testing new therapeutic approaches in human including stem cell area. To date, there are many successes of monkey ES cell lines establishment have been reported (Thomson et al., 1995; Thomson et al., 1996; Suemori et al., 2001; Cibelli et al., 2002; Vrana et al., 2003; Sasaki et al., 2005). The ES cell lines were established from *in vivo* flushed blastocyst (Thomson et al., 1995; Sasaki et al., 2005), *in vitro* produced blastocyst (IVF and ICSI) (Suemori et al., 2001), as well as parthenogenetic blastocyst (Cibelli et al., 2003; Vrana et al., 2003). However, the success rate of ES cell establishment was quite low and not all of the obtained ES cells retain stability of their ES cell morphology after long time subculture (Sasaki et al., 2005). There were approximately 70% of individually plated

ICMs usually attached to the feeder layer within 2–3 days and initiated outgrowths, and approximately 50% of those outgrowths were able to be subcultured. The ES cell like colonies could be observed within 3–7 days after subcultured, consisting of cells with a high nuclear to cytoplasmic ratio and prominent nucleoli. Finally, about 0-27% ± 6% of the isolated ICMs were exhibit the stable ES cell morphology. However, the higher blastocyst age contributed to a progressively increasing efficiency in ESC line derivation 14% ± 0%, 19% ± 7%, 26% ± 7%, and 41% ± 20% for day 6, 7, 8, and 9 blastocysts, respectively (Mitalipov et al., 2006).

There are several approaches of ES cell derivation have been used such as blastocyst dissection which can use either a small sharp needle or laser beam to dissect ICM from intact blastocyst (Tanaka et al., 2006), blastocyst outgrowth (Tabar et al., 2005) and immunosurgery (Solter, Knowles, 1975; Martin, 1981). Among these techniques, immunosurgery is the most commonly used for separate ICMs form trophectoderm cells (Thomson, et al., 1995; Suemori et al., 2001). A rabbit anti monkey spleen cell antiserum has been used as a primary antibody to bind with the trophectoderm cells then apply guinea pig compliment to lyses the trophectoderm cells. After the trophoblastic layer was cleared, individual ICM clump was isolated and plated onto feeder cells in monkey ES medium for two weeks (Thomson et al., 1995). The central mass of cell was removed from the outgrowth and dissociated into small clumps of cells before replated onto new feeder cells. After the first passaging, colonies with monkey ES cell like morphology were selected for further propagation, characterization and storage. However, xeno-contamination could occur when using immunosurgery because rabbit is a host animal for antiserum production. Morover, the good antiserum for immunosurgery is expensive. Therefore, some labs prefer to used others techniques such as blastocyst dissection and blastocyst outgrowth even

though the obtained colonies were not as clean as immunosurgery. As previously reported of primate ES cells, the limited dissociation during subculture was required to maintain ES cell clusters of 10–50-cells, to enable continued growth (Suemori et al., 2001). If monkey ES cells were dissociated into single or very few cells per clump, they could not survive. This is one of many differences in characteristic between primate ES cell and rodent ES cell. Presence day, monkey ES cells have been differentiated to be several cell types such as endothelium cells (Kaufman et al., 2004), neural progenitors and neurons (Calhoun et al., 2003; Kuo et al., 2003), hepatocyte like cells (Saito et al., 2006), and cardiomyocyte (Schwanke et al., 2006). Since the main purpose of ES cell establishment is stem cell based therapy, so, immunology compatibility between ES cell and patient is needed to be considered. Therefore patient specific ES cells need to be established. Nuclear transfer (NT) and blastomere separation can produce the patient specific ES cell. However, monkey NT is quite difficult and low success. There is only one study reported the birth of monkey life offspring produced by nuclear transfer of embryonic blastomeres into enucleated oocytes (Meng et al., 1997) and there has no success case have been reported until now. In case of somatic cell nuclear transfer (SCNT), there were many attempts to produce the monkey SCNT embryos but the blastocyst rate was quite low (Wolf et al., 1999; Mitalipov et al., 2002; Narita et al., 2007). There was only one report have been demonstrated recently that two monkey ES cell lines could be established from 20 SCNT produced blastocysts (http://blogs.nature.com /reports/theniche/2007/06/oregon_scientist_ reports_first.html). Despite the rarely success, few blastocysts could be produced by nuclear transfer and the in depth mechanisms of monkey nuclear reprogramming processes have not yet been clarified, the blastomere separation seems to have higher possibility to produce the patient

specific stem cell. Chan and colleagues (2000) reported a success of live monkey offspring born from blastomeres separation of eight cell stage embryo. The experiment has been demonstrated that two blastomeres from an eight cell embryo (quadruplets) contain sufficient information to support not only development to blastocyst, but also development to term. One monkey was born while the other three quadruplets miscarried or formed only placental tissue. Moreover, the study also demonstrated that the percentage of split embryos developing into blastocysts was reduced when blastomere separation was performed in more advanced cleavage stage (Chan et al., 2000). However, monkey blastomeres separation in earlier embryonic stage has been demonstrated two years later by Mitalipo and colleagues (2002). Individuals (from 2 cell embryos) and paired (from 4 cell embryos) blastomere were separated mechanically before transferred into empty zonae pellucida. The study suggested that embryos derived from two and four cell blastomeres separation could develop to blastocyst as similar as intact embryos. Although the ratios of ICM to TE and ICM to total cells in blastomere separation derived embryos are equivalent to those of intact blastocysts, the total cell numbers were reduced as a proportion to intact embryo. Interestingly, after differential staining each pair blastomere separation derived embryos, it was found that one embryo has quite different cell number from other (Mitalipo et al., 2002). The result of this study was similar to the previous report by Schramm and Paporcki (2004). They also demonstrated that blastomeres separation derived embryos could develop to blastocyst and the blastocyst rate was reduced if the blastomere derived from embryo at higher stage. According to the above review, it can be concluded that blastomere separation derived embryos could develop to blastocyst as similar as intact embryo and together with the success of mouse ES cells establishment from single blastomeres has been demonstrated in

previous two chapters. So, this experiment would like to study the possibility of ES cell establishment from monkey single blastomere break down derived embryos. However, the suitable protocol for ES cell establishment needs to be firstly established. Therefore, the first objective of this study was to determine the suitable protocol for monkey ES cell establishment from intact blastocyst (Experiment 1) and then use the most suitable protocol to determine the possibility of monkey ES cell establishment from 2CBD and 4CBD derived embryos (Experiment 2).

5.3 Materials and Methods

5.3.1 Monkey embryo culture

ICSI derived zygotes were cultured in HECM-9 medium + 1x AAP for two days, then 4-8 cell stage embryos were transferred to HECM-9 medium + 1x AAP + 5% FBS until reach blastocyst stage in 37°C, 5% CO_2, 5%O_2 and 90%N_2. Day 8 blastocysts were used for ES cell establishment. Some of the embryos at 2C and 4C stage were used for blastomere break down and ES cell establishment from single blastomere experiment.

5.3.2 Feeder cell preparation

Mouse fetal fibroblasts (MFF) were prepared from 13.5 dpc mouse fetuses. The MFFs were cultured in DMEM supplemented with 10% FBS, 200mM L-glutamine and 1x Penicillin/Steptomycin. The MFFs were inactivated with 5 µg/ml mitomycin C (Sigma) for two hrs followed by a thorough wash before plating. For ICM culture, MFF were cultured in 4 well dish with culture media 500 µl per well. The MFF should be prepared about 24h before used. The feeder cell culture media was replaced with monkey ES media at least 3 hrs before ICMs were plated.

5.3.3 Immunosurgery monkey embryo

Blastocysts were briefly incubated in acid tyrode solution to remove the zona-pellucida. Then the zona free embryos were washed in monkey ES medium for four times for a total of 10-15 min. The monkey ES medium contains DMEM/F12 supplemented with 20% FBS Hyclone), 1% Nonessential amino acid, 10 ng/ml basic Fibroblast Growth Factor (bFGF; Invitrogen) and 1000 IU/ml hLIF. The embryos were then move to mDPBS and wash 4 times before move to a drop of 1:250 of CD-3 antibody (Biosource: Cat No. APS0301) and incubate for 15 min in incubator. After incubation, embryos were transferred to mDPBS, washed six times then moved to a drop of 1:5 guinea pig complement and incubated for 30 min in incubator. Then, a small pulled pipette was used for transfer embryos into mDPBS and wash 4 times. Keep pipeting up-and-down several times in each drop with a small pulled pipette to remove lyses TE cells. At this step, the damaged TE cells were easy to remove from an ICM clump. The ICM clump was washed in monkey ES media for three times and transferred onto freshly prepared MFF which pre-incubated with monkey ES media and cultured for 2 days without any movement to allow the ICM attach well onto the feeder. On day 3, half of the media was removed by using a P200 pipette and replaced with fresh media. The media was changed everyday and the ICM outgrowth could be seen on day 3. The ICM outgrowths were continue cultured for 9-16 days before subcultured and spread onto new feeder cells.

5.3.4 Blastocyst dissection

Blastocysts were briefly incubated in acid tyrode solution to remove the zona-pellucida. Then the zona free embryos were washed in monkey ES medium for four times for a total of 10-15 min. Embryos were transferred to mDPBS and washed for

three times, then the insulin syringes, 28g, were used for dissociate the ICM clump from TE cells in mDPBS medium. The ICM was collected and washed three times in monkey ES media and cultured onto freshly prepared feeder cells. Then follow the previously described protocol in 5.3.2.

5.3.5 Blastocyst outgrowth

Blastocysts were briefly incubated in acid tyrode solution to remove the zona-pellucida. Then the zona free embryos were washed in monkey ES medium for four times for a total of 10-15 min and cultured zona free embryos onto freshly prepared feeder cells. Then follow the previously described protocol in 5.3.3.

5.3.6 Nomenclature

ICSI embryo means the embryo produced by intra cytoplasmic sperm injection technique. Embryos derived from the blastomere break-down of two-and-four cell stage embryos were named 2CBD and 4CBD, respectively.

5.3.7 Monkey blastomeres separation

Monkey embryos at 2C and 4C stage were briefly incubated in 0.5% Protease (Sigma) to remove the zona-pellucida. Then the zona free embryos were washed in TALP-Hepes + 10% FBS four times before transferred to a drop of the same medium in a glass chamber on the stage of an inverted microscope. The biopsy pipettes with 20μm of the inner diameter were set up and used for aspirate the individual blastomeres (Fig. 5.1). The separated blastomeres of the same embryo were washed three times and individually cultured alongside with their sister blastomeres.in HECM-9 medium + 1x AAP + 5% FBS in 72 well plate which pre-coated with MFF.

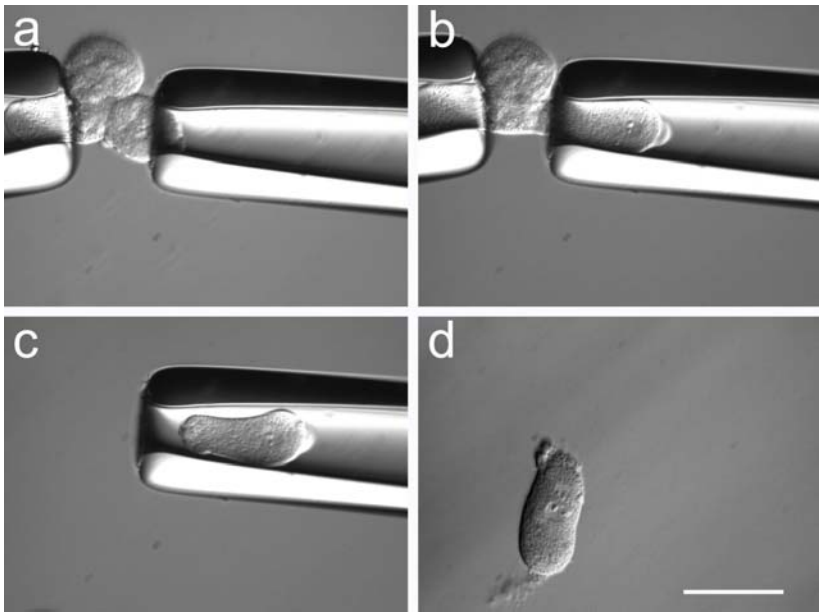


Figure 5.1 Monkey blastomere separation by using two biopsy pipettes. The zona free embryo was fixed with two biopsy pipettes with the appropriate position (a). The left pipette fix one blastomere while the right hand pipette gently suck another blastomere to separate them (b). The separated blastomere was released from its sisters (c), then released a biopsied blastomere into culture medium (d) and individually cultured in HECM-9 medium side by side with its sister blastomeres. Bar = 50 μm

5.3.8 Single blastomere culture

The blastomeres of the 2CBD and 4CBD were cultured individually in a 72-well plate pre-coated with 0.1% gelatin (Sigma). The MFFs were plated at 1,000 cells/well in 10 μl of culture medium 24 hours before use. The blastomeres derived from two cell and four cell embryo were cultured in HECM-9 medium + 1x AAP and after first cleavage, the 2CBD derived embryos were moved to culture in HECM-9 medium + 1x AAP + 0.5% FBS until reach blastocyst stage. While the blastomeres derived from four cell stage embryos were cultured in HECM-9 medium + 1x AAP +

0.5% FBS until reach blastocyst stage. Then monkey ES media was replaced after the blastocyst collapse or ICM clump was isolated.

5.3.9 ES cell establishment from single blastomere

Since the ICM clump of the 4CBD derived blastocysts was not clearly identified. Therefore, blastocyst dissection and blastocyst out growth were used for ES cell establishment. The procedure of blastocyst dissection and blastocyst outgrowth are similar as described above.

5.3.10 Subculture of the primary ICM outgrowths

The primary ICM outgrowths were subcultured using two different subculture techniques, 0.05%Trypsin/EDTA and 5 mg/ml Dispase. For trypsinization, the out growths were lift up from feeder cell and transferred to wash in drops of mDPBS for 4 times before incubating in 0.05% Trypsin/EDTA for 3 min at 37°C. Then gently pipeting up-and-down several times to break the out growth into small pieces which contains about 20-30 cells/piece. Then the small pieces were washed in monkey ES medium for four times before spread onto a new feeder cell pre-incubated with monkey ES medium. For dispase, the dispase was prepared to 5 mg/ml by PBS. The outgrowths were detached from feeder cell and washed for four times in mDPBS then follow the similar protocol as trypsinization procedure. The obtained colonies from both methods were further subcultured by manual dissociation.

5.3.11 Immunocytochemistry

The colonies were washed twice by PBS, fixed with 4% PFA for 30 minutes and incubated with a blocking buffer consisting of 0.2% triton-x 100, 3 mM sodium azide,

0.1% saponin, 2% BSA, and 5% house serum in PBS (-) for one hour. Then the colonies were incubated at room temperature using the following primary antibodies: either mouse monoclonal antibody against human Oct-4 (1:250), rabbit polyclonal antibody against mouse and human Sox-2 (1:100), mouse monoclonal antibody against SSEA-3 (1:50), mouse monoclonal antibody against SSEA-4 (1:50), mouse monoclonal antibody against TRA-1-81 (1:100) and alkaline phosphatase activity.for another two hours. The samples were then washed several times with PBS before being incubated at room temperature with the following secondary antibodies: Alexa fluor® 488 donkey anti mouse IgG (1:1000) and/or Rhodamine Red™- goat anti rabbit IgG (1:1000) for one hour. Finally, the samples were incubated with 5µg/mL Hoechst 33342 for five minutes before a thorough last wash with PBS. Then fresh PBS was added to cover the samples before microscopic examination.

5.3.12 Experimental design

Experiment 1: The monkey blastocysts derived from ICSI were used for monkey ES cell establishment. Immunosurgery, blastocyst dissection and blastocyst outgrowth were used for isolate the ICM from the intact blastocyst. The primary ICM outgrowths were subcultured with either 0.05%Trypsin/EDTA or 5 mg/ml Dispase before re-seeded onto a freshly prepared feeder cells pre-incubated with monkey ES medium. The efficiency of ICM isolation techniques and technique for sub-culturing the primary ICM outgrowth were determined.

Experiment 2: Two and four cell stages of monkey ICSI derived embryos were used for study the possibility of single blastomeres development after cultured *in vitro* system. Then, the blastocysts derived from 2CBD and 4CBD embryos were used for ES cell establishment. Since the ICM clump of single blastomere derived embryos

were small, therefore, only blastocyst dissection and blastocyst outgrowth techniques were used for ICM isolation. The primary ICM outgrowths were subcultured with 0.05% Trypsin/EDTA and re-plated onto a freshly prepared feeder cells pre-incubated with monkey ES medium. The obtained colonies were manually subcultured.

5.4 Results

5.4.1 Comparison of ICM isolation techniques

Twenty-six ICSI derived embryos were used for three isolation procedures, immunosurgery, blastocyst outgrowth and blastocyst dissection (Table 5.1). The result found that 14 ICM clumps from 14 embryos were isolated by immunosurgery (100%). Eight embryos were used for blastocyst outgrowth and all of 8 embryos could attach and form outgrowths on feeder cells (100%). Four embryos were used for blastocyst dissection, the result found that only 3 ICM clumps (75%) could attach on feeder cells while another ICM reformed as blastocyst and floated in the culture media. The ICM clumps from immunosurgery, blastocyst dissection and blastocyst outgrowths could attach the feeder cells at about 24 to 48h after culture. The outgrowths could be observed around day 3 after culture. Morphology of the outgrowths from three different procedures was showed in figure 5.2. The pictures showed that immunosurgery could generate less accumulated cell at the central of colony than blastocyst dissection and blastocyst derived outgrowths, respectively. The outgrowths from each treatment were cultured for 9-16 days before subculture to new feeder cells. Finally, there were only 12 outgrowths from immunosurgery (85.7%; 12/14) able to be subcultured while the outgrowths from blstocyst outgrowth and blastocyst dissection were unable to be subcultured (0%; 0/3 and 0%; 0/8,

respectively) because these outgrowths had differentiated morphology such as accumulated multi layers cell in the central of the colony or had mixture of differentiated cells from trophoblastic lineage.

Table 5.1 Comparison of ICM isolation techniques

Technique	No. embryo	No. Outgrowth (%)	Remark
Immunosurgery	14	14 (100)	-
Dissection	4	3 (75)	1 ICM reform blastocyst (25%)
Out growth	8	8 (100)	-

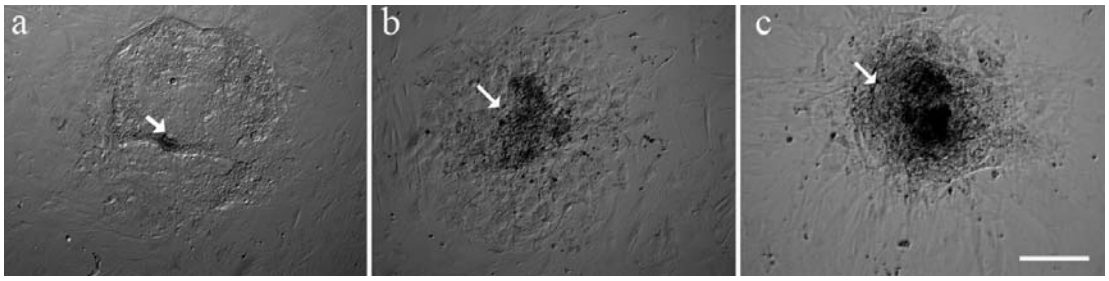


Figure 5.2 Comparison of the ICM outgrowth morphology (day 10) derived from immunosurgery (a) blastocyst dissection (b) and blastocyst outgrowth (c). The arrows point accumulated cells of each outgrowth. Bar = 100

5.4.2 Comparison of subculture techniques

Twelve ICM outgrowths from immunosurgery were subcultured with either 0.05% Trypsin/EDTA or 5 mg/ml Dispase before spreading onto new feeder cells, six outgrowths per treatment (Table 5.2). The new colonies were appeared approximately at 1-3 days after subculture. Among those, there were two cell lines from Trypsin/EDTA (33.3%) which showed ES cell like morphology (Fig. 5.3). Since this

was the first subculture, therefore, both clean (Fig. 5.3a and 5.3b) and colonies with some differentiated cells could also be observed. (Fig. 3c and d). Only the clean colonies were picked up for propagation by manual subculture technique. After passage four, only one cell line (16.7%) retained monkey ES cell like morphology (Table 5.2).

Table 5.2 Comparison of primary ICM outgrowth subculture technique

Technique	No. Outgrowth	Colony appearance	ES cell like morphology (%)	
			P1	P4
0.05%Trypsin/EDTA	6	6	2 (33.3)	1 (16.7)
5mg/ml Dispase	6	6	0 (0.0)	0 (0.0)

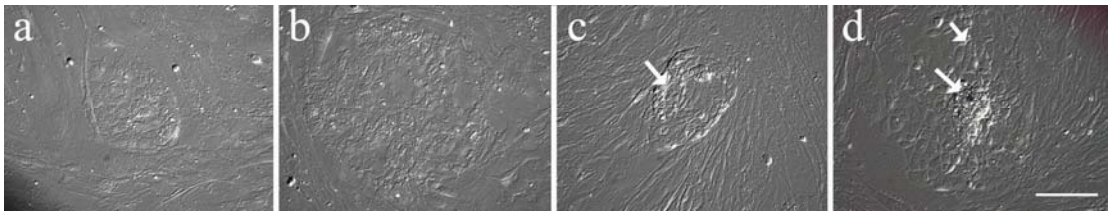


Figure 5.3 Morphology of monkey ES colonies on day 3 after trypsinization. After the first passage by 0.05% Trypsin/EDTA found that some colonies had ES like cell morphology (a-b) while some colonies had differentiated cells (arrow) mixture inside the colony (c-d). Bar = 50 μm.

Monkey ES like cell colonies could be observed approximately at 1-3 days after subculture and those colonies showed monkey ES like cell morphology such as monolayer, clear edge, high nuclear-cytoplasmic ratio and the cells constructed as honeycombed structure (Fig. 4-5).

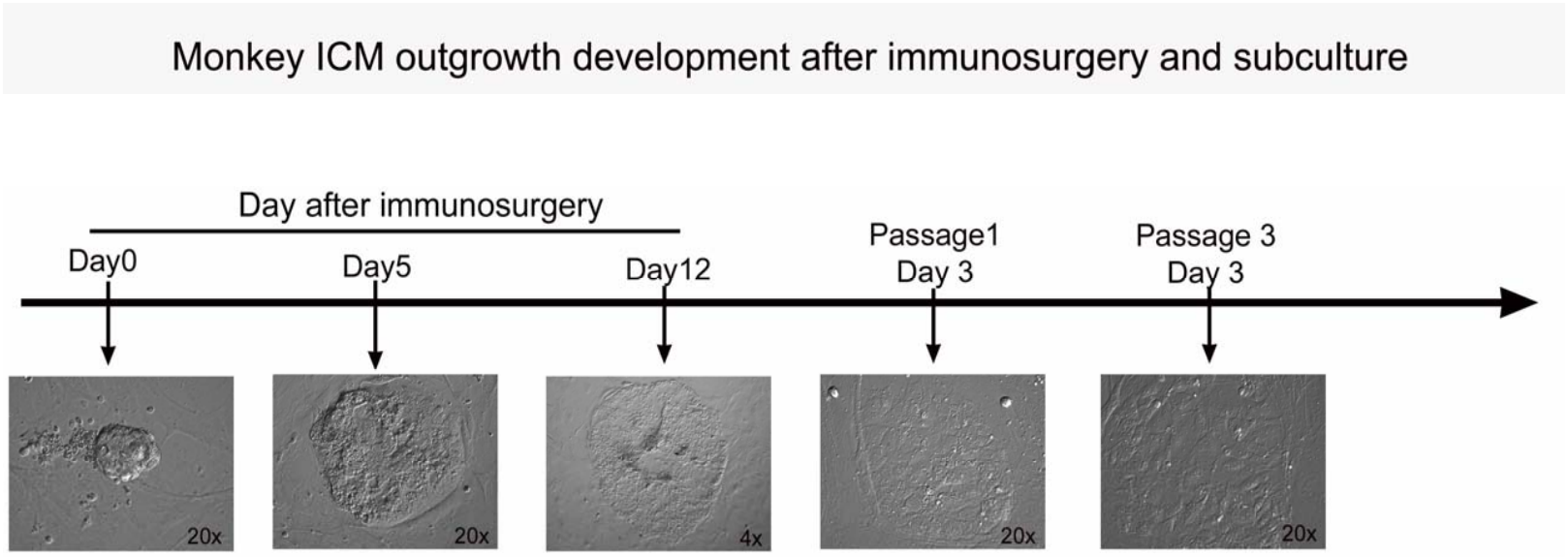


Figure 5. 4 Morphology of the primary ICM outgrowth development after immunosurgery (D0-D12). On day 12, the primary ICM outgrowth was subcultured by 0.05% Trypsin/EDTA but the later passages were performed by manual dissociation.

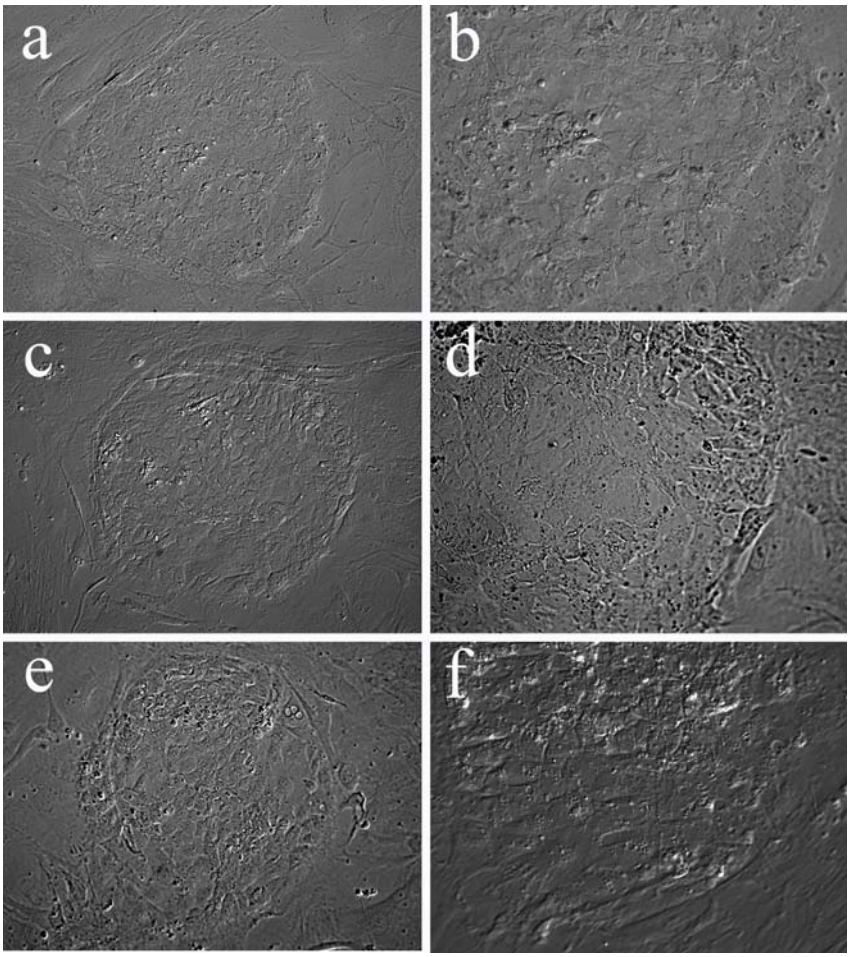


Figure 5.5 Monkey ES colonies at different passages. The colonies derived from passage 2-4 (a,c,e, respectively) showed monolayer and clear edge. All of these colonies had high nuclear-cytoplasmic ratio and cell construct as the honeycombed structure. (a,c and e were 10x magnification and b,d and f were 20x magnification)

5.4.3 Monkey ES markers expression

The ES like cell morphology was subcultured for four passages before determining ES markers expression. Firstly, the AP activity could be observed from every colony but the expression level was not consistent even in the same colony. Some areas of a colony showed strong AP activity while some areas showed less or no activity (Fig. 5.6). The immunofluorescent staining of other monkey ES specific markers had been done by Oct-4, Sox-2, SSEA-3, SSEA-4 and TRA-1-81. It was found that the colony expressed all ES markers but the expression levels were low (Fig. 5.7). Eventually, this ES like cell line could not be maintained for further longer. It was then spontaneous differentiated after longer subculture.

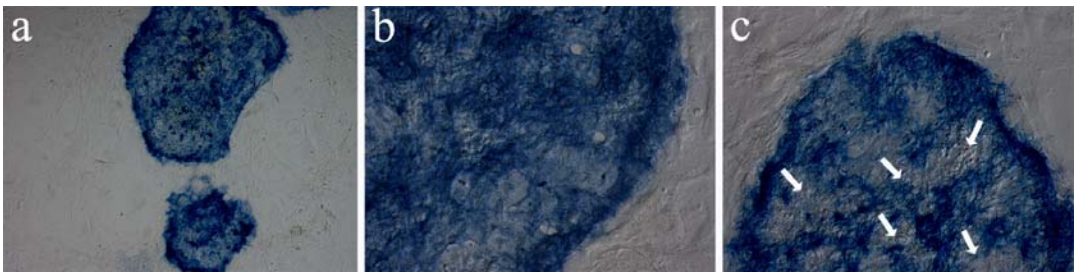


Figure 5.6 Alkaline phosphatase (AP) activity staining of monkey ES cell colony. The figures showed that the expression level of AP activity was not consistent (a-c). Especially, the colony in panel C has several areas showed paleness AP activity (arrows). (Fig a = 4x magnification, Fig b and c = 10x magnification)

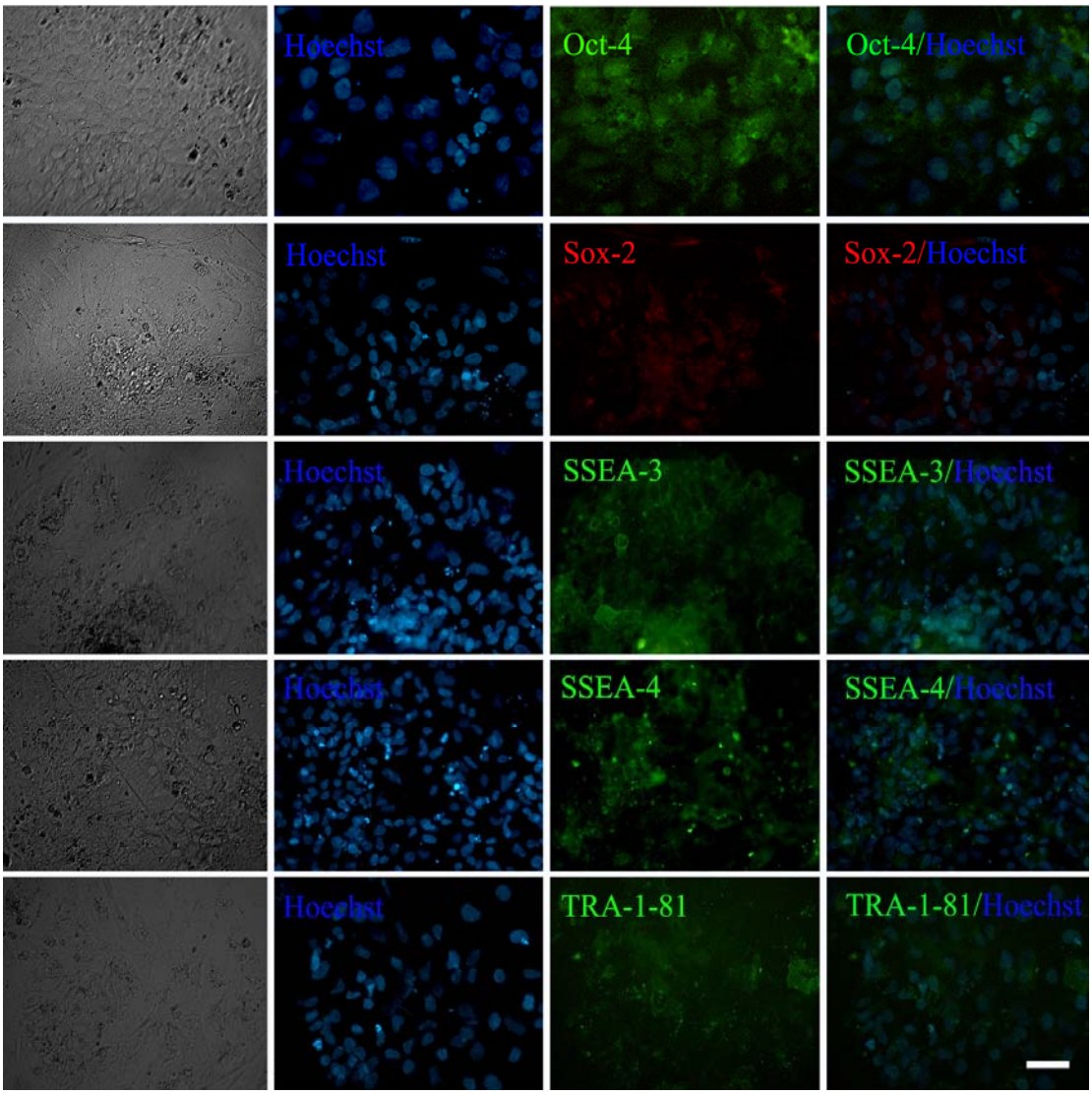


Figure 5.7 Monkey ES cell specific markers staining. Bar = 100μm.

5.4.4 Monkey single blastomeres development

Blastomeres of 2C and 4C stage embryos were separated into single blastomeres and cultured individually along with their sister blastomeres. Developmental rate had been daily recorded. The result found that blastomeres derived from 2C stage embryos could not have further division after separation (Table 5.3). However, the blastomeres derived from 4C stage embryos could divide and develop to blastocyst, consequently. There were 76 blastomeres from 20 embryos (three-four blastomeres/embryo) were separated and individually cultured in 10μl of culture media as described in 5.3.8. At 24h after cultured, there were 55 blastomeres (72.4%) which had one cell division to generate two blastomeres and 27 blastomeres (35.5%) which developed to 4C stage on day 2. Morula stage embryo was found on day 3 (18 blastomeres, 23.7%). After 5 days, six blastomeres (7.9%) could develop to blastocyst stage (Table 5.3 and Fig. 5.9). However, there was none of blastomeres from the same embryo could survive as a full set; some of them were lysed or stop development (Fig.

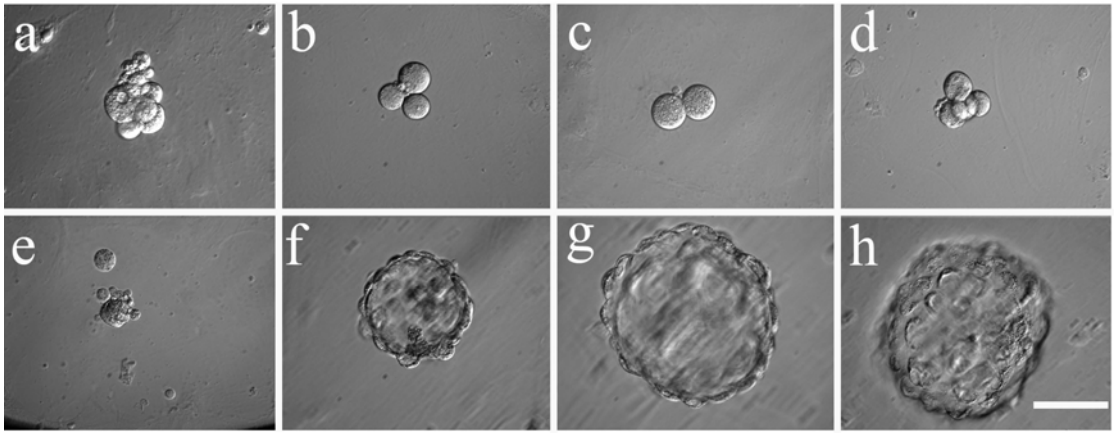


Figure 5.8 Development of 4CBD derived embryos which came from the same original embryo on day 1 (a-d) and day 5 (e-h) after cultured.

Bar = 50μm.

Table 5.3 Development of monkey blastomeres break down derived embryos

Type	No. embryo	No. blastomere	D1	D2		D3			D4				D5	
			1C	2C	2C	4C	8C	4C	8C	Mor	4C	8C	Mor	Mor	Blast
2CBD	2	4	4	0	0	0	0	0	0	0	0	0	0	0	0
		%	100	0	0	0	0	0	0	0	0	0	0	0	0
4CBD	20	76	21	55	22	27	15	19	9	18	17	7	19	15	6
		%	27.6	72.4	28.9	35.5	19.7	25.0	11.8	23.7	22.4	9.2	25.0	19.7	7.9

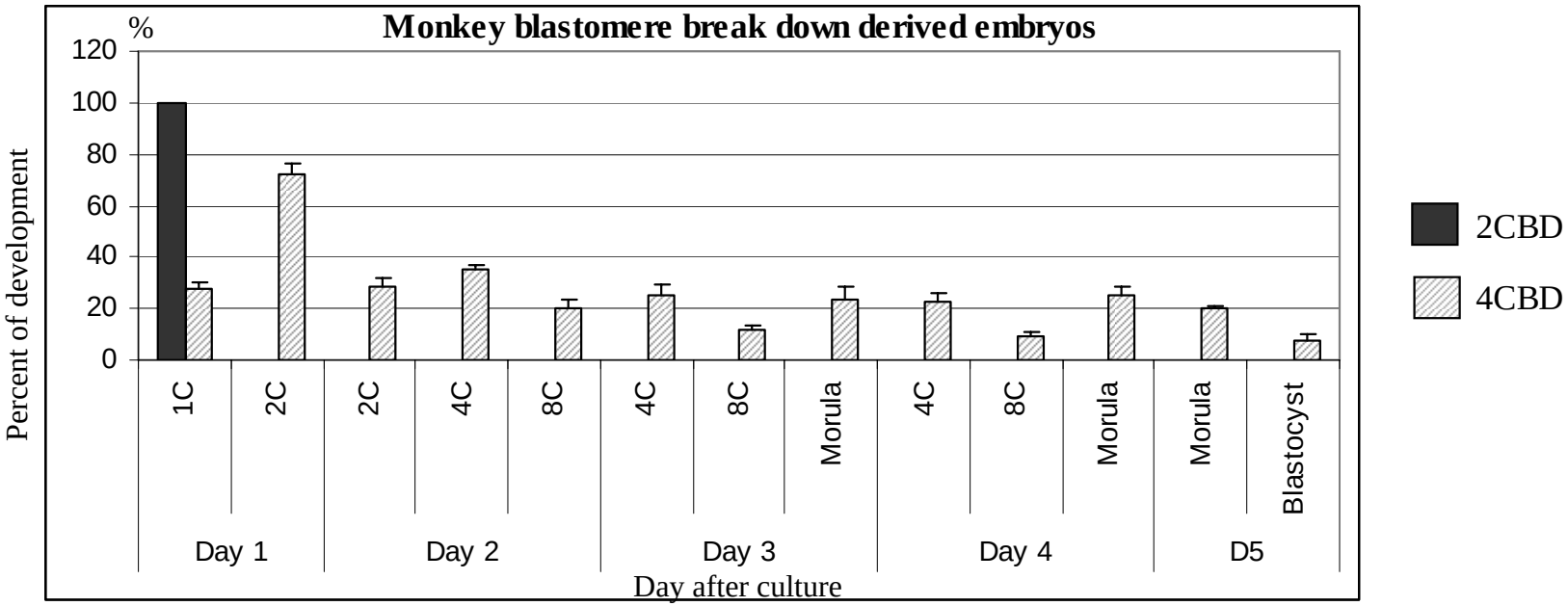


Figure 5.9 Histogram show development of 2CBD and 4CBD derived embryos

5.4.5 Monkey ES cell establishment from single blastomere derived embryo

Four out of six blastocysts derived from 4CBD were used for study the possibility of ES cell establishment from monkey single blastomere. Since the ICM clump of 4CBD derived blastocysts was not clear as the normal embryo (Fig. 5.10). Therefore, blastocyst dissection and blastocyst outgrowth techniques were used for ES cell establishment and two 4CBD derived blastocysts were used for each technique.

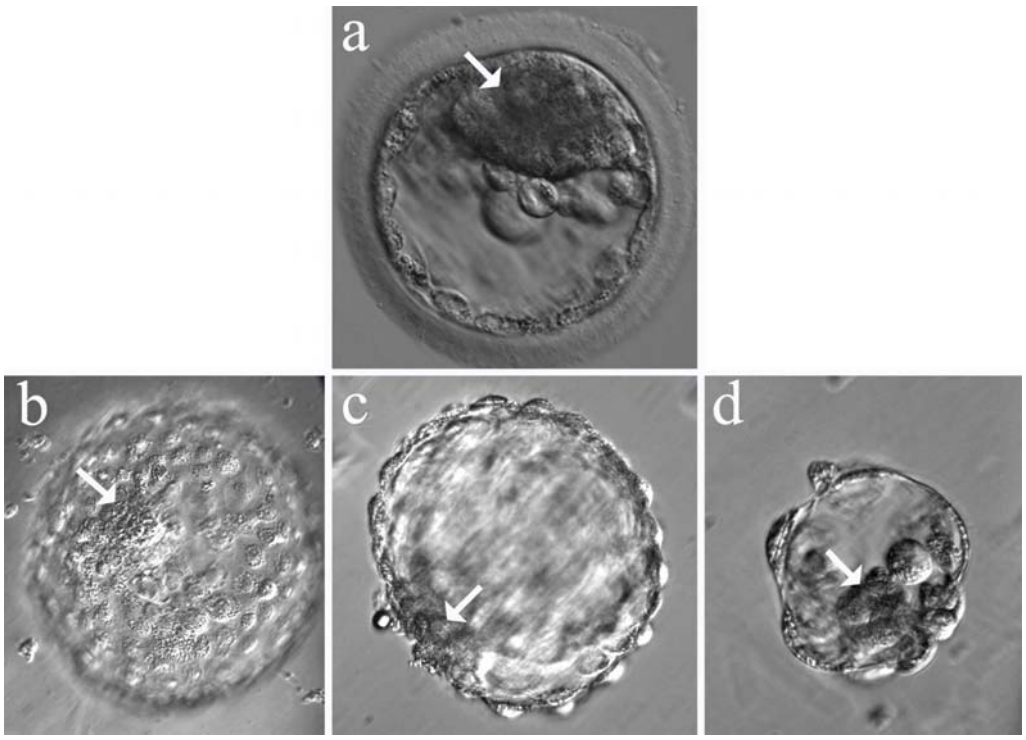


Figure 5.10 The ICSI derived intact blastocysts (a) has more ICM cells than 4CBD derived blastocysts (b-d). The ICM cells (arrow) inside the embryos. Bar = 100μm.

Table 5.4 Technical comparison of the ICM isolation from blastocyst derived from 4CBD embryos.

Technique	No. embryo	No. out growth (%)	ES-like cell morphology
Blastocyst dissection	2	2 (100)	Not honeycombed structure
Blastocyst outgrowth	2	2 (100)	Not honeycombed structure

The result found that all of the ICMs isolated by blastocyst dissection and blastocyst outgrowth could form primary ICM outgrowth at approximately 2-3 days after cultured on feeder cells (Table 5.4). The established outgrowths from two techniques had different morphology as shown in figure 5.11. All four primary ICM outgrowths were maintained for 9-16 days before subculturing with 0.05%Trypsin/EDTA and spreading onto a new feeder cells. New colonies from four outgrowths could be observed at 1-3 days later and were subcultured with manual dissociation technique for later passages.

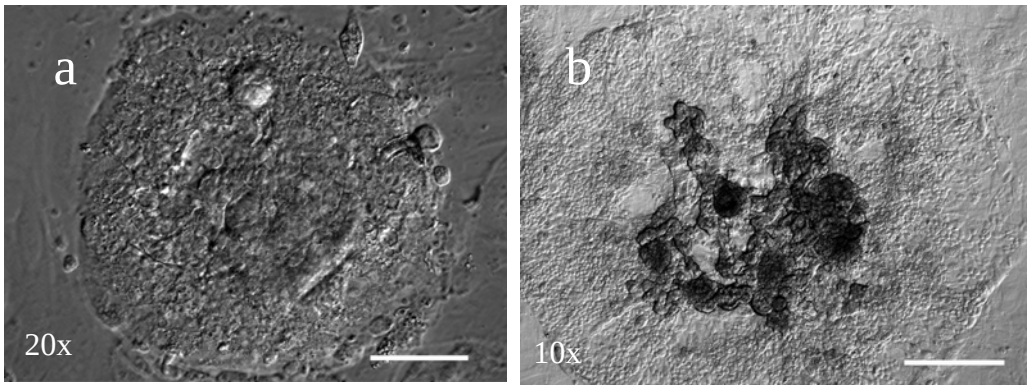


Figure 5.11 The ICM outgrowth of 4CBD derived embryo on day 3 after blastocyst dissection (a) and blastocyst outgrowth (b).

After subcultured four primary ICM outgrowths with Trypsin/EDTA found that, the colonies derived from blastocyst outgrowth always form as clumps with multilayer of cells while the outgrowths from blastocyst dissection form monolayer, clear edge colonies with some accumulated cell at the central of colonies (Fig. 5.12). However, structure of the cells inside colony was not a honeycombed. Some of the colonies had the mixture of differentiated cells inside. There were only the monolayer colonies with clear edge were selected for further subculture to study the cells morphology after a few more subcultures. After three times subculture, the established colonies had more homogenous cell morphology than the earlier passage but some colonies still had multi layers cells accumulated in the central of colony (Fig. 5.13).

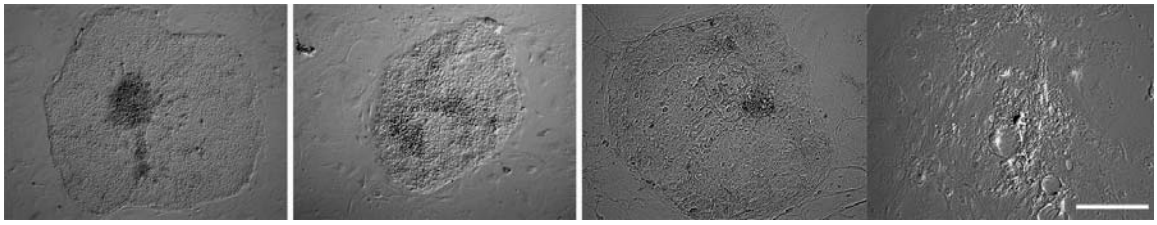


Figure 5.12 Morphology of the obtained colonies after break down the primary ICM out growth which derived from blastocyst dissection by trypsinization technique.

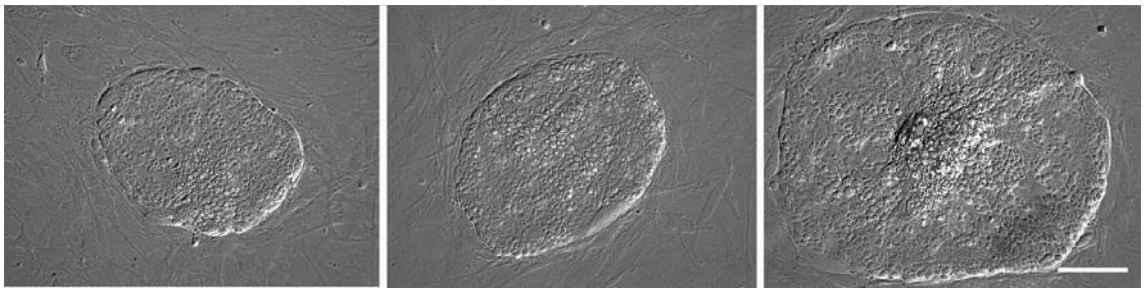


Figure 5.13 Morphology of the colonies obtained from 4CBD embryo at passage 3. Bar = 100μm

5.4.6 Monkey ES marker expression from the 4CBD derived colonies

The colonies established from blastocyst dissection technique were expanded and tested the monkey ES cell marker including AP activity and Oct-4 and Sox-2. The result found that only the accumulated cell at the center of colony showed AP positive while the monolayer cells showed AP negative. But every cell of the colony showed Oct-4 and Sox-2 positive (Fig. 5.14). Even thought, the colonies showed Oct-4 and Sox-2 positive but the expression of other monkey ES cell markers could not be observed after immunocytochemistry. Moreover, the morphology of the established colonies was not similar to the monkey ES cell. Therefore, it was hesitating to conclude that this experiment was successfully established monkey ES cell from single blastomere.

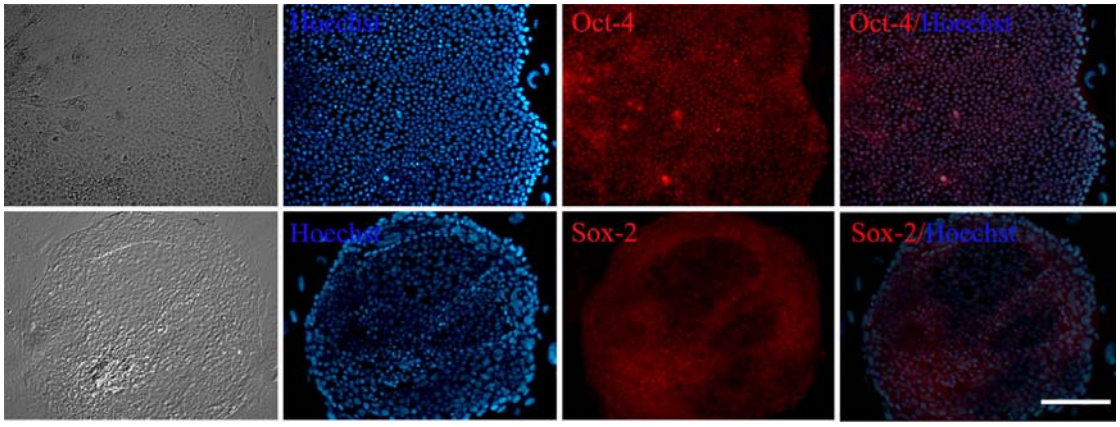


Figure 5.14 Oct-4 and Sox-2 staining on the colony derived from ICM outgrowth of 4CBD derived embryo. Bar = 100μm.

5.5 Discussion

These studies have been demonstrated that immunosurgery is a sufficient method to isolate monkey ICM cells and use of 0.05%Trypsin/EDTA for subculture the primary ICM outgrowths provided the higher chance to get monkey ES like cell morphology than use of 5 mg/ml dispase. Moreover, this study also demonstrated that the single blastomere derived from early preimplantation monkey embryo (2 and 4 cell stage) could be individually cultured *in vitro* and it could develop to blastocyst.

ICM isolation technique is an important step of the ES cell establishment successiveness. In intact embryo, trophoblastic cells induce ICM differentiation to be three embryonic germ layers by suppress Oct-4 and Nanog expression level (Robert et al., 2004). Therefore, completely removed trophoblastic cells provide more benefit to ICM cells turn to be ES cells. Immunosurgery is the most frequently used for isolating an ICM from an intact embryo because of the specificity of troproblastic cells' antigen and antibody complexes. Therefore, the obtained ICM cells were cleaned from trophoblastic cells. The results of experiment one have been demonstrated that 25% of ICM from blastocyst dissection could reform blastocyst and float in a medium instead of attached to feeder cells. That was because of blastocyst dissection technique could not remove all trophoblastic cells especially cells that aligned at sides and underneath the ICM clump. Therefore, the remaining trophoblast cells could reform blastocyst but smaller size. For blastocyst outgrowth, it is the easiest technique and 100% outgrowths were obtained after culture the zona free embryos onto feeder cells. However, in this study, there was no ES cell could be established from this technique. The expression of

caudal-related homeobox protein Cdx2, which is the trophoblast-specific transcription factor, suppressed Oct-4 expression of ICM cells. Oct4 is normally expressed in all blastomeres of the cleavage stage embryo, but becomes restricted to the ICM after initiation of blastocyst formation (Palmieri et al., 1994) and also ES cell maintain their pluripotentcy stage by the expression of Oct-4 (Nichols et al., 1998; Niwa et al., 2000). The conditional loss of Oct-4 expression in ES cells triggers trophoblast-like differentiation (Niwa et al., 2000; Hay et al., 2004; Strumpf et al., 2005; Tulkunowa et al., 2006). Once Cdx2 from trophoblast cells suppress Oct-4 expression of ICM, then the pluripotency stage of ICM was not occurred. Therefore, this is might be one of the possible reasons why we could not get ES cells from blastocyst outgrowth.

The primary ICM outgrowths were determined and choosed the least differentiated outgrowth for subculture with either 0.05%Trypsin/EDTA or 5mg/ml Dispase. There are several types of animal derived enzymes used for dissociating the monkey ES cells such as collagenase IV (Kuo et al., 2003), dispase (Li et al., 2005) and trypsin (Thomson et al., 1995; Cowan et al., 2004). Among those enzymes, dispase and collagenase activities result the less damage of cell membrane than trypsin, and both digestion activities are not different. Therefore, dispase and trypsin were chosen for subculture the primary ICM outgrowths. Our results have been demonstrated that ES like cell morphology (33.3%) could be obtained after using 0.05% Trypsin/EDTA for subculture the primary ICM outgrowths. While there was no ES cell like morphology obtained from 5mg/ml Dispase. This might because trypsin has higher digestion activity than dispase (Fig. 5.15). Trypsin could make ICM outgrowth became smaller clumps and single cells as well. This property probably benefit to ES cell formation in term of

differentiated cell screening, because the differentiated cells were easier to lose from attachment and might be discarded after broken down into single cells during subculture.

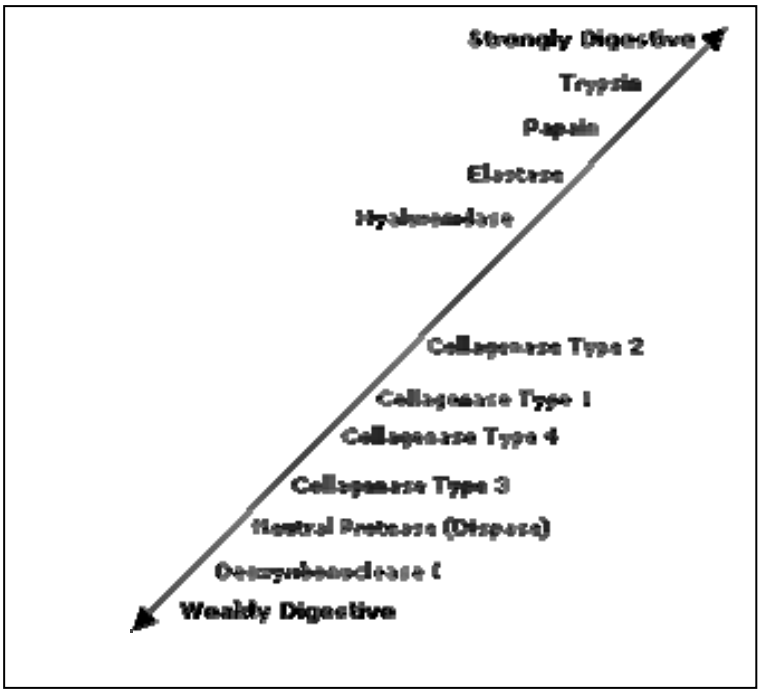


Figure 5. 15 Enzyme digestion scale shows that dispase has a minder digestion activity than trypsin (http://www.tissuedissociation.com/faq.html#2.1.1).

Since monkey ES cell can not survive if it was broken down into single cells or very small clump of cells and trypsin might cause excessive damage to the monkey ES cells (Suemori et al., 2001). Therefore, the suitable concentration and exposure time were needed to be determined. However, using 0.05%Trypsin/EDTA treated the primary ICM outgrowths for 3 min could get approximately 33% of ES like cell morphology. Mechanical or manual subculture is another preferably used, because with this method,

cells do not need to expose with other animal derived enzymes. But manual subculture needs to do one by one colony; it is not practical to do with a large scale culture. Usually, the manual subculture is applied when their ES cells are fewer than five passages. Then enzymatic passage with trypsin is used instead with a large scale and routine subculture. In rodent ES cell, Trypsin/EDTA can be used for subculture mouse ES cells because mouse ES cells are able to divide even broken down into very small clumps or even single cells. Further more, there are some differences between mouse and monkey ES cells such as ES cell morphology, ES markers set, cytokine factors control self renewal and maintaining undifferentiated stage. Moreover, the doubling time of ES cells is also different. Primate ES cells show a longer average population doubling time than that of mES cells (Carpenter et al., 2000). Therefore, monkey ES cell need to be subcultured every 5-7 days while mouse ES cell needs every 2-3 days interval.

For mouse ES cells, it is possible to substitute the feeder layer of embryonic fibroblasts with recombinant LIF, which controls self-renewal of mouse ES cells through the gp130 receptor subunit to activate STAT3. In contrast, LIF is insufficient to inhibit the differentiation of hES cells which continue to be cultured routinely on feeder layers of MFFs or feeder cells from human tissues. (Thomson et al, 1998; Reubinoff et al., 2000; Pera M.F., 2001; Daheron et al., 2004). The identity of the essential self-renewal signal(s) provided to monkey ES cells by MFF feeder cells remains not defined. Therefore, people who work with monkey ES cells still prefer to culture their ES cell on feeder cells as routine (Schwaken et al,. 2006; Saito et al., 2006). In monkey, bFGF has been used to maintain the undifferentiated stage but bFGF is expensive, so, this

requirement is obstacle to the use of monkey ES cells (Yamashita et al., 2006). Recently, Yamashita and colleagures (2006) suggested that, instead of using bFGF, the high density of MFF (1 to 1.5 x 10^5 cell/cm^2) could successful maintain undifferentiated monkey ES cell for two years. Culturing and maintaining monkey ES cell undifferentiated stage are difficult and much more complicated than those of mouse ES cell because monkey ES cell always spontaneous differentiate during *in vitro* culture. Therefore, not every cell lines could be maintained after long time subculture (Sasaki et al., 2005; Yamashita et al., 2006). In this experiment, two lines have been shown ES like cell morphology after primary subculture ICM outgrowth but they could not be maintained for longer time. One line of ES like cell morphology was differentiated after passage two while another line could be maintained for a little bit longer then higher spontaneous differentiation were observed finally. However, the further study and more replication of experiment are needed to confirm the efficiency of ES cell establishment.

Blastomere separation has been used for producing the monkey monozygotic twins' monkey (Chan et al., 2000; Mitalipov et al., 2002). One live offspring was born from a pair of blastomeres (quadruplet) separated from eight cells stage embryos (Chan et al., 2000). Later report by Mitalipov and colleagues (2002), two and four cell stage embryos had been used in producing monozygotic twin as well but there was no live offspring was born from both embryonic stage. However, there was only two cell embryos have been separated into single blastomere. The others were separated as a pair of blastomeres and the separated blastomeres were transferred to empty zona pellucida for further culture until blastocyst. The blastomere separation derived blastocysts of those two experiments were comparable to intact embryo. However, their results were

different from this study. That might because, in this experiment, all of the blastomeres from two and four cell embryos were separated into singles and co-cultured with feeder cells instead of transferred to empty zona pellucida. Even though, the single blastomeres cultured system has been proved by culturing mouse two and four cell embryo in previous two chapters, this culture system seems to be not suitable for monkey single blastomere. The single blastomeres development outside the zona pellucida might not appropriate for monkey blastomere. From this study also found that monkey blastomeres could not be separated from its sisters by briefly exposure to PBS (-) and gentle repeatedly pipetting to separate them as it was done in mouse blastomere separation. Instead of using PBS (-) and manual pipetting, manipulation under microscope with two biopsy pipettes were needed. Moreover, longer exposure of monkey blastomere in PBS (-) resulting in the shrinking of blastomere cell membrane. Separation by biopsy pipettes in TALP-Hepes + 10% FBS provide more healthy blastomeres after separation.

In conclusion, the results of preliminary study has been demonstrated that using immunosurgery to isolate ICM cells from intact blastocysts could get better quantity and quality of ICM outgrowths than blastocyst dissection or blastocyst outgrowth. Moreover, using 0.05% Trypsin/EDTA to subculture the primary ICM outgrowths could provide higher chance to get ES like cell morphology. From the first experiment, two ES like cell morphology could be observed after trypsinization but one of the differentiated after passage two and another has spontaneous differentiation after longer culture. The second experiment found that blastomeres of monkey two and four cell embryos could be separated into single blastomeres by biopsy pipettes. 2CBD derived blastomeres could not develop after individually culture while 7.9% of the 4CBD derived embryo

could develop to blastocyst on day five after separation. The 4CBD derived blastocysts were used for ES cell establishment but the morphology of the obtained colonies was not similar to normal monkey ES cell, even though the obtained colonies showed Oct-4 and Sox-2 positive. After this preliminary study found that there are many factors could influence the success of monkey ES cell establishment from single blastomere such as the suitable blastomere separation procedure, culture system, and more knowledge about the character of monkey single blastomere development. Therefore, the further studies about those subjects are also needed to be verified.

5.6 References

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Eldor, I.J. and Thomson, J.A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. **Dev. Biol.** 227: 271–278.

Calhoun, J.D., Lambert, N.A., Mitalipova, M.M., Noggle. S.A., Lyons, I., Condie, B.G. and Stice, S. (2003). Differentiation of rhesus embryonic stem cells to neural progenitors and neurons. **Biochemical and biophysical Research Communications** 306: 191-197.

Chan, W.S.A., Dominko, T., Luetjens, C.M., Neuber, E., Martinovich, C., Hewitson, L., Simerly, C.R. and Schatten G.P. (2000). Clonal propagation of primate offspring by embryo splitting. **Science** 287: 317-319.

Chan, W.S.A. (2004). Transgenic Nonhuman Primates. **Proceeding of post-conference symposium associated with the 2004 IETS annual meeting**. January 14-15,

2004. Portland,Oregon, USA.

Cibelli, J.B., Grant, K.A., Chapman, K.B., Cunniff, K., Worst, T., Green, H.L., Walker, S.J., Gutin, P.H., Vilner, L., Tabar, V., Dominko, T., Kane, J., Wettstein, P.J., Lanza, R.P., Studer, L., Vrana, K.E. and West, M.D. (2002). Parthenogenetic stem cells in nonhuman primates. **Science** 295:819.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D. and Melton, D. (2004). Derivation of embryonic stem cell lines from human blastocysts. **N. Eng. J. Med.** 350: 1353-1356.

Daheron, L., Opitz, S.L., Zaehres, H., Lensch, W.M., Andrews, P.W., Eldor, J. i. and Daley, G.Q. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. **Stem Cells** 22: 770–778.

DeWit, N. and Baker, M. (2007). Oregon scientist reports first ES cells from cloned primate embryos [On-line]. Available at: http://blogs.nature.com/reports/theniche/2007/06/oregon_scientist_reports_first.html.

Kaufman, D.S., Lewiz, R.L., Hanson, E.T.,m Auerbach, R., Plendl, J. and Thomson, J.A. (2004). Funtional endotherlial cells derived from rhesus monkry embryonic stem cells. **Blood** 103: 1325-1332.

Hay, D. C., Sutherland, L., Clark, J. and Burdon, T. (2004). Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. **Stem Cells** 22: 225-235.

Kuo, H.C., Pau, K.Y.F., Yeoman, R.R., Mitalipov, S.M., Okano, H. and Wolf, D.P. (2003). Differentiation of Monkey Embryonic Stem Cells into Neural Lineages.

Biol. Reprod. 68:1727–1735.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. **Proc. Natl. Acad. Sci. U.S.A**. 78:7634–7638.

Meng, L., Ely, J.J., Stouffer, R.L. and Wolf, D.P. Rhesus monkeys produced by nuclear transfer. **Biol. Reprod.** 57:454-459.

Mitalipov, S.M., Yeoman, R.R., Kuo, H.C. and Wolf, D.P. (2002). Monozygotic twinning in rhesus monkeys by manipulation of in vitro derived embryos. **Biol. Reprod.** 66: 1449-1455.

Mitalipov, S., Kuo, H.C., Byrne, J., Clepper, L., Meisner, L., Johnson, J., Zeier, R. and Wolf, D. (2006). Isolation and Characterization of Novel Rhesus Monkey Embryonic Stem Cell lines. **Stem Cells** 24:2177-2186.

Narita, J.O., Tsuchiya, H., Takada, T. and Torii, R. (2007). Cloned blastocysts produced by nuclear transfer from somatic cells in cynomolgus monkeys *(Macaca fascicularis).* **Primates** 48: 232-240.

NavdeBerg, J.L. and Blangero, W.S. (1996). Strategies for using nonhuman primates in genetic research on multifactorial diseases. **Lab. Anim. Sci.** 46: 146-151.

Nichols, J., Zevnik, B., Anastassiadia, K., Niwa, H., Nebenius, D.K., Chambers, I., Scholer, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the pou transcription factor Oct-4. **Cell** 95: 379-391.

Niwa, H., Miyazaki, J., Smith and A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. **Nat. Genet.**

24: 372–376.

Palmieri, S. L., Peter, W., Hess, H. and Scholer, H. R. (1994). Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. **Dev. Biol.** 166: 259-267.

Pera, M.F. (2001). Human pluripotent stem cells: a progress report. **Curr. Opin. Genet. Dev.** 11:595-599.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro.* **Nat. Biotechnol.** 18: 399–404.

Roberts, R.M., Ezashi, T. and Das, P. (2004). Trophoblast gene expression:Transcription factors in the specification of early trophoblast. **Reprod. Biol. Endo.** 2:24.

Saito, K., Yoshikawa, M., Ouji, Y., Moriya, K., Nishiofuku, M., Ueda, S., Hayashi, N., Ishizaka, S. and Fukui, H. (2006). Promoted differentiation of cynomolgus monkey ES cells into hepatocyte-like cells by co-culture with mouse fetal liver-derived cells. **World J. Gastroenterol.** 12: 6818-6827.

Sasaki, E., Hanazawa, K., Kurita, R., Akatsuka A., Yoshizaki, T., Ishii, H., tanioka, Y., Ohnishi, Y., Suemizu, H., Sugawara, A., Tamaoka, N., Izawa, K., Nakazaki, Y., Hamada, H., Suemori, H., Asano, S., Nakatsuji, N., Okana, H. and Tani, K. (2005). Establishment of novel embryonic stem cell lines derived from the common marmoset (Callithrix jacchus). **Stem Cells** 23: 1304-1313.

Schramm, R.D. and Paprocki, A.M. (2004). Stratergies for the productionof genetically identical monkeys by blastomere separation. **Proceeding of post-conference**

symposium associated with the 2004 IETS annual meeting. January 14-15, 2004. Portland,Oregon, USA.

Schwanke, K., Wunderlich, S., Reppel, M., Winkler, M.E., Matzkies, M., Groos, S., Itskovitz-Eldor, J., Simon, A.R., Hescheler, J., Haverich, A. and Martin, U. (2006). Generation and characterization of functional cardiomyocytes from rhesus monkey embryonic stem cells. **Stem Cells** 24:1423-1432.

Solter, D. and Knowles, B.B. (1975). Immunosurgery of mouse blastocyst. **Proc. Natl. Acad. Sci. U.S.A**. 72:5099–5102.

Strumpf, D., Mao1, C.A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. **Development** 132: 2093-2102.

Suemori, H., Tada, T., Torii, R., Hosoi, Y., Kobayashi, K., Imahie, H., Kondo, Y., Iritani, A. and Nakatsuji, N. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. **Dev. Dyn.** 222:273–279.

Tabar, M.H., Javadnia, F., Orazizadeh, M. and Baazm, M. (2005). Isolation and differentiation of mouse embryonic stem cells. **Iranian Journal of Reproductive Medicine** 3: 42-46.

Tanaka, N., Takeuchi, T., Neri, Q.V., Sills, E.S. and Palermo, G.D. (2006). Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model. **Journal of Translational Medicine** 4:20. [On-line]

Available at: http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=1479373&blobtype=pdf.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A. and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. **Proc. Natl. Acad. Sci. U.S.A**. 55:254–259.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P. and Hearn, J.P. (1996). Pluripotent cell lines from common marmoset (Callithrix jacchus) blastocysts. **Biol. Reprod.** 55:254–259.

Thomson, J.A., Eldor, J.I., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. **Science** 282: 1145–1147.

Tolkunova, E., Cavaleri, F., Eckardt, S., Reinbold, R., Christenson, L.K., Schöler, H.R. and Tomilin, A. (2006). The Caudal-Related Protein Cdx2 Promotes Trophoblast Differentiation of Mouse Embryonic Stem Cells. **Stem Cells** 24:139-144.

Vrana, K.E., Hipp, J.D., Goss, A.M., McCool, B.A., Riddle, D.R., Walker, S.J., Wettstein, P.J., Studer, L.P.,Tabar, V., Cunniff, K., Chapman, K., Vilner, L., West, M.D., Grant, K.A. and Cibelli, J.B. (2003). Nonhuman primate parthenogenetic stem cells. **Proc. Natl. Acad. Sci. U.S.A**. 100: 11911–11916.

Wolf, D.P., Meng, L., Ouhibi, N. and Zelinski-Wooten, M. (1999). Nuclear transfer in the rhesus monkey: Practical and basic implications. **Biol. Reprod.** 60: 199-204.

Yamashita, A., Takada, T., Yamamoto, G. and Torii, R. (2006). Stable Maintenance of Monkey Embryonic Stem Cells in the Absence of bFGF. **Transplantation Proceedings** 38: 1614–1615.

CHAPTER VI

OVERALL CONCLUSION

Personal ES cell is a valuable cells replacement for the patient who owns it because the graft rejection would not occur after transplantation. This experiment has been demonstrated that single blastomere could be used as a source of ES cell establishment by using ES supporting cells-free culture condition. However, the potential to establish ES cell is variable between each sister blastomeres. This statement has been demonstrated by the differences expression patterns of ES cell markers (Sox-2 and Oct-4), and trophectoderm marker (Cdx2). The patterns show that only some but not all sister blastomeres derived from embryos and embryonic outgrowth expressed ES cell markers when blastomere derived from two and four cell stage embryonic blastomeres were individually cultured. Moreover, the result of ES cell establishment has also been demonstrated that success rate of establishment was depending on embryo age; the success rate would decreased if the blastomeres were taken from higher stage embryos. However, using ACTH supplemented into blastomere culture media could support the ES cell establishment rate when the blastomeres were taken from high stage embryo.

APPENDIX

mDPBS

1) NaCl	4.0000 g.
2) KCl	0.1000 g.
3) KH_2PO_4	0.1000 g.
4) Na_2HPO_4	0.5750 g.
5) Glucose	0.5000 g.
6) Pyruvic acid	0.0180 g.
7) $CaCl_2.2H_2O$	0.0687 g.
8) $MgCl_2.6H_2O$	0.0500 g.
9) P-S (stock 100x)	500 µl.
10) Ultra pure water	500 ml.

PBS (-)

1) NaCl	10.0000 g.
2) KCl	0.2500 g.
3) Na_2HPO_4	1.4400 g.
4) KH_2PO_4	0.2500 g.
5) Ultra pure water	1.0000 L.

Trypsin/EDTA

1) Trypsin	0.2500 g.
2) EDTA	0.0400 g.
3) PBS (-)	100 ml.

0.1% Gelatin

1) Gelatin	0.1000 g.
2) PBS (-)	100 ml.

0.5% Pronase

1) Pronase	0.5000 g.
2) mDPBS	50.0000 ml.

Hoechst 33342 (stock)

1). Hoechst 33342	0.0020 g.
2.) DMSO	1.0000 ml.

α-MEM

1) α-MEM powder	1.0000 g.
2) $NaHCO_3$	2.2000 g.
3) Ultra pure water	1.0000 L.

L-glutamine (stock)

1) L-glutamine	0.1462 g.
2) Ultra pure water	10.0000 ml.

α MEM + 10% FBS

1.) α MEM (sterilized)	88.0000 ml.

2). FBS (sterilized)	10.0000 ml.
3). P/S (100x; sterilized)	1.0000 ml.
4). L-Glutamine (200x; sterilized)	1.0000 ml.

α MEM + 10%FBS + 20%DMSO

1.) α MEM + 10%FBS	8.0000 ml.
3). DMSO	2.0000 ml.

TCM 199

1) 199 powder	0.9990 g.
2) Na pyruvate	0.0056 g.
3) $NaHCO_3$	0.2200 g.
4) Ultra pure water	100 ml.

TCM199-Hepes

1) 199 powder	0.9990 g.
2.) Na pyruvate	0.0056 g.
3) HEPES (free acid)	0.5960 g.
4) $NaHCO_3$	0.1250 g.
5) Ultra pure water	100 ml.

199 + 10% FCS

1) 199H	18.0000 ml.

2) FBS	2.0000 ml.
3) P-S stock	20 µl.

mES medium

1). α MEM (sterilized)	40.5000 ml.
2). FBS for ES cell (sterilized)	7.5000 ml.
3). P/S (100x; sterilized)	0.5000 ml.
4). L-Glutamine (200x; sterilized)	0.5000 ml.
5). MEM-NEAA (100x; sterilized)	0.5000 ml.
6). Beta-Mercatoethanol (sterilized)	0.5000 ml.
7). LIF (5,000 iu/µl; sterilized)	5 µl.

Blocking solution

	Final conc.	**100 ml**
1). Triton X-100	0.2%	200 µl.
2). Sodium azide	3 mM	19.5 mg.
3). Saponin (mass/V)	0.1%	100 mg.
4). BSA (mass/V)	2%	2.0000 g.
5). Horse/Donkey serum (V/V)	5%	5.0000 ml.
6). PBS (-)		95.0000 ml.

BIBLIOGRAPHY

Chanchao Lorthongpanich was born in Ratchaburi, Thailand on April 29th, 1978. She finished high school from Naree Vitaya School in Ratchaburi. In 2000, she recived Bachelors Degree (B.Sc.) in Animal Production Technology from Suranaree University of Technology, Nakhon-ratchasima, Thailand. In 2003, she received a Master degree (M. Sc.) in Biotechnology from Institute of Agricultural Technology, Suranaree University of Technology. Her research topic was cloning of cat embryos by using ear fibroblast cells and granulosa cells as donor cell. Her master thesis was awarded as an outstanding master thesis from Thai Society of Biotechnology in 2004. During her Ph.D. study, she received a graduate student outstanding award from Suranaree University of Technology and also received a fellowship from National Center for Genetic Engineering and Biotechnology Research on "Production of bovine IVF-derived embryos from sexed-sorted semen" at Department of Animal Science, Faculty of Agriculture and Life Science, University of Wisconsin-Madison, USA during March 2005 - June 2005. Her Ph.D. study was supported by the Royal Golden Jubilee (RGJ) Ph.D. Program of Thailand Research Fund. Her Ph.D. thesis title was embryonic stem cell lines establishment from single blastomeres of a pre-implantation mouse embryo. Parts of this work have been presented as poster presentation in the 63rd Annual meeting of American Society for Reproductive Medicine on October 13-17, 2007 at Washington DC, U.S.A. and fourth Asian Reproductive Biotechnology Society on November, 24th – 26th, 2007 at Singapore.