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จุฬาลงกรณ์มหาวิทยาลัย

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DEVELOPMENT OF NUCLEAR TRANSFER AND EMBRYONIC STEM CELL TECHNOLOGY IN MOUSE

Mr. Ruttachuk Rungsiwiwut

สถาบันวิทยบริการ
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รัฐจักร รังสิวิวัฒน์ : การพัฒนาเทคโนโลยีการย้ายฝากนิวเคลียสและเซลล์ต้นกำเนิดตัวอ่อนในหนู (DEVELOPMENT OF NUCLEAR TRANSFER AND EMBRYONIC STEM CELL TECHNOLOGY IN MOUSE) อ. ที่ปรึกษา: ศ.น.สพ.ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาร่วม: ศ.กิตติคุณ นพ. ประมวล วีรุตมเสน, Prof. Andras Dinnyes, 80 หน้า

การศึกษานี้มีวัตถุประสงค์เพื่อ เปรียบเทียบประสิทธิภาพการสร้างเซลล์ต้นกำเนิดตัวอ่อนหนูเมาส์ จากตัวอ่อนที่เกิดจากการปฏิสนธิของหนูต่างสายพันธุ์ เปรียบเทียบประสิทธิภาพของการสร้างเซลล์ต้นกำเนิดตัวอ่อนและคุณลักษณะของเซลล์ต้นกำเนิดตัวอ่อนที่เกิดจากการปฏิสนธิและการย้ายฝากนิวเคลียส เปรียบเทียบการเปลี่ยนแปลงเป็นเซลล์ที่ทำหน้าที่จำเพาะระหว่างเซลล์ต้นกำเนิดตัวอ่อนที่สร้างจากตัวอ่อนที่เกิดจากการปฏิสนธิและการย้ายฝากนิวเคลียส โดยแบ่งออกเป็น 3 การทดลอง

<u>การทดลองที่ 1</u> ใช้หนูเมาส์ 3 สายพันธุ์ อินบริด (inbred), ไฮบริด (hybrid) และเอาท์บริด (outbred) เก็บตัวอ่อนที่ปฏิสนธิภายในร่างกายระยะเอกซแพนบลาสโตซิส (expanded blastocyst) จำนวน 40-44 ตัวอ่อนจากแต่ละสายพันธุ์ เปรียบเทียบจำนวนอินเนอร์เซลล์แมส (inner cell mass) ในบลาสโตซิส เก็บตัวอ่อนจำนวน 10-15 ตัวอ่อนจากแต่ละสายพันธุ์ตรวจหาเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 ในตัวอ่อนจากหนูทั้งสามสายพันธุ์ ผลการศึกษาพบว่าจำนวนอินเนอร์เซลล์แมสในตัวอ่อนระยะแอกซแพนบลาสโตซิสของหนูสายพันธุ์อินบริดไม่แตกต่างอย่างมีนัยสำคัญทางสถิติ กับสายพันธุ์ไฮบริด แต่มากกว่าสายพันธุ์เอาท์บริดอย่างมีนัยสำคัญทางสถิติ ตรวจพบเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 ในตัวอ่อนทุกสายพันธุ์ ประสิทธิภาพการสร้างเซลล์ต้นกำเนิดตัวอ่อนจากตัวอ่อนระยะแอกซแพนบลาสโตซิสของหนูสายพันธุ์อินบริด (16.7%) เท่ากับไฮบริด (31.6%) ไม่สามารถสร้างได้จากสายพันธุ์เอาท์บริด ทดสอบเพิ่มเติมเฉพาะกับสายพันธุ์เอาท์บริดโดยใช้ตัวอ่อนระยะดีเลย์บลาสโตซิส (delayed blastocyst) พบว่า มีจำนวนอินเนอร์เซลล์แมสมากกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับตัวอ่อนระยะแอกซแพนบลาสโตซิสในสายพันธุ์เดียวกัน ตรวจพบเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 และสามารถสร้างเซลล์ต้นกำเนิดตัวอ่อนจากสายพันธุ์เอาท์บริดได้ สรุปว่าประสิทธิภาพการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนระยะแอกซแพนบลาสโตซิสของสายพันธุ์อินบริดและไฮบริดไม่ต่างกันเพราะจำนวนอินเนอร์เซลล์แมสไม่ต่างกัน ระยะของตัวอ่อนที่เหมาะสมที่จะใช้สร้างเซลล์ต้นกำเนิดตัวอ่อนจากสายพันธุ์เอาท์บริดคือระยะดีเลย์บลาสโตซิส

<u>การทดลองที่ 2</u> ตรวจหาเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 ในตัวอ่อนระยะบลาสโตซิสที่เกิดจากการย้ายฝากนิวเคลียสและจากการปฏิสนธิอย่างละ 10 ตัวอ่อน เปรียบเทียบประสิทธิภาพการสร้างเซลล์ต้นกำเนิดตัวอ่อนและคุณลักษณะของการเป็นเซลล์ต้นกำเนิดที่สร้างจากตัวอ่อนทั้งสองชนิดในระดับเซลล์และการแสดงออกของยีนบางยีน ผลการศึกษาพบว่าสามารถตรวจพบเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 ทั้งในตัวอ่อนที่เกิดจากการย้ายฝากนิวเคลียสและจากการปฏิสนธิ ประสิทธิภาพการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่เกิดจากการย้ายฝากนิวเคลียสเท่ากับ 15.4% (4/26) ต่ำกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับตัวอ่อนที่เกิดจากการปฏิสนธิ 62.5% (15/24) เซลล์ต้นกำเนิดที่สร้างจากตัวอ่อนที่เกิดจากการย้ายฝากนิวเคลียสแสดงคุณลักษณะของการเป็นเซลล์ต้นกำเนิดและมีจำนวนโครโมโซมที่ปกติเช่นเดียวกับเซลล์ต้นกำเนิดจากตัวอ่อนที่เกิดจากการปฏิสนธิ สรุปว่าแม้ตัวอ่อนระยะบลาสโตซิสที่เกิดจากการย้ายฝากนิวเคลียสจะมีเซลล์ที่ให้ผลบวกต่อยีน Pou5f1 เช่นเดียวกับที่เกิดจากการปฏิสนธิ แต่ประสิทธิการสร้างเซลล์ต้นกำเนิดยังต่ำกว่า เซลล์ต้นกำเนิดจากตัวอ่อนที่เกิดจากการย้ายฝากนิวเคลียสแสดงคุณลักษณะของการเป็นเซลล์ต้นกำเนิดในระดับเซลล์และการแสดงออกของยีนจำเพาะบางยีนคล้ายกับเซลล์ต้นกำเนิดจากตัวอ่อนที่เกิดจากการปฏิสนธิ

<u>การทดลองที่ 3</u> เปรียบเทียบการเปลี่ยนแปลงเป็นเซลล์ที่ทำหน้าที่จำเพาะระหว่างเซลล์ต้นกำเนิดตัวอ่อนที่สร้างจากการย้ายฝากนิวเคลียสและจากการปฏิสนธิโดยศึกษาใน embryoid bodies (EBs) สร้าง EBs จากเซลล์ต้นกำเนิด เลี้ยงแบบแขวนลอย วัดขนาดทุก 3-4 วัน จนถึงวันที่ 26 เก็บ EBs ในสัปดาห์ที่ 1, 2, 3, 4 และ 5 เพื่อตรวจการลักษณะทางจุลกายวิภาคและ การแสดงออกของยีน พบว่าประสิทธิภาพการพัฒนาเป็น EBs ระหว่างเซลล์ต้นกำเนิดสร้างจากการย้ายฝากนิวเคลียสและจากการปฏิสนธิไม่แตกต่างกันทางสถิติ (95% และ 96.8% ตามลำดับ) เส้นผ่าศูนย์กลาง EBs จากเซลล์ต้นกำเนิดตัวอ่อนจากการย้ายฝากนิวเคลียสมีขนาดใหญ่กว่าจากการปฏิสนธิอย่างมีนัยสำคัญทางสถิติในสัปดาห์ที่ 2 และ 3 พบโครงสร้างระยะแรกของท่อระบบประสาทในสัปดาห์ที่สองของ EBs จากเซลล์ต้นกำเนิดตัวอ่อนทั้งสองชนิด มีความแตกต่างของการแสดงออกของยีน AFP ใน EBs ที่สร้างจากเซลล์ต้นกำเนิดตัวอ่อนจากการย้ายฝากนิวเคลียสและเซลล์ต้นเนิดตัวอ่อนจากการปฏิสนธิ ความแตกต่างระหว่างเซลล์ต้นกำเนิดตัวอ่อนที่สร้างจากการย้ายฝากนิวเคลียสและจากการปฏิสนธิที่ได้จากการศึกษานี้จะเป็นพื้นฐานในการศึกษาเชิงเปรียบเทียบระหว่างเซลล์ต้นกำเนิดตัวอ่อนทั้งสองชนิดต่อไป

ภาควิชาสูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์
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ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
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RUTTACHUK RUNGSIWIWUT: DEVELOPMENT OF NUCLEAR TRANSFER AND EMBRYONIC STEM CELL TECHNOLOGY IN MOUSE. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorate 3^{e} cycle THESIS CO ADVISOR: PROF. EMERITUS PRAMUAN VIRUTAMASEN, M.D., M.Sc., PROF.ANDRAS DINNYES, Ph.D, 80 pp.

The objectives of this study were to compare the efficiency of embryonic stem cells (ESCs) derived from fertilized blastocysts of different mouse strains (EXP. 1), to compare the efficiency of ESCs derivation and characterization of ESCs derived from cloned and fertilized blastocysts (EXP. 2) and to compare the potential of *in vitro* differentiation of ESCs derived from cloned and fertilized blastocysts (EXP. 3).

EXP 1. The strains of mouse used in this experiment were C57BL/6 (inbred), B6D2F1 (hybrid) and CD1(outbred). Firstly, 40-44 expanded blastocysts developed *in vivo* were collected and subjected to ICM number evaluation. Secondly, Pou5f1 positive cells were detected in 10-14 expanded blastocyts. Thirdly, Efficiency of ESC derivation from different mouse strains was compared. Expanded blastocysts of inbred showed no significant difference in ICM number compared to hybrid but significantly higher than outbred. The Pou5f1 positive cells were detected in all blastocysts of three examined strains. No significant difference of efficiency in ESCs derived from expanded blastocysts of inbred (16.7%) and hybrid strain (31.6%) but no ESC line was obtained from the outbred strain. Finally in additional experiments, delayed blastocysts of outbred strain were used to evaluate ICM number, detect Pou5f1 and establish ESC line. Delayed blastocyst of outbred showed significantly higher ICM number than expanded blastocysts of the same strain. Pou5f1 was detected in delayed blastocysts and outbred ESC lines can be derived from delayed blastocysts. This finding indicated that efficiency of ESC derived from expanded blastocysts of inbred and hybrid strain was not different regarded to no difference of ICM number. Outbred ESC lines can be derived from delayed blastocysts but not expanded blastocysts.

EXP 2. Cloned and fertilized blastocysts were subjected to Pou5f1 positive cells detection. Efficiency of ESC derived from cloned and fertilized blastocysts were compared. Pluripotency of ESCs derived from cloned and fertilized blastocysts were detected by specific markers and gene expression of ESCs. Pou5f1 positive cells were detected in cloned (10/10) and fertilized blastocysts (10/10). Efficiency of ESCs derived from cloned [15.4%; 4/26)] was significantly lower than fertilized blastocysts [62.5%; 15/24]. ESCs derived from cloned displayed specific markers, gene expression and normal chromosome number similar to those derived from fertilized blastocysts. This study indicated that although Pou5f1 positive cells were detected in cloned as in fertilized blastocysts, the efficiency of ESC derivation of the former was inferior. By detecting specific markers, gene expression and chromosome number, ESCs derived from cloned and fertilized blastocysts display similarity.

EXP 3. Nuclear transfer-derived ESCs (NT-ESCs) and fertilization-derived ESCs (F-ESCs) derived EBs were produced in hanging drops, cultured in suspension and *in vitro* differentiation potential were compared. Mean of diameter of EBs were measured every 3-4 day up to Day 26. Histomorphology and gene expression were investigated in 1, 2, 3, 4 and 5-week-old EBs. EBs formation efficiency of NT-ESCs did not significantly differ from F-ESCs (95% vs 96.8% respectively). NT-ESCs derived EBs showed significantly greater diameter than F-ESCs derived EBs in Day 7 and 10. Primitive neural tubes were found in NT-ESCs and F-ESCs derived EBs at Day 14. Expression pattern of AFP in NT-ESCs and F-ESCs derived EBs was different. This study showed some differences between NT-ESCs and F-ESCs derived EBs which will be useful for further study of comparative characteristics of NT-ESCs and F-ESCs.

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LIST OF ABBREVIATION

AP	alkaline phosphatase
BSA	bovine serum albumin
h	hour
hCG	human chorionic gonadotropin
DAPDH	glyceraldehyde phosphate dehydrogenase
DAPI	4′- 6′-diamidino-2-phenylindole
dpc	day post coitus
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
EBs	embryoid bodies
ESCs	embryonic stem cells
F	fertilize
FBS	fetal bovine serum
H&E	hematoxyline and eosin
ICM	inner cell mass
IU	international unit
KCl	potassium chloride
KSOM	potassium simplex optimized medium
LIF	leukemia inhibitory factor
MEF	mouse embryonic fibroblast
min	minute
ml	milliliter
mRNA	messenger ribonucleic acid
NT	nuclear transfer
NEAA	non essential amino acid
PFA	paraformaldehyde
PMSG	pregnant mare serum gonadotropin
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S	second
SSEA	stage specific embryonic antigen
SD	standard deviation

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

TE	trophectoderm
µg	microgram
µm	micrometer
µs	microsecond
wk	week

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

The history of stem cell research began in 1964 when stem cells were discovered from the analysis of a type of cancer called a “teratocarcinoma”. It was found that a single cell in teratocarcinomas could be isolated and remained undifferentiated in culture. These types of stem cells became known as embryonic carcinoma cells (ECCs) (Andrews et al., 2005). Later, the primordial embryonic germ cells (EGCs) and embryonic stem cells (ESCs) can be cultured and stimulated to be different cell types.

Stem cells have the remarkable potential to develop into many different cell types in the body. Theoretically, they can divide without limit to replenish other cells as long as the animal or human is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell or become another type of cell with a more specialized function, such as a nerve cell, a muscle cell, or a cardiac cell. There are two important characteristics that distinguish stem cells from other types of cells. Firstly, they are unspecialized cells that renew themselves for long periods through cell division. Secondly, under certain physiologic or experimental conditions, they can be induced to become cells with special functions such as the insulin-producing cells of the pancreas or the beating cells of the heart muscle. Stem cell can principally derive into two types; adult and embryonic stem cells, each has different functions and characteristics.

The adult stem cells are the undifferentiated cells found among differentiated cells in a tissue or organ. It can renew itself and can differentiate to yield the major specialized cell types of the tissue or organ (Fig. 1). The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Unlike ESCs, which are defined by their origin [the inner cell mass (ICM) of the blastocyst], the origin of adult stem cells in mature tissues is unknown. Research on adult stem cells has recently generated a great deal of excitement. They have found adult stem cells in many more tissues than they once thought possible. This finding has led to the question whether adult stem cells could be used for cell transplants.

In fact, adult blood forming stem cells from bone marrow have been used in transplants for more than 30 years. Certain kinds of adult stem cells seem to have the ability to differentiate into a number of different cell types, given the right conditions. If this differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of cell therapies.

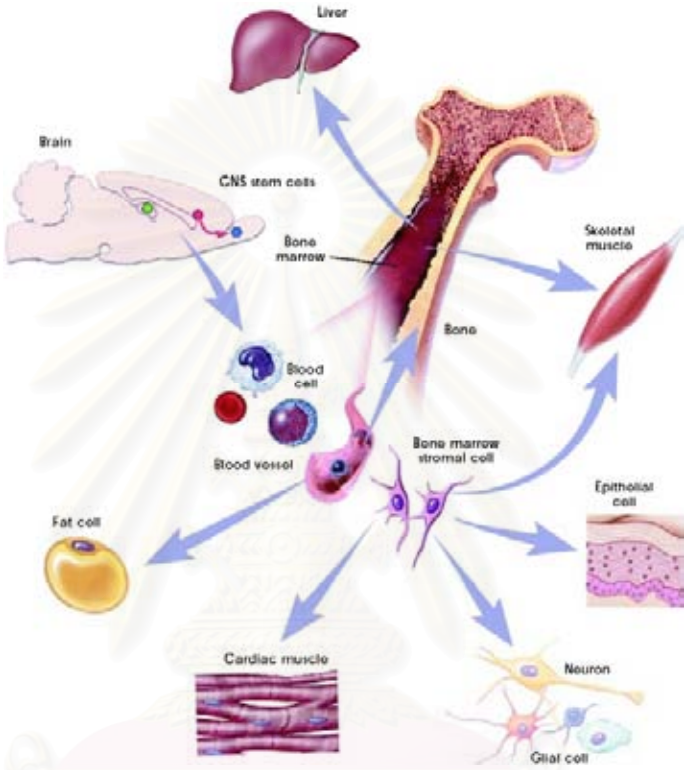

Figure 1 A schematic picture to illustrate the stem cells in bone marrow

This picture shows the ability of stem cells in bone marrow to differentiate into multiple cell types, called plasticity or trans-differentiation. Adapted from stem cells information, NIH; http://stemcells.nih.gov

ESCs are derived from a group of cells in pre-implantation embryo (Fig. 2). These cells have the ability to become almost any kind of cells in the body. The establishment of mouse ESCs from the ICM of blastocysts was firstly described over 20 years ago (Evans and Kaufman, 1981; Martin, 1981). Since then, technical advances in the propagation and manipulation of ESCs have improved the understanding of their growth and differentiation. ESCs have found a number of important applications in basic research, opening the door to potential applications in

drug discovery and cell replacement medicine (Smith, 1998; Solter and Gearhart, 1999). Traditionally, ESCs are stem cells derived from ICM of the pre-implantation stage embryo, blastocyst (Evans and Kaufman, 1981; Martin, 1981). However, it has been proved recently that ESCs can be isolated from single blastomere (Wakayama et al., 2007) or compacted stage (Tesar, 2005) of embryos. ESCs have the potential to differentiate into all derivatives of three primary germ layers: ectoderm, endoderm and mesoderm. The ability of differentiation potential of ESCs referred to "pluripotency". When ESCs were grown *in vitro* and given no stimuli for differentiation, they are able to maintain their pluripotency through multiple cell divisions, known as "self-renewal". ESCs are capable of maintaining an undifferentiated or pluripotent state that remains karyotypically normal and phenotypically stable *in vitro* (Evans and Kaufman, 1981; Thomson et al., 1995). The *in vitro* differentiation of ESCs provides a basis both for detailed studies of developmental mechanisms, the generation of specific cell types for tissue engineering and regenerative medicine applications. Thus, ESC therapies have been proposed for regenerative medicine and tissue replacement after injury or diseases.

Although no approved medical treatments have been derived from ESC research, ESCs have been differentiated into specific cell type for the studies of cell replacement or cell therapy. In the mouse, ESCs have been differentiated in functional-gut-like organ (Yamada et al., 2002), genetically stabilized into heart cells (Klug et al., 1996), modified into nerve cells with retinoic acid (Deacon et al., 1998) or separated *in vitro* and directed into insulin-producing cells *in vitro* (Kahan et al., 2003). These cells released insulin in the presence of blood sugar. When mouse ES-derived nerve cells were transplanted into mice with spinal cord injuries and brain disorders, engraftment of the injected cells into the diseased sites occurred with improvement of nerve function (Deacon et al., 1998). Similarly, the transplantation of ES-derived heart cells into the scar tissue of ischaemic adult mouse hearts showed improvement of new blood vessel formation and improvement of heart function (Klug et al., 1996).

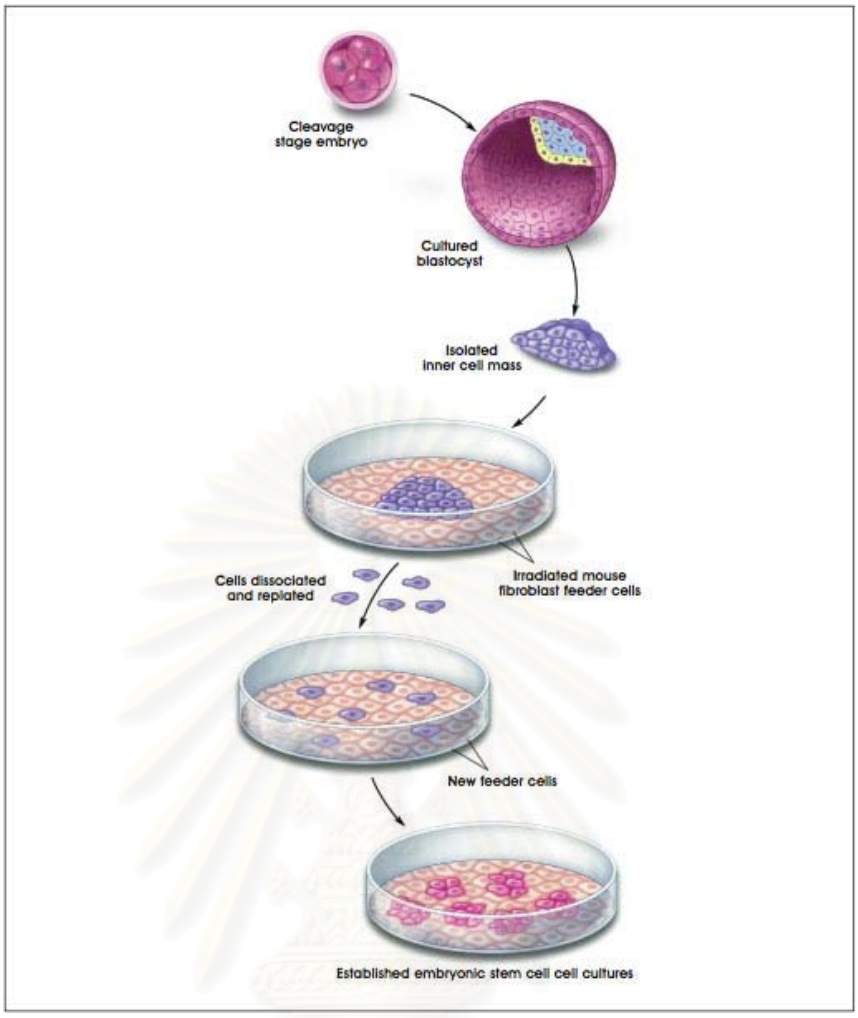

Figure 2 An overview of embryonic stem cell derivation

The ICM is removed from the blastocyst and the large clumps of ICM cells are transferred to feeder layer. After several days, ICM outgrowth will appear. The outgrowth will be dissociated and replated on the new feeder layer. Within several days the "putative ESC line" will appear. Adapted from stem cells information, NIH; http://stemcells.nih.gov

A major technical problem of using ESCs in cell transplantation is graft-versus-host-disease associated with allogeneic stem cell transplantation. However, these problems associated with histocompatibility may be solved by using autologous donor cell via somatic nuclear transfer (SCNT). The SCNT approach, known as "**therapeutic cloning**," the DNA from any cell in the body of a patient, usually a skin or muscle cell, could be removed and transferred through nuclear transfer technique into an enucleated oocyte that previously had its own DNA removed. When transfer the embryos generated by SCNT so called, "**cloned embryo**" into the uterus of a surrogate mother, the life offspring can be generated by this way called **"reproductive cloning".** To date, the cloned animals were produced by SCNT included sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998a), goat (Baguisi et al., 1999), pig (Polejaeva et al., 2000), rabbit (Chesne et al., 2002) as well as mouse (Wakayama et al., 1998) etc. Alternatively, when the cloned embryo is explanted in culture, it can produce ESCs that have the potential to become any or almost any type of cell present in the adult body (Holchedlinger and Jaenisch. 2003).

The ESCs can be also isolated from nuclear transfer derived embryos known as "**nuclear transfer-derived ESCs (NT-ESCs)**", which were derived from somatic cells. NT-ESCs derived by this pathway would have nuclear DNA identical to the cell owner or patient's and, therefore, would likely not to be subjected to immune rejection (Solter and Gearhart, 1999; Lanza et al., 1999; Colman and Kind, 2000) (Fig. 3). In cows and mice, NT technology and ESC derivation have been used successfully in combination to establish ESCs or ES-like cell lines from reprogrammed somatic cell nuclei (Cibelli et al., 1998b; Munsie et al., 2000; Kawase et al., 2000; Wakayama et al., 2001) and mouse NT-ESCs have been shown to be fully pluripotent (Wakayama et al., 2001). The NT-ESCs expressed characteristic ESC markers, had a normal karyotype, also could be differentiated in vitro into neural and myogenic cells. Furthermore, when injected those NT-ESCs into mice, they formed teratomas containing differentiation into all three germ layers and extensively contributed to numerous organs in chimeric fetuses and pups after injected into host blastocysts (Munsie et al., 2000). Recently, it have been proved that NT-ESCs are identical to those ESCs derived from fertilized embryos as they exhibit the similar phenotypes, normal ranges of phenotypes, DNA methylation pattern as well as gene expression (Wakayama et al., 2006)

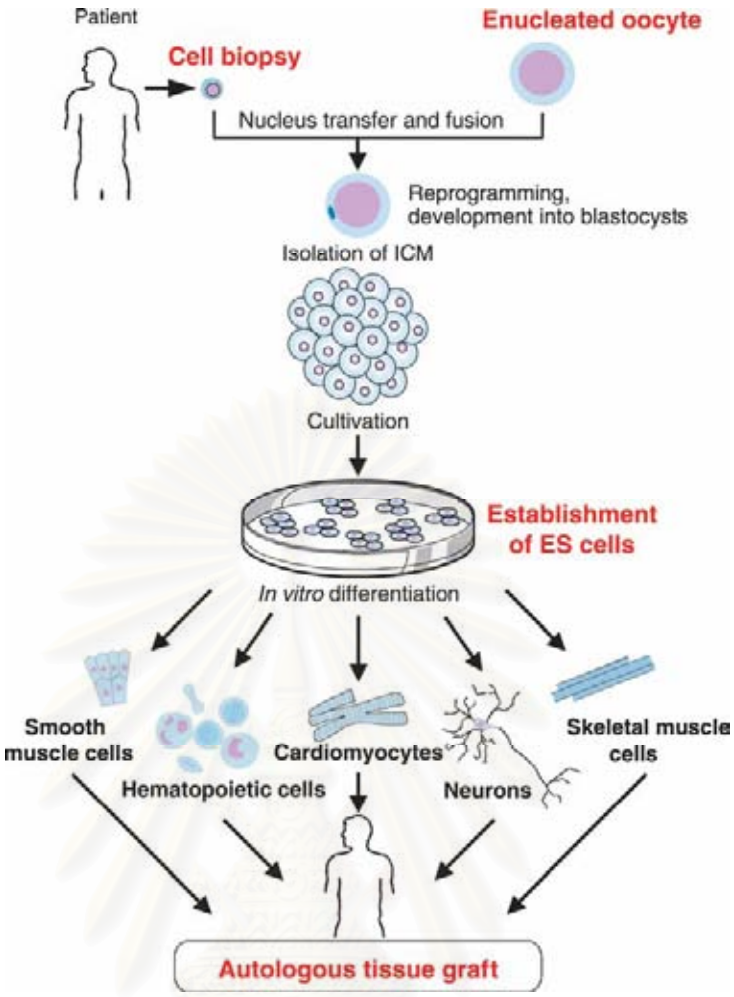

Figure 3 A Strategy of "therapeutic cloning" to generate autologous tissue grafts via somatic cell nuclear transfer (SCNT)

Somatic donor cell nuclei is fused to enucleated oocyte. In the context of the oocyte cytoplasm, the genome of adult cell is reprogrammed to an embryonic status. From this embryo, blastocyst is developed that is used to establish nuclear transfer-derived-embryonic stem cells (NT-ESCs). These ESCs are subsequently differentiated *in vitro* into the desired cell type to generate an autologous tissue graft for transplantation. (Adapted from Lanza et al., 1999)

CHAPTER II

LITERATURE REVIEW

Embryonic stem cell (ESC) lines are termed continuous cell lines that the cell cultures can be maintained indefinitely by continuous passages (Amit et al., 2000). The term “cell line” implies the homogeneity of phenotype within the population of cells which show the ability of maintenance of the cell culture’s original phenotype during continuous culture. The phenotypic definition of ESC lines is that most of the cells should be capable of giving rise to somatic cells representative of the three primary embryonic germ layers (Evans and Kaufman, 1981; Martin, 1981) and should be able to differentiate into the germ cells (Bradley et al., 1984; Geijsen et al., 2004).

2.1 Derivation of ESCs

The aspirations of ESCs for biotechnological and medical exploitation will require efficient establishment of stable ESCs (Smith, 2001). There are several ways to obtain ESCs in laboratory; firstly, requesting already established and characterized ESC lines from the laboratory in which they were generated. Secondly, purchasing ESCs from commercial sources or the last, establishing and characterizing in housed ESC lines from blastocyst embryos. The process of mouse ESC derivation from blastocysts requires expertise and skills in handling mouse embryos. To overcome these limitations, researchers have introduced many improvements to the conventional protocol. Such improvements include use of specifically conditioned medium (Schoonjans et al., 2003), genetically modified blastocysts (McWhir et al., 1996), microdissection of the blastocyst (Brook and Gardner, 1997), treatment with pharmacological drugs (Buehr and Smith, 2003) and use of serum replacement (SR) (Cheng et al., 2004). Although the derivation of ESCs from certain mouse strains is relatively straightforward, the maintenance of pluripotency during and after derivation is often less successful (Buehr and Smith, 2003).

2.1.1 Effect of genetic background on ESC derivation

It is clear that it is possible to derive ESCs from a variety of mouse strains and not just from 129, inbred strain (Table 1). However, to generate in housed ESC lines should note that genetic background can significantly influence the ease of ESC

derivation and developmental potential. To date, most available ESC lines are derived from various 129 mouse strains. However, given the wide range of 129 substrains and the genetic variability of these strains, many researchers backcross gene-targeted mice to other inbred strains. Other than 129 mouse strains, another inbred mouse, the C57BL/6J has been extensively used as a reference strain (Ware et al., 2003; Cheng et al., 2004). The phenotypes of many mouse mutants have been studied in this strain, including a variety of immunological and behavioral mutants (Crawley et al., 1997). Additionally, because the genome of C57BL/6J is being sequenced as part of the Human Genome Project, extensive mapping, sequence, and genetic data are available for this strain (Nadeau et al., 2001). Derivation of mouse ESCs from blastocysts is a process that is often very inefficient, and even in the most favorable strain,129 mouse strain, a success rate of 30% is regarded as high (Robertson, 1987). Practically, the efficiency of derivation in strains other than 129 strains does not usually exceed 10% (McWhir et al., 1996). Suzuki et al. (1999) examined strain dependency for the establishment of ESCs by culturing embryos crossed by 12 combinations of mouse strains and found that ESC lines could be obtained from inbred, hybrid but not outbred strain, suggesting that outbred strain might have inhibitory genetic factor(s) for the ESC formation.

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Table 1. Example of mouse ESC lines from variety of genetic background

ES cell lines	Genetic background	References
CCE	129/SV	Robertson et al. (1986)
O3	129/SV	Gossler et al. (1986)
E14	129/SV	Hooper et al. (1987)
AB1	129/SV	McMahon et al. (1990)
MBL-5	129/SV	Pease et al. (1990)
EFC-3	129/SV	Nichols et al. (1990)
J1	129/SV	Li et al. (1992)
PJ15	129/SV	Johnson et al. (1992)
BL6-3	C57BL/6	Ledermann and Borki (1991)
F1/1	C57BL/6xCBA	Tokunaga and Tsunoda (1992)
TT2	C57BL/6xCBA	Saga et al. (1992)

Adapted from Kawase et al. (1994)

2.1.2 Method for ESC derivation

The protocols for ESC derivation are relatively straightforward; the starting materials are dissociated morulae or intact blastocysts (Evans and Kaufman, 1981), the ICM (Martin, 1981) or even the epiblasts in delayed blastoysts (Brook and Gardner, 1997). However, the standard protocol of ESCs derivation (Fig. 4) which ESCs can be isolated efficiently and less complicated was reported by Robertson (1987) and it has been referenced by several studies. The derivation of ESCs by the standard protocol including; (1) culturing intact blastocyst on the feeder layers until the ICM outgrowth appear (2) dissociation of ICM outgrowth and re-plating the ICM-derived cells on the new feeder layer (3) expansion and identification of ESCs for further studies. The success might depend on three critical factors: the exact stage at which pluripotent cells, capable of growth in tissue culture existing in the embryo,

explantation of a sufficiently large number of these precursor cells from the embryo and culture conditions most conducive to multiplication rather than differentiation of these embryonic cells (Evans and Kaufman, 1981). As ICM compartment is the source of pluripotent precursor cells for ESC establishment, it is important to consider the method for ICM isolation. The original method was immunosurgery, reported by Solter and Knowles (1975). Immunosurgery referred to an exposure of blastocyst to anti-serum followed by the complement. The resulting is the death of trophoblastic cells and then the ICM can be separated from the remnants of trophoblastic cells. The schematic of immunosurgery method was shown in Fig. 4. At present, this method for isolation of ICM is not only applied for mouse but also for other species, for example, pig (Kim et al., 2007), primate (Thomson et al., 1995) as well as human (Thomson et al., 1998) and ESC lines can be obtained by this ICM isolation method.

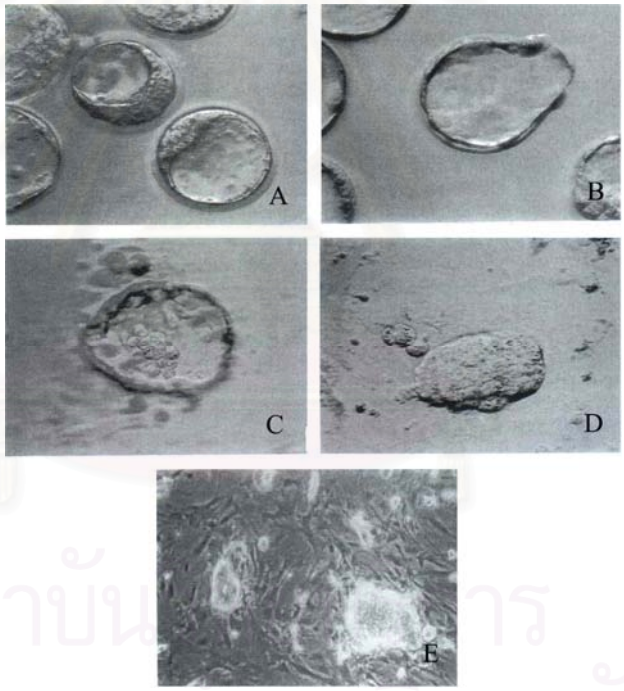

Figure 4 Derivation of mouse ESCs from blastocysts

Blastocyst-stage embryos (A). Hatching blastocyst (B). Blastocyst attached to feeder layer 2 days after hatching, ICM is apparent inside the blastocyst (C). Clump of ICM outgrowth appeared 4-6 days after culturing and ready to be dissociated (D). Primary ES like colony grown after dissociation of ICM outgrowth (E).

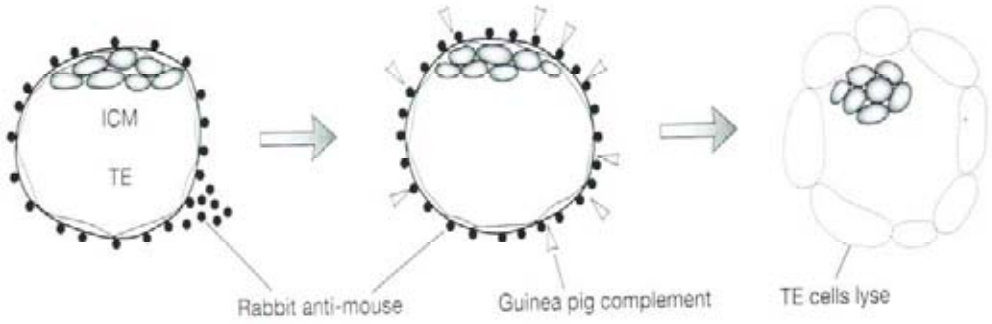

Figure 5 A schematic diagram to illustrate the ICM isolation by immunosurgery method

Blastocysts are incubated with rabbit anti-mouse serum (*closed circles*), washed thoroughly, and exposed to Guinea pig complement (*triangles*). Only the outer trophectoderm (TE) cells are lysed; the ICM cells are protected from exposure to the rabbit antibodies by the tight permeability seal of the trophectoderm. Adapted from Nagy et al., 2003

Besides removal of ICM by immunosurgery, the new approach is to isolate ICM from trophoblastic cells by laser assisted blastocyst dissection (Tanaka et al., 2006). Laser assisted ICM isolation can reduce the risk of contamination with animal products via immunosurgery. This method is based on the micromanipulation technique (Fig. 6) and has been successfully applied for human (h) ESCs derivation (Turesky et al., 2007).

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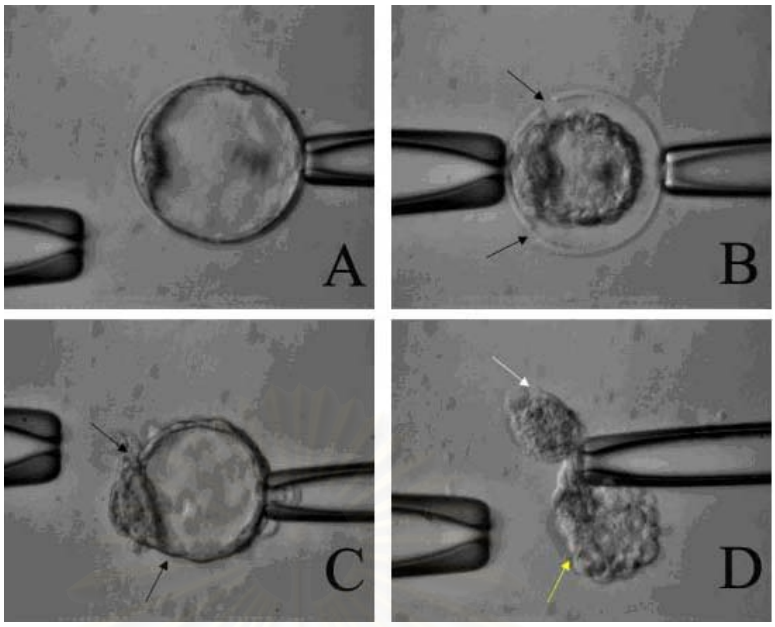

Figure 6 Isolation ICM by laser dissection

Blastocyst holded by two holding pipettes with ICM being positioned at 9 O'clock before (A) and after being sectioned by laser with (B) and without (C) zona pellucida. Arrows (B, C) indicate the resected area by laser beam. The smaller blastocyst fragment (white arrow) contains the ICM while the larger (yellow arrow) is exclusively trophoblast (D). Adapted from Tanaka et al. (2006)

In mouse, it has shown that it is possible to isolate ESCs from a limit window of early development and it would be recommended that explantation of implantationally delayed blastocysts (the stage that blastocyst is already hatched from zona pellucida), is the most effective method for ESC isolation as it involved no microdissection or immunosurgery (Brook and Gardner, 1997). Other than ICM, the epiblast (Fig. 7) in late stage of blastocyst as well as ESCs express the POU transcription factor, Pou5f1, a specific marker of pluripotent cells *in vivo* and *in vitro* (Rosner et al., 1990; Scholer et al., 1990). To obtain the epiblast cells in delayed blastocysts, the involving method has to be considered. Surgical method has been successfully applied for obtaining delayed blastocysts. This surgical method involves ovariectomy followed by injection of progesterone. Interestingly, non invasive technique, an alternative method of using the antiestrogenic effect of tamoxifen together with progesterone and this method show less invasive, simpler and considerably improved condition (MacLean and Evan, 1999). Previous work

showed that ESCs can be derived from the late ICM originating exclusively from the epiblast rather than the primitive endoderm or trophectoderm (Brook and Garner, 1997). Although starting with epiblast isolated from advanced blastocysts greatly increased the ease of obtaining ESCs, certain mouse strain such as ICR and the NOD strain derived genome therefore still proved to be largerly refractory with this method (Brook et al., 2003).

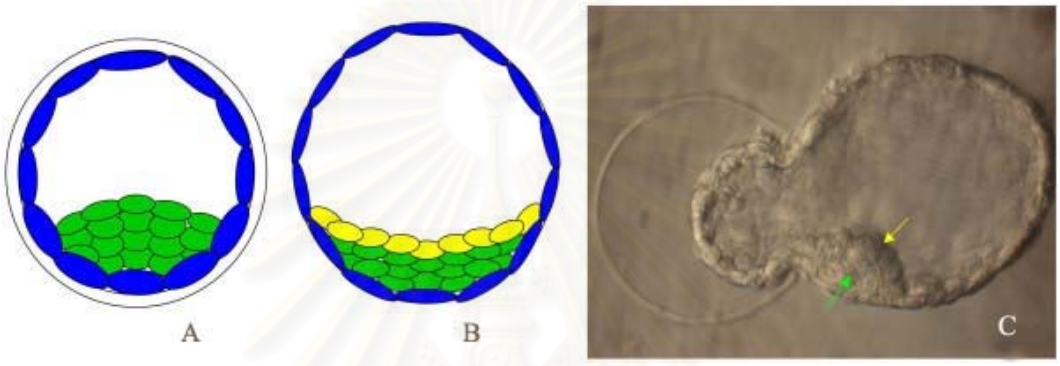

Figure 7 A schematic of primitive endoderm in expanded blastocyst

Blastocysts with absence (A) and presence (B) of primitive endoderm (hypoblast) in a day 4 expanded blastocyst and day 6 hatched blastocyst, respectively. In B, ICM remnant is defined as the epiblast (green) and the hypoblast (yellow). Hatching blastocyst (C) with epiblast (green arrow) and hypoblast (yellow arrow). Adapted from Tanaka et al. (2006)

2.1.3 Cultivation and expansion of ICM-derived ESCs and stable ESC lines

The cultivation of ESCs generally aims to accomplish three objectives: sustaining the self regenerating ESCs properties, maintain the capability of differentiation, and enable cryopreservation for maintaining the established ESC lines. Mouse ESCs were originally derived and maintained using inactivated mouse embryonic fibroblast (MEFs) as feeder layers in serum containing medium (Evan and Kaufman, 1981; Martin, 1981). Mouse ESCs grow on the feeder layer as colonies. They are normally subcultivated every two to three days and the conolies are

enzymatically dissociated with trypsin to obtain single cells which are then plated onto MEFs. Recently, different cultivation protocol of ICM-derived ESCs and stable ESC lines were improved. Different sources of feeder cells were used instead of MEFs. STO fibroblasts were isolated from SIM (S, SIM; T, 6-thioguanine resistant; O, ouabain resistant) mouse and can maintain the pluripotency of ESCs during cultivation. Because STO cells are immortal cell line and easily cultured for a long period, they have been then often used as feeder cells. Despite of using as feeder layer for mouse ESCs, it has been reported that human ESCs can be established and cultured on STO feeder layer (Park et al., 2003). This indicated that STO cells provided some factors to promote self-renewal and suppress differentiation similar to those MEFs. Interestingly, alternative source of feeder layer can be isolated from ESCs themselves. The isolation of ESCs-derived fibroblasts can be performed via differentiation of ESCs. As the differentiation potential of ESCs, they can differentiate also into fibroblast cells and these fibroblat cells can support the growth of their own ESCs. It has been shown recently that mouse and rhesus monkey ESCs can be maintained their pluripotentcy as they are able to expressed specific markers for pluripotent stem cell after cultured on fibroblast cells derived from themselves (Shi et al., 2006; Li et al., 2005).

Furthermore, feeder-freed culture system was applied for ESC cultivation to avoid the complication of feeder cell preparation and contamination to ESCs via feeder layer. Although, feeder cells may provide some factor (s) that promote self renewal and maintain ESCs pluripotency, mouse ESCs have recently been cultured in the presence of leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) or serum replacement (SR) on gelatin coated dish in feeder free system. Alternatively, conditioned medium (CM) derived from STO cells or rabbit fibroblast cells transduced with genomic rabbit LIF, enhanced proliferation of undifferentiat
ed ESCs through the promotion of ESC attachment on gelatin-coated dishes (Schoonjans et al., 2003).

2.2 Characterization of ESCs

Once ESCs were established, ESCs must be characterized for checking their pluripotency. Characterization of ESCs can be performed by morphology analysis of ESCs or ES colonies, molecular characteristics and cytogenetic appearances. ESCs isolated from different species might show differences in colony morphology, for

example, colonies of human and bovine ESCs are more flat and consisting only one or two layers of growing ESCs while mouse ESCs normally consist more than two layers. However, ESCs from several species share similar important typical characteristic of ESCs that are a large clear nucleus containing one or more prominent nucleoli and little cytoplasm.

Maintenance of pluripotency is normally assessed by morphology, where ESCs form compact colonies of cells with high nucleus to cytoplasm ratio. In molecular level, ESCs are characterized by high levels of intracellular alkaline phosphatase (AP), presentation of specific cell surface glycoproteins such as the stage-specific embryonic antigen (SSEA)-1, presence of the transcription factor Pou5fl (formerly known as Oct- 4), a high telomerase activity and a short G1 phase of the cell cycle (Thomson et al., 1998), Several markers mentioned above were used to identify the pluripotency of ESCs, however, there are different markers expressed in different species of where ESC lines were derived as shown in Table 2. The identification of these markers has been important for monitoring and quantifying pluripotency of ESCs when testing different culture conditions.

Characterization of pluripotent markers of ESCs can be performed by immunocytochemistry (ICC), immunohistochemistry (IHC), fluorescent activated-cell sorting (FACS) or gene expression by reverse transcriptase polymerase chain reaction (RT-PCR). However, these properties of pluripotency, except the presence of Pou5f1, are characteristic of, but not specific for, pluripotent stem cells. Other essential characteristics include growth as multicellular colonies, normal and stable karyotypes, prolonged undifferentiated proliferation, and the potential to differentiate into derivatives of all three embryonic germ layers even after prolonged culture (Shamblot et al., 1998; Thomson et al., 1998). The karyotypic stability of ESC lines is one major concern for characterization of ESCs especially for biomedical and transgenic research. There have been reported that abnormalities of chromosome arise during long term culture of ESCs (Drapper et al., 2004).

Table 2. Cell-surface markers for pluripotent stem cells

Markers	Mouse EC, ES cells	Human EC cells	Human ES cells	Human ICM cells
SSEA-1	+	-	-	-
SSEA-3	-	+	+	+
SSEA-4	-	+	+	+
TRA-1-60	-	+	+	+
TRA-1-81	-	+	+	+
GCTM-2	-	+	+	?
TG343	?	+	+	?
TG30	?	+	+	?
CD9	+	+	+	?

Adapted from Laslett et al. (2003)

2.3 Differentiation of ESCs

ESCs have the potential to differentiate both *in vitro* and *in vivo.* Differentiation can be stimulated experimentally either *in vitro*, by detachment from their substrate into a suspension culture or *in vivo*, by their inoculation into syngenic host animal to form chimera. ESCs spontaneously form three dimensional aggregate after withdrawal of LIF and transferring to a non-adherent surface. These three dimensional aggregated, called **embryoid boides (EBs)**, recapitulate early embryological development in the mouse and allow derivatives of three germ layers *in vitro*. In mouse ESCs, spontaneous differentiation toward ectoderm, endoderm and mesoderm has also been reported when cultivated as EBs (Moritoh et al., 2003; Choi et al., 2005). EB formation from mouse ESCs is normally achieved by dissociating colonies into single cells and promoting agglomeration by seeding at high cell densities in non-adherent petri dishes. Another way to form EBs is to suspend cells in small droplets hanging from the underside of a culture plate, often referred to as the

hanging drop method (Dang et al., 2002). Differentiation of ESCs via EBs formation can be detected by gross morphology, histology as well as germ-lineage gene expression in growing EBs.

2.4 Derivation of nuclear transfer-derived embryonic stem cells (NT-ESCs)

Since the first report of a single mouse NT-ESCs line being developed (Munsie et al., 2000), several reports have documented the establishment, characterization and germline transmission (Kawase et al., 2000; Wakayama et al., 2001; Wakayama et al., 2005; Eggan et al., 2004). To date, proven NT-ESCs have been obtained from mice and bovine SCNT embryos (Munsie et al., 2000; Kawase et al., 2000; Cibelli et al., 1998b). In mouse, such NT-ESCs have been established from a variety of donor cell types, including cumulus cells (Munsie et al., 2000), tail tip fibroblast (Wakayama et al., 2001) and neuronal cells (Kawase et al., 2000) but the efficiency of NT-ESCs derivation is still low (Table 3). Similar to ESCs derived from fertilized embryos (F-ESCs), NT-ESCs can be established by the same derivation protocol. The impact of mouse strain on the NT-ESCs derivation was not detected as the genotype of cloned embryos was no longer influence on the efficiency of NT-ESCs establishment once when embryos reached the blastocyst stage (Wakayama et al., 2005). Characterization and differentiation of NT-ESCs were usually followed the same protocol and NT-ESCs show similar pluripotency to those F-ESCs. Interestingly, the transitional profile of NT-ESCs is indistinguishable from F-ESCs, suggesting that selection of epigenetic erasure in NT-ESCs occurs (Brambrink et al., 2006). Although ESCs can be established from cloned embryos, but the efficiency of NT-ESCs establishment in comparison to ESCs derived from fertilized embryos is not widely investigated. The lack of these studies contains the control data for ESC lines derived in parallel from fertilized blastocysts and there is not much information available about the growth characteristic from cloned blastocysts until NT-ESCs were established.

Table 3.Efficiency of nuclear transfer-derived embryonic stem cells (NT-ESCs)

References	Blastocysts	NT-ESC lines	Efficiency (%)
Munsie et al. (2000)	10	1	10
Wakayama et al. (2001)	398	35	8.8
Hochedlinger and Jaenish (2002)	41	2	5
Rideout et al. (2002)	27	1	4

Adapted from Mombaert, 2003.

2.5 Objectives

In order to develop and establish ESC and NT research in our laboratory, derivation of mouse ESCs from fertilized blastocysts was firstly studied. The knowledge and results from derivation of ESCs from fertilized blastocysts were transferred and adapted for derivation of ESCs from cloned blastocysts. Finally, differentiation potential of ESCs derived from fertilized and cloned blastocysts were compared.

Due to it never been stated elsewhere that the ICM number and pluripotent cells in blastocysts from different strains related to ESC line establishment, thus the effect ICM number and pluripotent positive cells in blastocysts from different mouse strains on the efficiency of ESC establishment was investigated in Chapter III. The lack of information of NT-ESCs derivation in comparison to F-ESCs, the efficiency of ESC establishment as well as the characteristics of ESCs during the derivation step from both type of embryos were investigated in Chapter IV. More specifically, Chapter V investigated the characteristics during differentiation of EBs derived from NT-ESCs and F-ESCs. Finally, the specific questions raised during these studies and the information are summarized and discussed in Chapter VI.

CHAPTER III

DERIVATION OF EMBRYONIC STEM CELLS FROM FERTILIZED BLASTOCYST OF DIFFERENT MOUSE STRAINS

3.1 Abstract

This experiment was designed to compare the efficiency of ESC derivation from fertilized blastocysts of different mouse strains using conventional protocol. The mouse strains used in this study were, C57BL/6, B6D2F1 and CD-1 as common representatives of inbred, hybrid, and outbred strains, respectively. The effect of strain on the cell numbers of inner cell mass (ICM) in blastocysts, Pou5f1 positive cells in blastocyst and ESC derivation efficiency were compared. Firstly, blastocysts were differentially stained to evaluate the cell numbers of ICM and immunostained to detect the Pou5f1 positive cells. Secondly, expanded blastocysts from different strains were cultured and ESCs were isolated. Due to CD-1 strain is generally referred to non-permissive strain for ESC derivation, additional experiment of ESC derivation from delayed-blastocysts of this strain was included. ESCs were characterized by alkaline phosphatase (AP) assay, immunostaining (SSEA-1, Pou5f1 and Nanog) and chromosome analysis. The results of blastocyst staining showed that expanded blastocysts of inbred, hybrid and delayed blastocysts of outbred showed no significant difference in cell numbers of ICM, but significantly higher from expanded blastocyst of outbred. The Pou5f1 positive cells in blastocysts were detected in three examined strains. The results of ESC line derivation from expanded blastocyst showed that in case of the inbred strain, 16.7% (2/12) of embryos gave rise to ESCs versus 31.6% (12/38) of a hybrid; while no ESC line was obtained from the outbred. Interestingly, ESC lines of outbred strain were able to be isolated by culturing delayed-blastocysts with 4.5% (3/66) of efficiency. Newly established cell lines from three strains were positively stained for specific ESC markers, and had normal karyotype with at least 60% euploidy. To our knowledge, this is the first report that the rates of ESC derivation from different mouse strains, regarded to cell numbers of ICM and Pou5f1 positive cells in blastocysts was studied. Moreover, this is one of a few reports that derivation of outbred ESC lines were successfully performed.

3.2 Introduction

Embryonic stem cells (ESCs) are pluripotent cells, obtained from the inner cell mass (ICM) compartment of a blastocyst-stage embryo (Evan and Kaufman, 1981; Martin, 1981). These cells have been extensively used for providing a tool for the study of developmental biology and gene function as well as animal model for human diseases. The advantage of generation of new, pluripotent and germ-line competent ESC lines in the laboratory is that novel cell lines can be obtained from a given mouse strain (e.g. with certain mutations or with the required genetic background), and used in very early passages. The usage of early passage cell lines is strongly advantageous in case of non-129 mouse strain derived cell lines, were they have a high tendency to loose their ability of germline transmission in late passages (Auerbach et al., 2000).

There are several factors that have an influence on the success of ESC line derivation and need to be considered before the process, for instance, the strain of the mice (Kawase et al., 1994; Suzuki et al., 1999), the composition of medium and culture conditions (Schoonjans et al., 2003; Baharvand and Mattaei, 2004) as well as the protocol for ICM isolation (Tanaka et al., 2006). Theoretically, mouse ESCs from various inbred and mutant strains are desirable for several utilizations. In previous reports, most of the ESC lines were derived from 129/SV strain because of its relative ease of establishing ESC lines, its stability in culture and the efficiency of germ-line transmission. Because of their inconsistent backgrounds with varying strain history (Simpson et al., 1997), other inbred strains became more preferable. For example, C57BL/6 strain is a reference of mouse genome. Many mutants are maintained on this background and widely used in genetic, immunological and behavioral studies, which provide significant advantages of producing knock-out mice directly in the C57BL/6 strain. It is generally accepted that the efficiency of ESC derivation is greatly strain-dependent (Kawase et al., 1994). By the conventional protocol of ESC establishment, about 30% of the embryos obtained from strain 129 can give rise to ESCs (Robertson, 1987), while this efficiency is much lower in other strains (Kawase et al., 1994). Interestingly, a hybrid genetic background, referred as hybrid vigor, is more efficient to generate ESC lines (Suzuki et al., 1999). These hybrid ESC lines can be produced most effectively by crossing with 129/SV or C57BL/6 strains. Germ-line competent ESC lines from the F1 hybrid of non-obese diabetic (NOD) and 129 strain mice were previously created by Brook et al (2003). Consequently, they created F1 hybrid ESC lines that contain particular regions of NOD genome known to contain disease loci.

These results showed the possibility of derivation and using hybrid ESC lines in the study of a given disease. On the other hand, outbred strains are widely used in many experiments in the field of reproductive biology but ESC lines from outbred strains were not easily established. There are probably some features of genetic background of these strains that are inimical to the derivation and propagation of ESCs (Suzuki et al., 1999; Brook et al., 2003).

The cell number of inner cell mass (ICM), trophectoderm (TE) and the ICM/TE ratio are well accepted as parameters to evaluate the development and quality of mouse blastocyst. Especially the ICM population size correlates significantly with the implantation potential (Leppens et al., 1996; Van Soom et al., 2001). However, no studies have been conducted so far on the proportion of cell numbers of ICM in blastocyst from different mouse strains and Pou5f1 (POU domain, class 5, transcription factor 1; formerly known as Oct-4) positive cells although this might have a major influence on subsequent ESC isolation potential.

This study was designed to investigate the effect of mouse strains on the efficiency of ESC derivation, regarded to cell numbers of ICM and Pou5fl positive cells in blastocysts. The strains of mouse included in this study were C57BL/6, B6D2F1 and CD-1 as common representatives of inbred, hybrid, and outbred mouse strains, respectively.

3.3 Materials and Methods

3.3.1 Mouse strains and blastocyst collection

Blastocysts were obtained from inbred (C57BL/6), hybrid (B6D2F1: C57BL/6 x DBA2/J) and outbred (CD1) mice (Charles River Hungary Ltd., Hungary). Mice were used under the ethical approval of the Agricultural Biotechnology Center, Godollo, Hungary. Female mice at 6 to 8 wks of age were superovulated with 5 IU PMSG (Folligon®, Intervet, B.V., Hungary) followed by 5 IU hCG (Choragon®, Richter Gedeon Rt., Hungary) given 48 h later. After mating with males of the same strain, the vaginal plugs were checked the following day. Blastocyst-stage embryos (Fig.8A) were flushed out from uteri at 3.5 days post coitus (dpc) with M2 medium. Embryos were subjected to derivation of ESCs, counting of cell numbers of ICM or Pou5fl immunostaining.

Delayed blastocyst embryos (Fig.8B) from outbred CD1 mice were produced by non-invasive technique as previously described (MacLean and Evan, 1999).

Briefly, the day of finding the vaginal plugs was assigned as Day 0. Intra-peritoneal injection with tamoxifen (10 μg/animal diluted in sesame oil; Sigma-Aldrich Chem. Inc. St. Louis, USA) and subcutaneous injection with progesterone (10 mg/animal; Depo-Provera, Phadia AB, Uppsala, Sweden) were performed at Day 2. The delayed blastocysts were flushed from the uteri at Day 6 with ES medium supplemented with HEPES (Sigma-Aldrich Chem. Inc. St. Louis, USA).

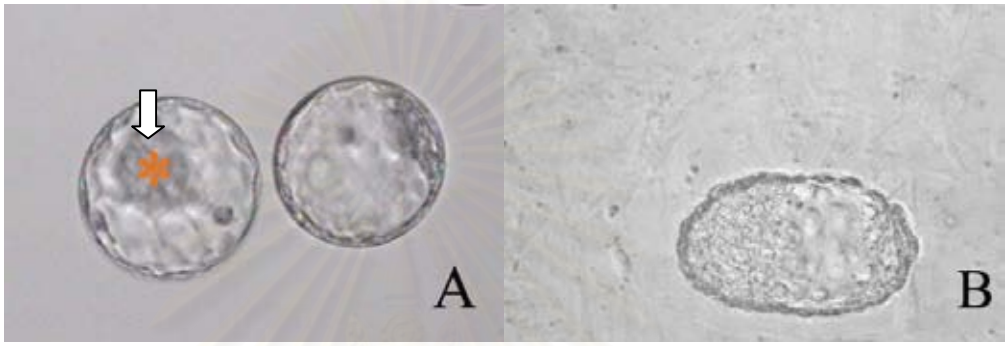

Figure 8 Blastocyst-stage embryos for ESC derivation, counting of cell number of ICM and Pou5fl detection

Expanded blastocysts of hybrid strain which ICM region indicated by the arrow (A) and delayed-blastocyst of outbred strain (B) (magnification: 200×)

3.3.2 Differential staining of inner cell mass (ICM) and trophectoderm (TE) nuclei

ICM and TE cells of blastocysts were stained differentially with chromatin-specific dyes for counting the cell number as previously reported (Thouas et al., 2004) with slight modification. Briefly, zona-intact blastocysts were incubated in BSA-free Hepes-buffered CZB-medium, supplemented with 1% Triton X-100 and 10 μg/ml propidium iodide (Sigma-Aldrich Chem. Inc. St. Louis, USA) for 10 s. Blastocysts were then immediately transferred into 100% ethanol with 25 μg/ml bisbenzimide (Hoechst 333258, Sigma-Aldrich Chem. Inc. St. Louis, USA) and then stored at 4°C overnight. Stained and fixed blastocysts were transferred into a drop of glycerol, mounted onto a glass slide, gently flattened with a cover slip and visualized for cell counting on an inverted microscope fitted with an ultraviolet lamp and excitation filters.

3.3.3 Expression of Pou5f1 in blastocysts

Zona pellucida of blastocyst was removed by brief incubation in acid tyrode solution (Sigma-Aldrich Chem. Inc. St. Louis, USA) and the zona-free embryos were fixed for 20 min in 4% PFA. Further steps of the immunostaining procedure were performed similarly as described in ESC characterization step. The nuclei of cells were counter-stained with DAPI (Vector Laboratories, Burlingame, CA).

3.3.4 Derivation and culture of mouse ESC lines

Blastocysts were plated individually on a 24-well dish covered with mitomycin-C treated (Sigma-Aldrich Chem. Inc. St. Louis, USA) mouse embryonic fibroblast (MEF). MEF cells were obtained from 13.5 dpc mouse fetuses, with standard protocol (Robertson, 1987). The blastocysts were allowed to hatch from the zona pellucida and attached to the feeder and left for the first 48 h without observation. The cultured blastocysts were fed daily with ESC establishment medium [Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 20% FBS (Hyclone, Logan, UT, USA), 0.1 mM non-essential amino acid (Sigma-Aldrich Chem. Inc. St. Louis, USA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Chem. Inc. St. Louis, USA), 50 IU penicillin/ml, 50 mg streptomycin/ml (Invitrogen, Carlsbad, USA), 2,000 IU/ml ESGRO-LIF (Chemicon-Millipore, Billerica, MA, USA), nucleosides (Sigma-Aldrich Chem. Inc. St. Louis, USA) and Insulin-Transferrin-Selenin supplementation (ITS Supplement 100x; Invitrogen, Carlsbad, USA)]. After 4 to 5 days in culture, the ICM outgrowths were mechanically removed from the trophectoderm cells and dissociated with 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, USA). The small clumps of ICM were re-plated on fresh feeder. The ESCs were grown to subconfluence and were gradually replated on a larger culture dish until freezing or characterization. Passage No. 1 was considered when cells first reached the confluence in a 6-cm culture dish.

Delayed blastocysts were washed though several drops of ESC establishment medium and plated immediately on the feeder layer. Further culture steps were identical with non-delayed blastocyst culture conditions described above.

The culture dishes were kept at 37°C in a humidified atmosphere of 5% CO_2. After the first freezing, the medium was changed back to the conventional ESC medium [(Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, 0.1 mM non-essential amino acid, 0.1 mM 2-mercaptoethanol, 50 IU

penicillin/ml, 50 mg streptomycin/ml, 1000 IU/ml ESGRO-LIF)]. ESCs were fed daily and passaged every 3 to 4 days onto fresh feeder.

3.3.5 Characterization of ESCs

3.3.5.1 Immunostaining

The isolated ESC lines were characterized enzymatically and immunocytochemically using antibodies against markers of undifferentiated mouse ESCs. ESCs were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich Chem. Inc. St. Louis, USA) for 15 min at room temperature (RT), and washed three times with PBS (Invitrogen, Carlsbad, USA). Samples were blocked in PBS supplemented with 1% BSA (Sigma-Aldrich Chem. Inc. St. Louis, USA), 5% FBS and 0.1% Triton-X (Sigma-Aldrich Chem. Inc. St. Louis, USA) for 1 h at RT. Primary antibodies [mouse monoclonal SSEA-1 (MC-480, Developmental Studies Hybridoma Bank, Iowa City, IA; 1:300), mouse polyclonal Pou5f1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:400), goat polyclonal Nanog (Santa Cruz Biotechnology; 1:400] were diluted in blocking solution and incubated overnight at 4°C. Fluorescent-labeled secondary antibodies [anti-mouse IgM-Cy3 (1:200); anti-mouse IgG-FITC (1:200) and anti-goat IgG-Cy3 (1:200), respectively, from Jackson ImmunoResearch Europe Ltd. Newmarket, Suffolk, UK] were incubated with the ESCs for 1 h at RT and visualized by fluorescent microscopy. Alkaline phosphatase (AP) assay was performed by using the standard methodology published earlier (Nagy et al., 2003).

3.3.5.2 Chromosome Number Counting

Chromosome preparations of established ESC lines were prepared as described by Nagy et al (2003). Briefly, demecolcin (Sigma-Aldrich Chem. Inc. St. Louis, USA) was added to subconfluent ESCs, grown on gelatin, with a final concentration of 10 μg/ml for 1.5 h. ESCs were then trypsinized, pelleted by centrifugation, re-suspended in 1 ml culture medium. To hypotonize the cells, 10 ml of 0.55% KCl (Sigma-Aldrich Chem. Inc. St. Louis, USA) was added to the cell suspension and incubated for 15-20 min in a 37°C water bath. After that the pellet cells were fixed in fresh ice-cold acetic acid and methanol (1:3) fixative (Sigma-Aldrich Chem. Inc. St. Louis, USA). This step was repeated at least three times. Cell suspension was dropped on clear slides. After drying the slides, chromosomes were stained with Giemsa (Sigma-Aldrich Chem. Inc. St. Louis, USA) or DAPI (Vector).

At least 50 metaphases from each cell lines were studied in order to examine their chromosome numbers.

3.4 Statistical analysis

Percentage of hatched blastocysts, attached blastocysts, isolated ICM outgrowth and ESC lines derived from different strains were calculated from number of cultured blastocysts and compared using the chi-square test analysis. Data regarding cell numbers in blastocyst were subjected to analysis of variance (ANOVA). *P*-values less than 0.05 were considered to be statistically significant.

3.5 Experimental design

Expanded blastocysts of three different mouse strains and delayed blastocysts of outbred strain were collected and subjected to cell number of ICM counting, Pou5fl detection and ESC derivation. A schematic diagram of experimental design was shown in Fig. 9.

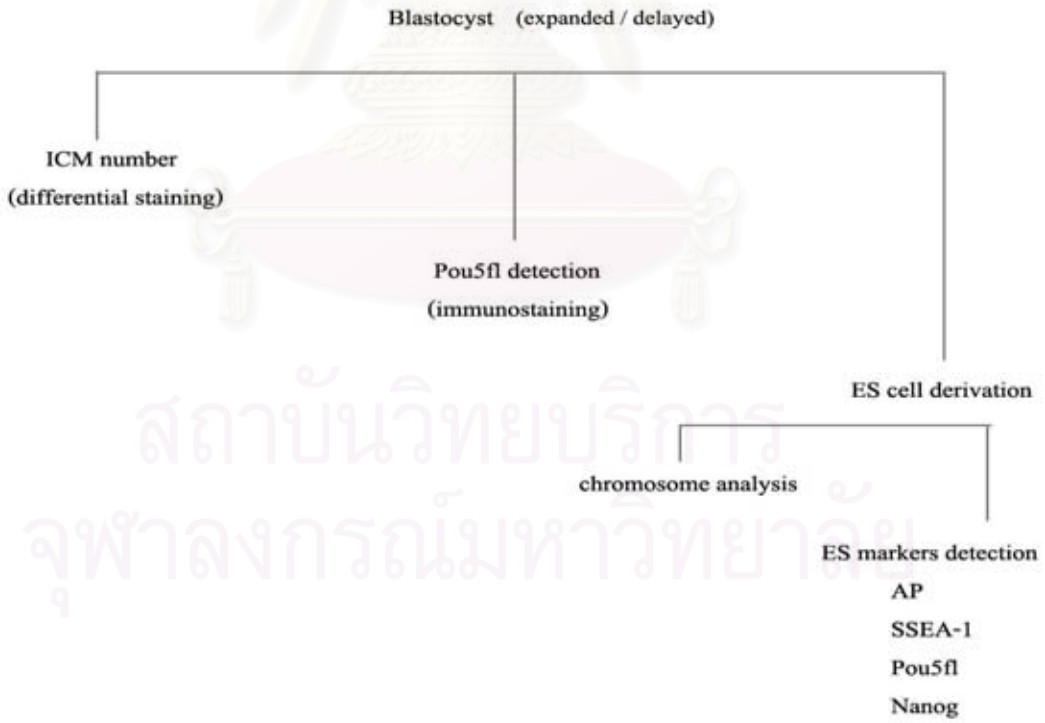

Figure 9 A schematic diagram of experimental design for derivation of ESCs from fertilized blastocysts of different mouse strains

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3.6 Results

3.6.1 Differential staining of ICM and TE

TE and ICM cells were distinguished in expanded blastocysts by differential staining. Among the three examined strains, cell numbers of ICM and TE as well as ICM:TE cell ratio of inbred were not significantly different (P>0.05) from the hybrid. But inbred and hybrid showed a significantly higher (P<0.05) of cell numbers of ICM, TE and ICM:TE cell ratio than the outbred embryos (Table 4). When the delayed blastocysts of outbred strain were compared, only the total (83.0±6) and cell numbers of TE (58.2±6) was significantly different (P<0.05) from all the others, while the ICM cell number differed significantly (P<0.05) only from the average of non-delayed CD1 blastocysts (Table 4).

Table 4. Cell number of ICM, TE and ICM:TE ratio of blastocysts in different mouse strains (mean ± SD)

Strain	Blastocyst (n)	ICM	TE	Total	ICM : TE ratio
Inbred	44	22.1±4 [a]	51.5±5 [a]	73.6±6 [a]	1:2.3 [a]
Hybrid	40	22.0±3 [a]	52.9±4 [a]	74.9±6 [a]	1:2.4 [a]
Outbred	42	18.9±2 [b]	50.2±4 [b]	69.1±5 [b]	1:2.7 [b]
Outbred delayed blastocyst	14	24.8±4 [a]	58.2±6 [c]	83.0±6 [c]	1:2.3 [a]

[a,b,c] Different superscripts denote statistically significant difference within a column (P <0.05)

3.6.2 Pou5fl positive cells in blastocysts

The pluripotency of blastocysts was investigated by immunostaining for the presence of Pou5f1 protein. The pluripotent cells positive to Pou5f1 were normally found in ICM in case of all the examined strains as shown in Fig. 10.

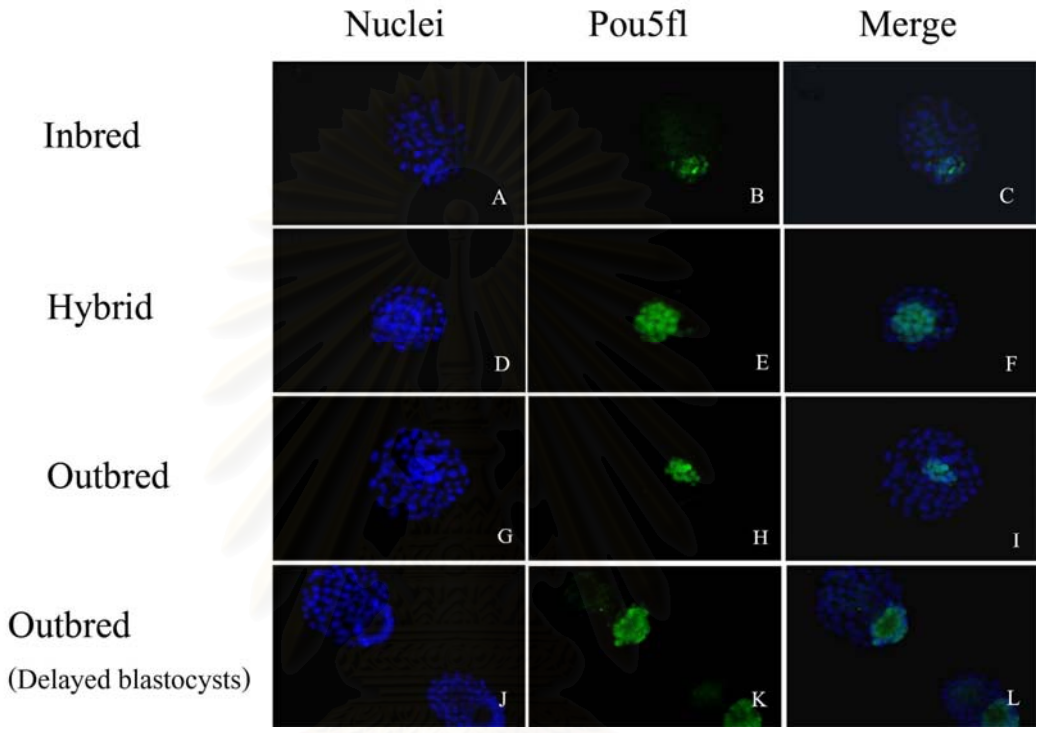

Figure 10 Expression of Pou5f1 protein in blastocyst-stage embryos of different mouse strains

Fluorescent microscopic analysis revealed expression of Pou5f1 localized in the ICM (green). The nuclei of trophectoderm and ICM were stained with Hoechst 333258 (blue). Pou5f1 positive cells in ICM were detected in blastocyst-stage embryos of all examined strains; inbred (A-C), hybrid (D-F), outbred (G-I) and delayed-blastocyst of outbred (J-L) (magnification 200×).

3.6.3 Derivation of ESC lines from blastocysts

Expanded blastocysts from the examined strains hatched and attached onto the feeder layer within 48 h after initial plating. Five to six days after plating, they formed ICM outgrowths and were ready for dissociation. The dynamic growth of blastocyst to ESC line was shown in Fig. 11. The efficiency of ESC establishment from hybrid mice was 31.6% (12/38) while that from inbred was 16.7% (2/12). However, no ESC lines were obtained from expanded blastocysts of outbred (Table 5). When the delayed blastocysts of outbred mice were subjected to ESC establishment, 4.5% (3/66) of efficiency were obtained (Table 5).

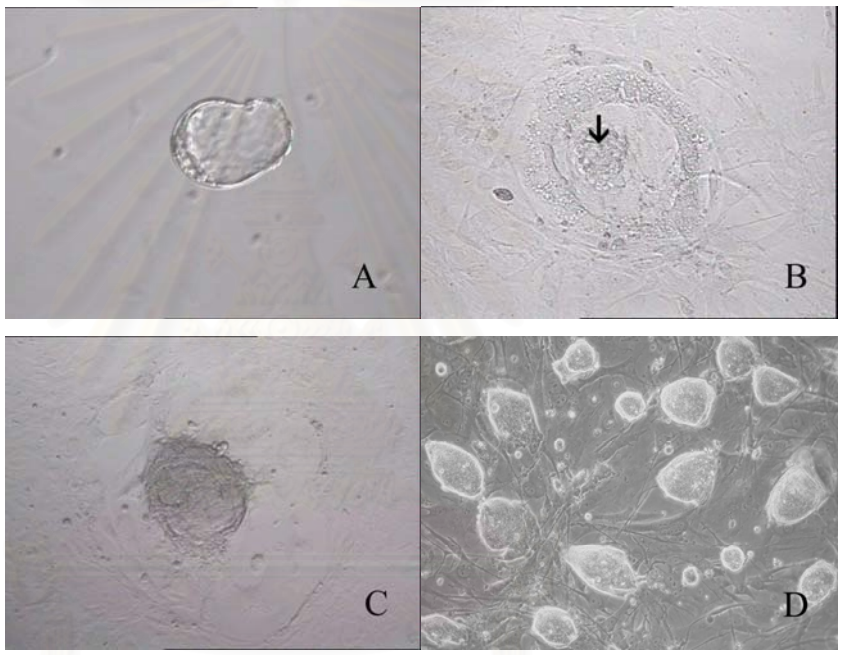

Figure 11 Dynamic growth of a blastocyst to ESCs

Expanded blastocyst hatching from zona pellucida within 24 h after plating on feeder layer (A) (magnification: 100×). Blastocyst attached to feeder layer and formed outgrowth. The harbor large and distinct inner cell mass indicated by the arrow and circularly surrounded by trophectoderm (B) (magnification: 100×). Inner cell mass and trophectoderm outgrowth on day 5 after initial plating just before trypsinization (C) and stable ESC line (D) (magnification 200×)

Table 5. Influence of strains on blastocyst hatching, attachment and ESC line derivation

Strain	Blastocyst cultured	Blastocyst hatched-attached n (%)	ICM outgrowth dissociated n (%)	ES cell lines n (%)
Inbred	12	10 (83.3)	10 (83.3)	2 (16.7) [a]
Hybrid	38	33 (86.8)	33 (86.8)	12 (31.6) [a]
Outbred	22	20 (90.9)	20 (90.9)	0 (0.0) [b]
Outbred delayed blastocyst	66	64 (97.0)	64 (97.0)	3 (4.5) [b]

[a,b] Different superscripts denote statistically significant difference within a column ($P < 0.05$)

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3.6.4 Characterization of ESC lines

The established ESC lines from the three mouse strains assessed for their pluripotency were all positive for AP, SSEA-1, Pou5f1 and Nanog staining. Also, the chromosome number counting showed normal karyotype and at least 60% euploidy in case of all examined ESC lines, from all strains. The immunostaining results and typical metaphase spread chromosome are shown in Fig. 12.

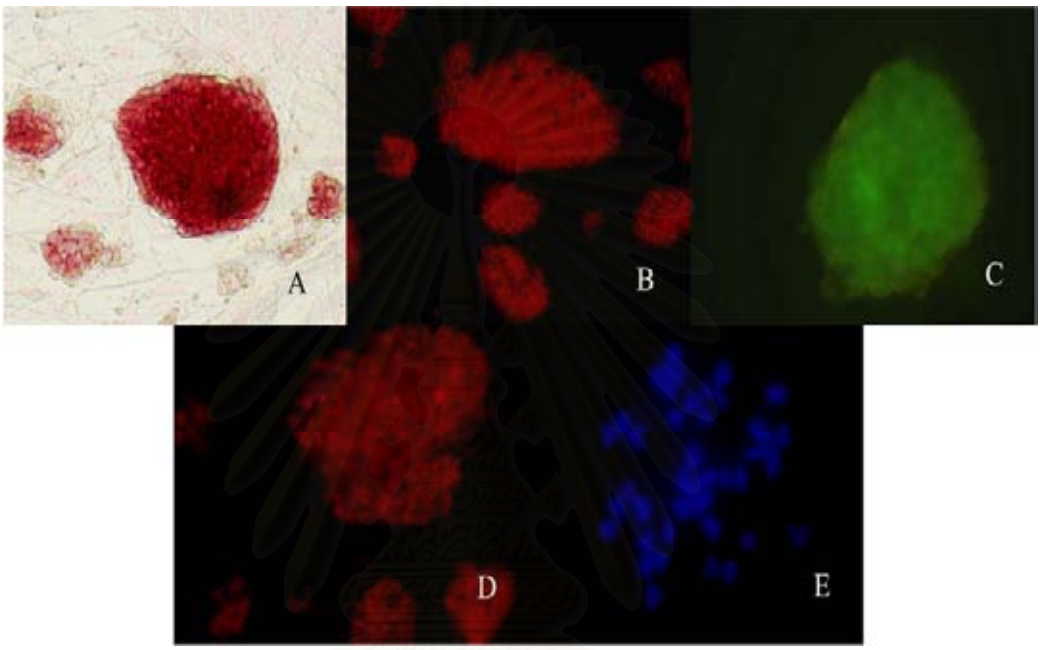

Figure 12 Characterization of ESC line (F1ES#9) established from hybrid strain

ESCs show positive results for undifferentiated mouse ESC markers; AP (A), SSEA-1 (B), Pou5f1 (C) and Nanog (D) (magnification 200×). ESC line shows normal chromosome number after the metaphase spreads were stained with DAPI (E) (magnification 200×).

3.7 Discussion

This experiment shows that mouse ESCs can be isolated and developed successfully by conventional protocol. The success was depended on mouse strains. The rates of ESC derivation are different among the three strains and that outbred expanded blastocysts are not permissive for ESC derivation unless they are delayed.

It has been reported that crossing the non-permissive with a permissive strain can improve the efficiency of ESC derivation, suggesting that beneficial heterosis effect can be expected in ESC line derivation in mice (Suzuki et al., 1999). According to the results from this study, the success rate of established ESC lines was not significantly improved by the crossing of inbred C57BL/6 to another inbred strain, the DBA. However the level of ESC establishment efficiency from hybrid strain is in general agreement with previous reports (Robertson, 1987; Tielens et al., 2006; Liu et al., 2006). In case of outbred, no ESC line was established from expanded blastocysts. Although the embryos were able to hatch from the zona pellucida, attached onto the feeder layer and formed ICM outgrowths similar to those from inbred and hybrid, but they tended to loose their pluripotency after ICM dissociation by showing differentiated cells instead of ES-like cells. It has been suggested that starting with epiblast isolated from delayed blastocysts greatly increased the ease of obtaining ESCs, certain mouse strain such as outbred ICR and the NOD strain derived, therefore, still proved to be largerly refractory with this method (Brook et al., 2003). Surprisingly, in the additional experiment where delayed blastocysts of the outbred were used, the primary ES-like colonies appeared after ICM outgrowth dissociation and finally ESC lines were obtained. This result supported the previous report that the delayed-implantation is beneficial for isolation of ESCs (Brook and Gardner, 1997; Buehr and Smith, 2003). Diapause can be induced experimentally by removal of the ovaries after fertilization or with non-invasive hormonal treatment by administering tamoxifen and progesterone about 55-60 h post fertilization (MacLean and Evan. 1999). In this case, embryos progress through blastocyst formation, hatched from the zona pellucida, then arrested in blastocyst stage but not implanted. When they are transferred to a recipient such embryos can implant and develop normally. This alteration of the normal schedule of development necessitates extended maintenance of the epiblast. Epiblast is regarded as a possible cell source of ES progenitor cells in the late blastocyst, however recent publications showed that the establishment of epiblast stem cells (EpiSCs) from implanted embryos are not

equivalent with the epiblast cells of late or delayed blastocyst-derived epiblast cells. These EpiSCs are not capable to form germline chimeras at all (Brons et al., 2007; Tesar et al., 2007) in contrast with diapaused embryos that generally appears to facilitate the derivation of germline competent ESCs in mice (Brook and Gardner, 1997). Nevertheless, the extended gene expression studies (Hamatani et al., 2004) gave no evidence why the delayed blastocyst method can enhance the success rate of ESC line establishment in mice.

Based on the results described above and that ESCs can normally be isolated from the ICM of blastocysts, the influence of cell numbers of ICM on the efficiency of ESC establishment was examined. It has been found that cell numbers of ICM of inbred did not differ from hybrid and this finding related to ESC derivation from both strains showed no significant difference in efficiency. Although cell number of ICM in expanded blastocysts of inbred and hybrid show no difference to those delayed blastocysts of ourbred, the rates of ESC lines derived from delayed blastocysts of outbred displayed lesser. The lack of some factor(s) to enhance the formation of ESCs or some features of the genetic background of these strains affected to the derivation and propagation of ESCs (Suzuki et al., 1999; Brook et al., 2003). In mouse, during the formation and maturation of ICM and TE, the expression of protein Pou5f1 at the blastocyst stage becomes restricted to the cells of the ICM (Pesce and Sholer, 2001). The expression of Pou5f1 is correlated with the undifferentiated cell type, suggesting that Pou5f1 is a marker of pluripotency (Buehr et al., 2002). Thus, the presence of ICM cells expressing Pou5f1 in the blastocyst should be related to the obtainable ES precursor cells. By immunostaining, the three different strains showed Pou5f1 expressing cells in the ICM of expanded and delayed blastocysts, suggesting that there are some ES precursor cells in those strains. The difference in number of ESC lines established from different strains may be related to their differences in the ability to maintain the Pou5f1 positive cells during the ESC establishment. A sudden loss of Pou5f1 might be behind an inefficiency of ESC derivation: if an ICM outgrowth loses all Pou5f1 expressing cells during the first few days of culture, it is likely to have lost all ESC precursors (Buehr et al., 2002).

All established ESC lines from inbred and hybrid strains showed the same colony morphology: round shape, clear edge and compact, dense colonies appeared, while outbred presented flatter and oval-shaped colonies. However, all ESC lines showed the typical characteristic of ESCs which have a high nucleus/cytoplasm ratio

with one or more distinct nucleoli. The established ESC lines were positive for the markers of undifferentiated pluripotent mouse ESCs, including AP, SSEA-1, Pou5f1 and Nanog. ESC lines having more than 50% of cells with normal chromosome numbers have been suggested to be efficient for germ-line transmission due to successful segregation of chromosomes throughout meiosis (Longo et al., 1997; Suzuki et al., 1997).

In summary, the difference of ESC derivation efficiency is related to the difference in cell numbers of ICM present in blastocysts. Inbred and hybrid strains can give rise to ESC lines more effective than outbred strain using the conventional ESC derivation protocol. In case of outbred strain, ESC lines were successfully established from delayed but not expanded blastocysts. To our knowledge, this study is one of a few reports that have been succeeded in the isolation of ESC lines from outbred strain.

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CHAPTER IV
DERIVATION OF EMBRYONIC STEM CELL LINES FROM CLONED BLASTOCYSTS

4.1 Abstract

Embryonic stem (ES) cells can be derived from embryos generated via nuclear transfer (NT-ESCs). Although NT-ESCs are identical to ESCs derived from fertilized embryos (F-ESCs), the efficiency of ESC derivation, the characteristics from blastocysts culture until stable ESC lines were established as well as the pluripotency of NT-ESCs and F-ESCs are lacking. This experiment was designed to investigate the efficiency of ESCs derived from cloned blastocysts in comparison to fertilized blastocysts. The characteristic of blastocysts growing until the appearance of stable ESC lines were observed. Cloned blastocysts were firstly produced by electrofusion NT technique, using cumulus cells as the donor cells. However, cloned blastocysts were inadequately obtained for ESC derivation. Then piezo-driven technique was applied instead and that cloned blastocysts were successfully generated. Cloned blastocysts were detected for Pou5fl positive cells and subjected to ESC derivation. Fertilized blastocysts were used as the control in this experiment. Newly established ESC lines were characterized by immunostaining for AP, SSEA-1, Pou5f1 and Nanog. Furthermore, pluripotency of ESC lines were also examined by embryoid body formation, expression of pluripotent and germ-lineage genes as well as the cytogenetic analysis. The results showed that Pou5fl positive cells were detected in cloned blastocysts, similar to those fertilized ones. During ESC derivation, ICM outgrowths of cloned blastocysts were relatively small, the modified disaggregation of ICM outgrowths was used and NT-ESC lines were established. Four independent NT-ESC lines were derived in this study. The efficiency of ESC lines derived from cloned blastocysts displayed significantly lower compared to those fertilized blastocysts (15.4% (4/26) and 65.2% (8/24); $P < 0.05$). NT-ESCs displayed similarly of pluripotency to those F-ESCs by expression of specific markers of ESCs and showed normal chromosome number. It can be concluded that even cloned blastocysts expressed Pou5fl similar to those fertilized blastocysts, the efficiency of ESC derivation was still inferior. Modification of the method to disaggregate ICM

outgrowth enhances the success of NT-ESC derivation. The pluripotency of NT-ESCs exhibited similarly to those F-ESCs.

4.2 Introduction

In cell replacement experiments, conventional ESCs, which were derived from fertilized blastocysts, inevitably face the risk of immuno-rejection when transplanted. It has been proposed that ESCs which are genetically identical to the patient's own cells represent a potential solution to the rejection problem (Smith, 1998). To prove the feasibility of creating ESCs using the patient's own cells, ESC lines derived from somatic cell via nuclear transfer (NT-ESCs) provide a good model for the development therapies that rely on histocompatible stem cells. It has previously shown that NT-ESC lines possess the same characteristics for self-renewal and differentiation as those ESC lines derived from fertilized blastocysts (Munsie et al., 2000; Kawase et al., 2000; Wakayama et al., 2001). More studies in the molecular biology showed that the transcriptional profiles, DNA methylation regions, DNA microarray profiles of mouse NT-ESCs are almost identical to those fertilized embryo derived ESCs (Brambrink et al., 2006; Wakayama et al., 2006). In the mouse model, NT-ESCs lines have been proved that they can be differentiated *in vitro* (Munsie et al., 2000; Wakayama et al., 2001). Although establishment of mouse ESCs from fertilized blastocysts is generally accepted that the efficiency is strain dependent (Kawase et al., 1994), the efficiency of NT-ESC establishment was no longer depended on genotype of donor nuclei once when embryos reached the blastocyst stage (Wakayama et al., 2005). In comparison to fertilized blastocysts, the success rate of ESC lines established from cloned blastocysts is normally lower (Wakayama et al., 2005). This difference probably related to the quality of cloned is inferior to those fertilized blastocysts as seen by total cell numbers as well as the mean proportion of ICM to TE cells of cloned blastocyst, which is usually lower. Also the proportion of dead cells in TE and ICM was significant higher for cloned blastocyst (Rybouchkin et al., 2002). Furthermore, it has been found that Pou5f1 transcription factor, a specific marker of pluripotent cells related to ESC derivation efficiency displayed abnormally in cloned embryos (Scholer et al., 1990). It has been suggested that the efficiency of NT-ESC derivation can be improved by using pluripotent cells, for instance, ESCs or embryonic germ (EG) cells as the donor cells (Blelloch et al., 2004). However, the use

of pluripotent cells as the donor cells is not exactly represented the principle of creating NT-ESCs for therapeutic cloning.

This study was designed to derive ESCs from cloned blastocysts generated via NT using cumulus cells as the donor cells. The presence of Pou5fl positive cells in cloned blastocysts, the efficiency of ESC derivation and pluripotency of ESCs derived from cloned blastocysts were investigated by using fertilized blastocysts as the control.

4.3 Materials and Methods

4.3.1 Animal

Six to eight weeks old B6D2F1 (C57BL/6 x DBA) hybrid and ICR outbred mice (Charles River Hungary Ltd., Hungary) were housed on a standard 12 h light/dark cycle. Superovulation by intraperitoneal injection of 7.5 units of PMSG (Folligon®, Intervet, B.V., Hungary) followed 48 h later by intraperitoneal injection of 7.5 units hCG (Choragon®, Richter Gedeon Rt., Hungary). Superovulated B6D2F1 mice were used to provide mature oocytes for NT and natural mating of B6D2F1 mice were used to provide zygotes for *in vitro* culture using as the control. Natural mating of ICR was used for providing fetuses.

4.3.2 Preparation of feeder layers

Thirteen to fourteen days old fetuses of ICR outbred strain were used as a source of primary mouse embryonic fibroblasts (PMEFs). PMEFs treated by mitomycin C for inactivation of cell division and cultured on 0.01% gelatin-coated plates in serum-supplemented medium overnight before plating ICM or ESCs.

4.3.3 Producing of blastocyst-stage embryos

4.3.3.1 Fertilized blastocyst

In vitro produced blastocysts were obtained by collecting zygote embryos from oviductal ampulae and cultured *in vitro* until the embryos reached blastocysts stage. Zygotes were freed from cumulus cells by brief exposure to 0.1% hyaluronidase in M2 medium (Specialty Media) followed by three times of washing in M2 before culture *in vitro* in potassium simplex optimized (KSOM) medium.

4.3.3.2 Cloned blastocyst

Freshly isolated cumulus cells were used as nuclear donors. NT was performed with two different techniques, electrofusion (Ogura et al., 2000) and piezo-driven (Wakayama et al., 1998).

4.3.3.2.1 Electrofusion technique

Zona pellucida of MII oocytes were cut by laser beam (Zylos tk® Hamilton trone, USA) and the chromosomes of oocytes were removed. A single cumulus cell was injected into an oocyte under the zona pellucida, and then membrane fusion was induced by a direct current of 200 V/mm pulsed for 15 μs in 0.3M mannitol solution containing 0.1mM $MgCl_2$, 0.5 mg/ml BSA, 0.5mM HEPES. Immediately after electrofusion, the oocytes were cultured in the KSOM medium and the fusion of couplet were checked 1 h after electrofusion. The fused oocytes were selected and then activated in Ca^{2+}-free KSOM medium containing 10 mM $SrCl_2$ and 5 μg/ml cytochalasin B (Sigma-Aldrich Chem. Inc. St. Louis, USA) for 4–6 h (Wakayama et al., 1998). The oocytes with two pronuclei were considered to have diploid chromosomes, and were cultured for 4 days in KSOM in 5% CO_2 in air at 37 °C.

4.3.3.2.2 Piezo-driven technique

In brief, a group of oocytes was transferred to a droplet of HEPES-CZB containing 5 μg/ml cytochalasin B placed under mineral oil in the operation chamber on the inverted microscope stage (Olympus IX71, Olympus). Zona pellucida was penetrated by using piezo-driven microcapillary (PMM-150FU; PrimeTech). The metaphase II chromosome-spindle complex was drawn into the pipette with a small amount of accompanying ooplasm and gently pulled away from the oocyte until a stretched cytoplasmic bridge was pinched off. The enucleated oocytes were transferred into cytochalasin B-free CZB and kept there for up to 2 h at 37.5°C. For donor nuclei injection, the cumulus cell was drawn in and out of the injection pipette until the cell membrane was broken. Then the cumulus nuclei were injected into an enucleated oocyte at room temperature. After NT, the reconstructed oocytes were activated by 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium in the presence of 5 μg/ml cytochalasin B and 1% dimethyl sulfoxide (DMSO; Sigma-Aldrich Chem. Inc. St. Louis, USA) and cultured for 4 days in KSOM.

Cloned blastocysts were subjected to detection of Pou5fl positive cells and ESC derivation.

4.3.4 Detection of Pou5f1 positive cells in blastocysts

Zona pellucida of blastocysts were removed by brief incubation in acid tyrode solution (Sigma-Aldrich Chem. Inc. St. Louis, USA) and the zona-freed embryos were fixed for 20 min in 4% PFA. Further steps of Pou5f1 staining were performed similarly as described in the part of immunostaining of ESCs below. The nuclei of cells were counter-stained with DAPI (Vector Laboratories, Burlingame, CA).

4.3.5 Cultivation of inner cell mass and ESC establishment

The conventional method to establish mouse ESC line (Robertson, 1987) was used with slight modification. Cloned and fertilized blastocysts were treated with acid tyrode solution to remove the zona pellucida and plated individually on a 24-well dish pre-coated with mitomycin-C inactivated PMEFs. Embryos were then cultured in ES establishment media [(Dulbecco modified Eagle medium (DMEM) high glucose (Invitrogen, Carlsbad, USA), supplemented with 2,000 IU/ml recombinant mouse leukemia inhibitory factor (ESGRO-LIF, CHEMICON), 20% fetal bovine serum (Hyclone, Logan, UT, USA), 1x nonessential amino acids (Invitrogen, Carlsbad, USA), 0.2 mM 2-mercaptoethanol (Sigma-Aldrich Chem. Inc. St. Louis, USA), 50 IU penicillin/ml, 50 mg streptomycin/ml (Invitrogen, Carlsbad, USA), nucleoside mixture and insuline-transferine-selenium supplementation (ITS; Invitrogen, Carlsbad, USA)]. Five to six days after initial plating, the ICM outgrowths were picked mechanically with a fine pipette and disaggregated by gentle pipetting in 50 µl of 0.5% trypsin-EDTA solution (Invitrogen, Carlsbad, USA). The small clumps of ICM were re-plated on fresh feeder. After few days the growing ES-like colonies were disaggregated and cells were replaced on fresh feeder layer until a stable cell line was established.

The culture dishes (COSTAR, Sigma-Aldrich Chem. Inc. St. Louis, USA) were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. The ESCs were fed daily with ES medium and passaged every 2-3 days onto fresh feeder.

4.3.6 Characterization of ESCs

4.3.6.1 Immunocytochemistry

Pluripotency of the established ESC lines were characterized *in vitro*. ESCs were fixed in 4% PFA for 15 min at room temperature (RT), washed three times with PBS, and blocked in blocking solution (PBS supplemented with 1% BSA, 5% FBS and 0.1% Triton-X) for 1 h at RT. Primary antibodies were incubated with the fixed and blocked ES cells overnight at 4°C, while fluorescent secondary antibodies were incubated 1 h at RT. The nuclei of ESCs were counter stained with DAPI and visualized by fluorescent microscopy. Antibodies used are as follows: mouse monoclonal SSEA-1 (MC-480, Developmental Studies Hybridoma Bank, Iowa City, IA. USA; 1:300), mouse polyclonal anti-Oct4 IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA,USA; http://www.scbt.com; 1:400), goat polyclonal anti-nanog IgG (Santa Cruz Biotechnology; 1:400)**.** Fluorescent-labeled secondary antibodies [anti-mouse IgM-Cy3 (1:200); anti-mouse IgG-FITC (1:200) and anti-goat IgG-Cy3 (1:200), respectively, from Jackson ImmunoResearch Europe Ltd. Newmarket, Suffolk, UK].

Alkaline phosphatise (AP) staining of ESCs and chromosome number analyses was made as described by Nagy et al. (2003).

4.3.6.2 Chromosome number analysis

Chromosomes of established ESC lines were prepared as described by Nagy et al (2003). Briefly, demecolcin (Sigma-Aldrich Chem. Inc. St. Louis, USA) was added to subconfluent ESCs, grown on gelatin, with a final concentration of 10 µg/ml for 1.5 h. ESCs were then trypsinized, pelleted by centrifugation, re-suspended in 1 ml culture medium. To hypotonize the cells, 10 ml of 0.55% of KCl was added to the cell suspension and incubated for 15-20 min in a 37°C water bath. After that the pelleted cells were fixed in fresh ice-cold acetic acid and methanol (1:3) fixative. This step was repeated at least three times. Cell suspension was dropped on clear slides. After drying the slides, chromosomes were stained with DAPI stain. At least 50 metaphases from each cell lines were studied in order to examine their chromosome number.

4.3.6.3 Embryoid body formation

Embryoid bodies (EBs) formation was used for estimate the potential of *in vitro* differentiation of newly established ESC lines. ESCs were treated with 0.25% trypsin, single cells suspension (300 cells/ 20 μl drop) were cultured by hanging drop method for 2 days in ES culture medium with LIF withdrawal. The resulting ESC aggregates were transferred into bacteriological dish (Greiner Bio-One GmbH) in suspension culture to allow them to form a better shape. EBs were cultured in suspension continuously up to two weeks and their ability to form the structures termed simple EB (SEB), cystic EB (CEB) and beating EB (BEB) were observed. At least 20 embryoid bodies per dish were observed daily to determine the appearance of SEB, CEB or BEB

4.3.7 RNA preparation, RT-PCR

For cell cultures, the TRI reagent was used to obtain RNA samples using the preparation method of the manufacturer. The RNA was suspended in DEPC treated water (0.1% DEPC) and the amount was calculated using a spectrophotometer. EBs were collected by sedimentation in centrifuge tubes and lysed by TRI reagent. The RT-PCRs were made using the 'Titan One Tube RT-PCR Kit' and the protocol of the manufacturer.

The samples were amplified in a Perkin Elmer 9600 thermocycler using 40 amplification cycles of 30 sec 95°C denaturation, 60 sec 60°C annealing, 90 sec 72°C elongation. For final extension, one cycle of 10 min at 72°C were used. The PCR products were visualized on 2% agarose gel electrophoresis in 1xTAE buffer containing 0.5 mg/ml ethidium bromide. Oligonucleotide primers used for RT-PCR reaction are listed in Table 6.

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Table. 6 Polymerase chain reaction primers

Gene product	Primer sequence	Product size
Pou5fl	Forward: GGCGTTCTCTTTGGAAAGGTGTTC	293 bp
	Reverse: CTCGAACCACATCCTTCTCT	
α-Fetoprotein	Forward: AGTGCGTGACGGAGAAGAAT	494 bp
	Rererse: TGTCTGGAAGCACTCCTCCT	
Albumin	Forward: TGCTGCTGATTTTGTTGAGG	500 bp
	Reverse: GCTCACTCACTGGGGTCTTC	
Nestin	Forward: GGACAGGACCAAGAGGAACA	599 bp
	Reverse: TCCCACCTCTGTTGACTTCC	
GAPDH	Forward: ACCTCAACTACATGGTCTAC	801 bp
	Reverse: TTGTCATTGAGAGCAATGCC	

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4.4 Statistical analysis

The results were evaluated by chi-square test, with a *P*-values less than 0.05 were considered statistically significant. Immunocytochemistry results were confirms in at least three replications.

4.5 Experimental design

Experiment I was designed to produce cloned blastocysts by electrofusion method using cumulus cells as the donor cells. To generated cloned blastocyst, the metaphase II was removed from mature oocyte. The cumulus cell was transferred into the perivetilline space of enucleated oocyte. The couplet were electrically fused and the reconstructed oocyte were activated and cultured *in vitro* until reach blastocyst stage. Approximately 20-30 mature oocytes were used for each replication.

Experiment II was designed to investigate the efficiency of ESC derived from cloned and fertilized blastocysts. Ten to 24 from each type of blastocysts were cultured on the feeder layer for ESC derivation. Established ESC lines were characterized for ESC markers by immunostaining, RT-PCR. Chromosome analysis and *in vitro* differentiation via EBs were investigated in both NT-ESCs and F-ESCs.

4.6 Results

4.6.1 Experiment I; Production of cloned blastocysts by electrofusion technique

Two hundred and twelve MII oocytes were used in this experiment, 90.6% (192/212) of MII oocytes survived after removal of metaphase plate and 53.7% (94/175) of couplets were successfully fused after electrical stimulation. Fused oocytes were able to be activated by forming pseudo-pronuclei with 67.0% (63/94) of efficiency. However, most of activated oocytes arrested at two-three cell stage. *In vitro* development of cloned embryos produced by electrofusion was shown in Fig. 13.

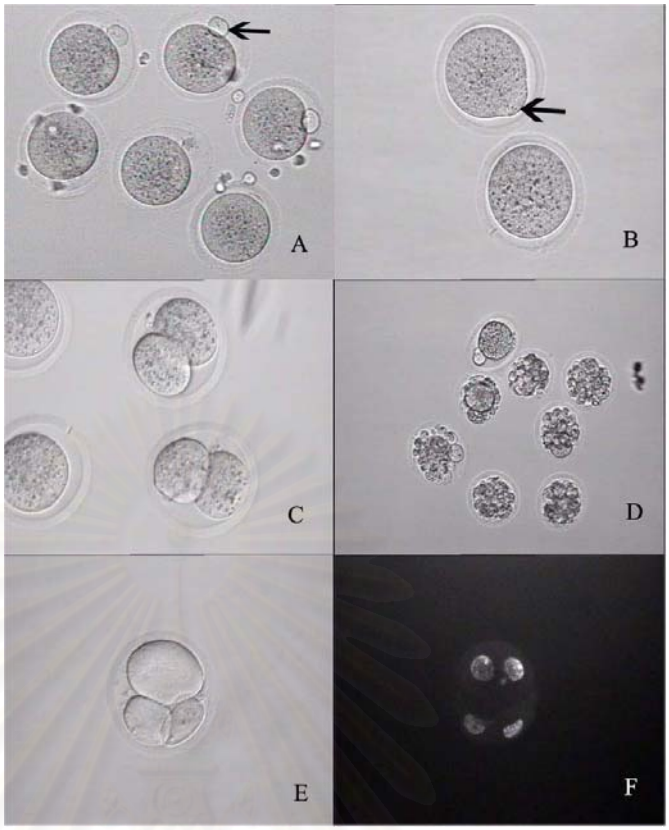

Figure 13 *In vitro* development of cloned embryos generated by electrofusion technique

Mature oocytes (A; magnification 40x) presenting the first polar body (arrow) were selected for NT experiment. Couplets were fused with cumulus donor cells by electrofusion (B; magnification 100x), the arrow indicates the fusing area. Cloned embryos developed *in vitro* into 2-cells stage (C; magnification 100x). Fragmented embryos were seen 12 h after activation (D; magnification 40x). Cloned embryo arrested at 3-cell stage after culture for 72 h (E; magnification 100x). Fluorescent picture of the same embryo in (E) shows the large non-divided blastomere containing two nuclei (binuleation) (F; magnification 100x)

Several replications of NT experiment were performed at the laboratory in Thailand. The success rate of blastocyst development was extremely low. The more appreciate technique to produce cloned blastocysts, piezo-driven, was used instead by the researchers at laboratory of Prof. Andras Dinnyes in Hungary. Thus, NT-ESCs lines in Exp. II were established at the laboratory in Hungary and then sent to Thailand for further studies.

4.6.2 Experiment II; Derivation of ESCs from cloned blastocysts produced by piezo-driven technique

4.6.2.1 Pou5fl positive cells in cloned and fertilized blastocysts

Blastocysts were investigated for the presence of Pou5f1 protein by immunostaining. The pluripotent cells positive to Pou5f1 were normally found in ICM in case of all the examined blastocysts as shown in Fig. 14.

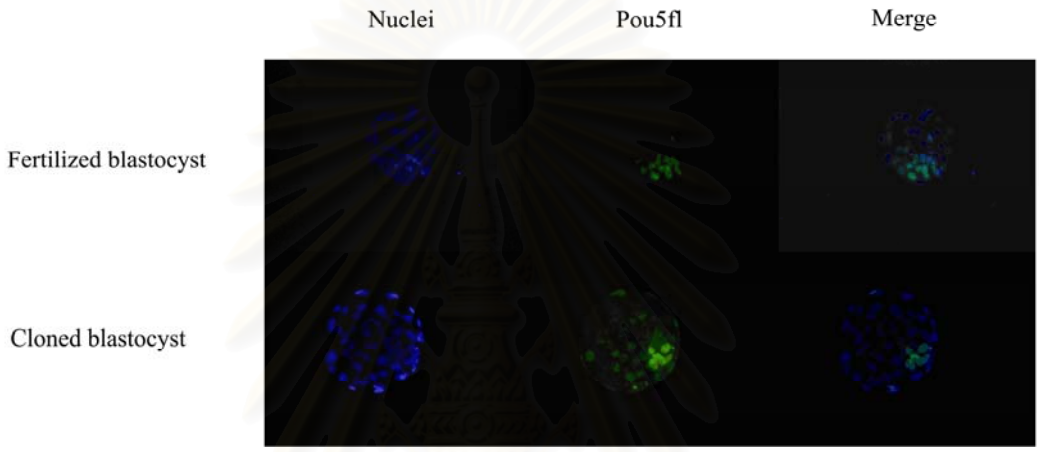

Figure 14 Expression of Pou5f1 positive cells in fertilized and cloned blastocysts

Fluorescent microscopic analysis revealed expression of Pou5f1 in the ICM (green) and nuclei were stained with DAPI (blue). Pou5fl positive cells were localized in ICM of fertilized blastocyst (A-C; magnification 100x) while some Pou5fl positive cells were seen in both the trophoblast cells and ICM of cloned blastocyst (D-F; magnification 200x).

4.6.2.2 Comparison of initial outgrowth formation and establishment of ESC lines from cloned and fertilized blastocysts

ICM attachment ability and consequent ESC colonies harvested from cloned and fertilized embryos are summarized in Table 7. Cloned blastocyst showed the significantly lower rate ($P<0.05$) of attachment (53.8%) than fertilized blastocyst (83.3%). The attachment to the feeder layer was seen from Day 2 of blastocyst culture. The attachment occurred by a gradual flattening of blastocysts followed by spreading out of trophoblastic cells. At Day 5 of blastocyst culture, the outgrowth, mostly from fertilized blastocyst, contained a central core of putative ICM-derived

cells, appearing large enough for enzymatic isolation and further subculture (Fig. 15A). After first trypsinization of ICM outgrowth, the ES-like colonies were formed (Fig. 15C) and the stable ESC line was established within 2-3 wks after initial plating (Fig. 15D). In the case of the outgrowth from cloned blastocyst, the putative ICM-derived cells seem much smaller than the outgrowth from fertilized blastocyst (Fig. 15B). According to the small size of putative ICM-derived cells, they were only mechanically removed from the trophectoderm outgrowth but not trypsinised and plated on a fresh feeder layer. Four to five days later, the putative ICM-derived cells grown much bigger (Fig. 15C) and ready to be trypsinized. Three out of four NT-ESC lines (Fig. 15F) were generated by this modification.

The data of ESC lines derived from cloned and fertilized blastocysts were summarized in (Table 7).

Table. 7 Establishment of ESC lines from fertilized and cloned blastocysts

Source of blastocyst	Blatocyst cultured	Outgrowth formation n (%)	ESC lines N	Efficiency (%)
Cloned	26	14 (53.8)	4	15.4 [a]
Fertilized	24	18 (75.0)	15	62.5 [b]

[a,b] Different superscripts indicate statistically significant difference within a column ($P<0.05$)

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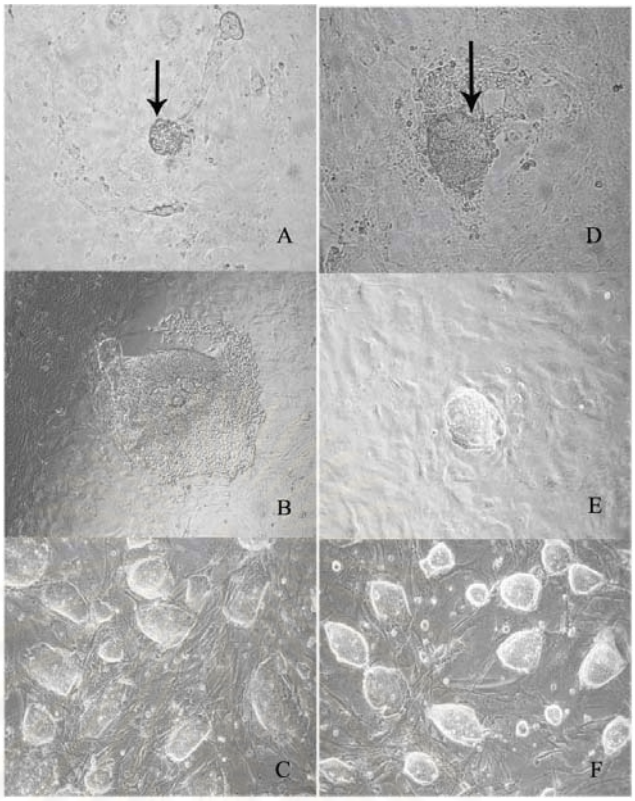

Figure 15 Derivation of ESCs from cloned and fertilized blastocysts

This picture shows the characteristic of dynamic growth of cloned blastocyst (A-C) and fertilized blastocyst (D-F) to ESC lines. Five days after initial plating, ICM outgrowth of cloned blastocyst (A) showed relatively smaller than ICM outgrowth of fertilized blastocyst (D). The primary ES like cells appeared after dissociation of ICM outgrowth. The primary ES like cells established from cloned blastocyst (B) contaminated with differentiated cells while the primary ES like cells from fertilized blastocyst (E) showed nice and typical ESC morphology. After several passages, ESCs can be obtained from cloned (C) and fertilized blastocyst (F) (magnification 200x)

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4.6.3 Characterization of ESC lines

By using morphological determinants, the colonies of stable ESC lines had a smooth outline and round or slightly oval in shape. Established ESC lines exhibited positive results for AP, SSEA-1, Pou5f1 and Nanog staining as shown in Table 8. The ability of NT-ESCs and F-ESCs differentiation *in vitro* was examined by EB development, was shown in Table 8. In early passage after establishment,

chromosome number analysis demonstrated that ESC lines derived from either cloned or fertilized blastocysts had the normal chromosome number at least 50% (Fig. 16).

NT-ESCs showed similar pluripotency and germ-lineage gene expression to those F-ESCs as shown in Fig. 17.

Table. 8 Characterization of NT-ESC and F-ESC lines by immunostaining and EB formation

ESC lines	Immunocytochemistry				EB Formation
	AP	SSEA-1	Pou5f1	Nanog	
NTES1	+	+	+	+	CEB
NTES2	+	+	+	+	CEB
NTES3	+	+	+	+	CEB
NTES4	+	+	+	+	BEB
FES1	+	+	+	+	CEB
FES2	+	+	+	+	CEB
FES3	+	+	+	+	BEB
FES4	+	+	+	+	BEB
FES5	+	+	+	+	CEB
FES6	+	+	+	+	CEB
FES7	+	+	+	+	CEB
FES9	+	+	+	+	BEB

Abbreviation: AP, alkaline phosphatise; SSEA-1, stage specific embryonic antigen-1; CEB, cystic embryoid body; BEB, beating embryoid body

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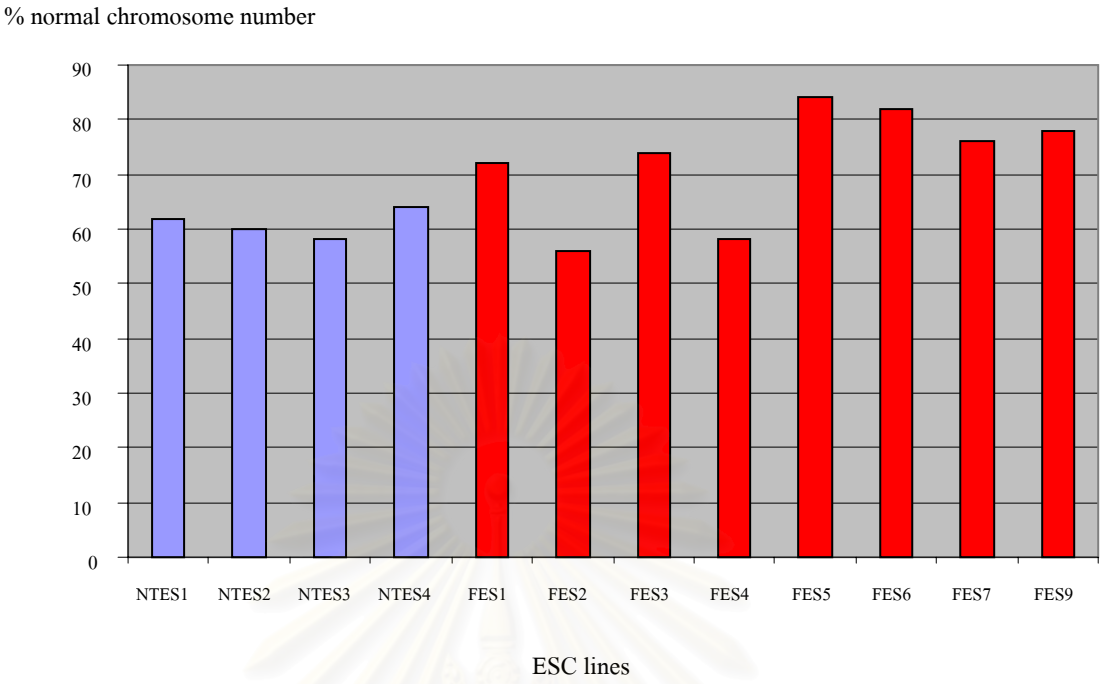

Figure 16 The percentage of normal chromosome number (40) of established ESC lines from cloned and fertilized blastocysts

Chromosome numbers were determined by cytogenic analysis of metaphase spread. NT-ES and F-ESC lines show normal chromosome number more than 50% of total counted metaphase.

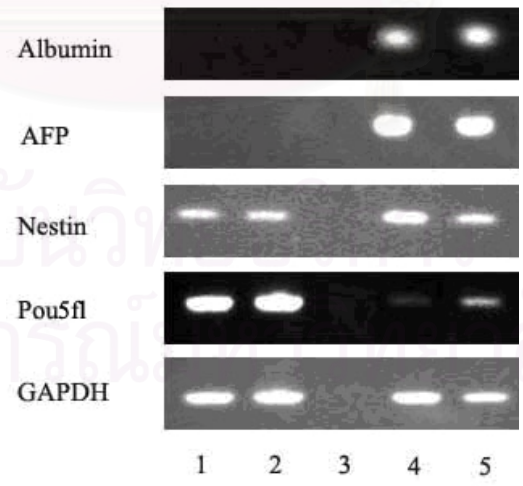

Figure 17 Expression of specific genes of ESCs of 14-day-old EBs.

Gene encoding transcription factors, markers of germ layers; ectoderm (Nestin), mesoderm (α- Fetoprotein; AFP) and endoderm (Albumin) expressed in F-ESCs (lane 1), NT-ESCs (lane 2), EBs derived from F-ESCs (lane 4) and EBs derived from N-TESCs (lane 5)

4.7 Discussion

ESC lines are conventionally established from cloned blastocysts produced by piezo-driven technique (Wakayama et al., 2001; Munsie et al., 2000; Wakayama et al., 2005). Derivation of NT-ESCs from cloned blastocysts generated by electrofusion technique was reported by Amano et al (2006) using ESCs as donor cells, however, no report so far using cumulus cells. In mouse, NT using electrofusion is inherently defective because the electrical stimulus activates the recipient oocytes. Consequently, the exposure time of the donor nucleus to the inactivated ooplasm is reduced (Kono, 1997). Also, the inefficient fusion can be caused by the little contact surface between cumulus cell and ooplasm due to the size of cumulus cell is relatively small. In this study, the fusion rate was comparable to the previous reports (Yu et al., 2005; Yu et al., 2007) but most of cloned embryos stopped their development at two cells stage, suggesting that the reprogramming of donor cell was not completed (Heindrykx et al., 2001). Cumulus cell is considered as somatic cells and that the epigenetic state of somatic cell genomes can be less efficiently reprogrammed (Blelloch et al., 2004).

However, the results in Exp II demonstrated that cloned blastocysts can be generated by piezo-driven technique using cumulus cells as the donor cells and NT-ESC lines can be established from those cloned blastocysts. The rates of NT-ESC derivation displayed significantly lower than F-ESCs. At a molecular level, the examined NT-ESC lines established from this study were positive for characterization of AP, SSEA-1, Pou5f1 and Nanog. Also NT-ESCs and EBs derived from NT-ESCs show the expression of Pou5fl, pluripotent gene and germ lineage gene as detected by RT-PCR. NT-ESC lines showed at least 60% of normal euploidy after chromosome number counting and they were able to form EBs as well as beating areas after differentiation *in vitro*, suggesting that these NT-ESC lines would have been pluripotent (Wakayama et al., 2001) and resembling these derived from fertilized blastocysts (Wakayama et al., 2006).

Cumulus cells known as somatic cells type showed the less efficiency in NT-ESCs establishment compared to other donor cell types, for example embryonic carcinoma (EC) cells and ES nuclei (Wakayama et al., 1999; Rideout et al., 2000; Blelloch et al., 2004). However, the efficiency from this experiment was 15.4% which was comparable to the previous report using the cumulus nuclei as the donor cells (Wakayama et al., 2005). In general, the efficiency of ESCs derived from cloned

blastocysts was lower than fertilized blastocysts due to the quality of cloned blastocysts, which is inferior to those fertilized ones as seen by less total cell numbers, high propotion of dead cells and abnormality of Pou5f1 expression in blastocyst embryos (Rybouchkin et al., 2002; Scholer et al., 1990). Although in this study, the Pou5f1 positive cells were detected in both fertilized and cloned blastocysts, NT-ESC establishment was still less efficient in comparison to fertilized blastocysts. It is possibly due to Pou5fl positive cells in cloned blastocysts not be able to maintain their pluripotency in ESC culture condition and this phenomenon needs to be proven.

The characteristic of dynamic growth from blastocyst to ESCs was observed in this experiment. Cloned and fertilized blastocysts show similar rate of attaching to the feeder layer. After initial plating, cloned blastocysts gave a lower rate of outgrowth formation compared to the fertilized ones. At the early stage of ESC derivation, cloned blastocysts formed a smaller size of ICM outgrowth compared to fertilized blastocysts. In some cases, there was no ICMs in the central core of the outgrowth, only the trophotodermal cells were present, this means those ICM in cloned blastocysts probably either degenerated or not propagated during the few days of culturing. The stage at which an individual ICM outgrowth is selected for disaggregation is fairly critical. Under the suitable conditions of culture, embryos will normally attend a suitable morphology following a 5 days of culture period (Robertson, 1987). However, there is no absolute correlation between phenotype of the colony and successful isolation of ESC lines. ESC lines can be obtained from both vigourous growing ICM and from the small outgrowth (Robertson, 1987). Thus, the small outgrowth of cloned blastocyst can give rise to NT-ESC lines. Due to the ICM outgrowths of cloned blastocysts were relatively small they were mechanically isolated by fine glass pipette without trypsinization and replated on the new feeder layer. This similar mechanical isolation has been proved beneficially to establish human ESCs (Kim et al., 2005; Strom et al., 2007) and buffalo parthenogenetic ESCs (Srithanaudomchai et al., 2007). Three out of four NT-ESC lines were generated by this modified method.

In this experiment, both NT-ESCs and F-ESCs showed the similar colony morphology: round shape, clear edge, compact and dense colonies. The established NT-ESC lines were positive for the markers of undifferentiated pluripotent mouse ESCs, including AP, SSEA-1, Pou5f1 and Nanog. After freezing, thawing and contineous culturing, these NT-ESC lines are able to maintain the undifferentiated

status similar to F-ESC lines. It has been proved that ESC lines which had normal chromosome number in more than 50% of cells are efficient for germ-line transmission due to successful segregation of chromosomes throughout meiosis (Longo et al., 1997, Suzuki et al., 1997). However, established NT-ESC lines were not determined for their ability of contribution to all three germ layers through germ-line chimera. Nevertheless, this study shows that EBs derived from both NT-ESCs and F-ESCs can express markers of all three germ layers, indicating their utility for the study of differentiation *in vitro*. The ability of ESCs to form simple, complex or beating EBs is one of the most popular methods to differentiate ESCs *in vitro*. Established NT-ESC lines with at least 60% of euploidy showed the ability to form simple and cystic EBs after differentiation *in vitro*. In contrast, ESC lines which showed less than 60% of euploidy were able to form only simple EBs but not cystic EBs. Thus, chromosome number analysis is a prerequisite for ensuring the normality of NT-ESC lines, as with F-ESCs. It is considered that ESC lines able to form only simple EBs have restricted pluripotency (Sukoyan et al., 2002) and the developmental potential of ESCs was lost easily due to the accumulation of profound epigenetic modifications by long term *in vitro* cultivation (Amano et al., 2006). This study demonstrated that NT-ESCs were identical to F-ESCs in their expression of pluripotency markers, pluripotency gene and germ lineage expression. Chromosome analysis demonstrated that most examined NT-ESC lines had the normal chromosome number.

In summary, NT-ESC lines were successfully established from cloned blastocysts using a simple derivation method. NT-ESCs displayed the morphological and molecular characteristic of conventional F-ESCs. Although the derivation efficiency of NT-ESCs is inferior to F-ESCs but the information on the characteristic of cloned blastocysts to ESC lines during the ESC derivation step, reported in this study is profitable. This might be useful for improving the efficiency of NT-ESC derivation by modification of the culture conditions and procedure.

CHAPTER V

IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS DERIVED FROM CLONED AND FERTILIZED BLASTOCYSTS

5.1 Abstract

Embryonic stem cells derived from cloned blastocysts (NT-ESCs) and fertilized blastocysts (F-ESCs) showed the potential to differentiate into all three germ layers, suggesting that both type of ESCs provide a new perspective for embryonic development study and application in regenerative medicine. Although the differentiation potential of NT-ESCs has been previously reported, not many studies were undertaken on the differentiation via EBs of NT-ESCs in comparison to those F-ESCs. This experiment was designed to investigate the differentiation of EB derived from NT-ESCs and F-ESCs in suspension culture through morphological analysis and examination of gene expression. EBs were generated via hanging drop method and collected at specific time points for up to five weeks. The diameters of EBs were measured and germlineage gene expression was detected by RT-PCR. A morphological study revealed that NT-ESCs and F-ESCs are able to form simple and cystic EBs. EBs continuously increased their size with time but reached a peak around Day 18-20. NT-ESCs derived EBs showed significantly greater diameter than those F-ESCs derived EBs at Day 7 and Day 10. The histological analysis indicated that NT-ESCs and F-ESCs derived EBs exhibited the similar structure according to the period of examinations. Solid round structures consisting of cells of uniform appearance were found in 7-day-old, EBs which contained most of differentiated structures. The primitive neural tube appeared by day 14 in both EBs derived from NT-ESCs and F-ESCs. Gene expression pattern of Pou5f1, ectoderm (Nestin and Pax-6), mesoderm (Flk-1 and PECAM) and endoderm (GATA-4) markers were similar in NT-ESCs-derived EBs and F-ESCs-derived EBs. Nevertheless, AFP (endoderm) mRNA expression of F-ESCs decreased while NT-ESCs derived EBs increased through the culture period. This study indicated NT-ESCs derived EBs represented differentiation similar to F-ESCs derived EBs. The main difference between NT-ESCs and F-ESCs derived EBs was AFP expression. The comparative study of *in vitro* differentiation of NT-ESCs and F-ESCs derived EBs was reported in this study for the first time.

5.2 Introduction

Nuclear transfer-derived embryonic stem cells (NT-ESCs) and fertilization - derived embryonic stem cells (F-ESCs) have the potential to differentiate into cells of various lineages upon proper stimulation, providing new perspectives not only for embryonic development but also for the application in regenerative medicine (Munsie et al., 2000; Kawase et al., 2000). Recently, ESCs have been shown to differentiate into insulin-secreting B cells treating diabetic animals (Soria et al., 2000; Assady et al., 2001; Lumelsky et al., 2001). Also transplantation of neuron cells derived from mouse ESCs successfully rescued defective neurons in the central nervous system, providing their potential value in the treatment of neuron disease (Brustle et al., 1999; McDonald et al., 1995). Furthermore, NT-ESCs show similar differentiation potential to those F-ESCs as germline transmission can be obtained, suggesting that NT-ESCs can differentiate into all embryonic tissues (Wakayama et al., 2005; Wakayama et al., 2006).

ESCs can be differentiated readily in the presence or absence of chemical inducers such as retinoic acid (RA) and hexabisacetamide (Robertson, 1987). A synchronous pattern of differentiation can be obtained by the technique of suspension culture to induce the formation of three dimensional structures. By *in vitro* system, differentiation of ESCs generally occurs after formation of three-dimentional cell aggregates, so called embryoid bodies (EBs). Upon aggregation, differentiation is initiated and the cells begin to a limited extent to recapitulate embryonic development. The aggregate, while first simply appearing as a ball of cells, takes on an increasingly more complex appearance, becoming after a few days a hollow ball, so called cystic EBs, and next forms internal structures such as a yolk sac, and cardiomyocytes, heart muscle cells which beat in a rhythmic pattern to circulate nutrients within the increasing EBs. Various types of differentiated cells, such as neural cells, cardiac and skeletal muscle cells, hematopoietic cells, adipocytes, chondrocytes and osteoclasts, are found in the EBs and in the molecular level, EBs are able to express a set of germ-lineage genes (Itskovitz-Eldor et al., 2000). In mouse, gene expression in EBs showed similarity to those detected in embryos and adult animals (Choi et al., 2005)

Among specific markers of germ layers, Nestin and Pax-6 are expressed in the neuroectodermal area. Flk-1 and PECAM in expressed in the mesodermal area, GATA-4 and α-fetoprotein (AFP) are considered endodermal markers initially

expressed in the primitive endoderm during early postimplantation stages and are maintained in the visceral and parietal endoderm of the yolk sac during gastrulation. These markers are useful for identifying germ layers in EBs. Currently, numerous research efforts have been put forth to examine the ESC differentiation via EBs, limited knowledge about differences or similarity of growth and gene expression between EBs derived from NT-ESCs and F-ESCs.

The aim of this experiment was to investigate the differentiation of NT-ESCs and F-ESCs via EB development, particularly with respect to selected developmental periods at 7, 14, 21, 28 and 35 days. This was achieved through the examination of gross morphology, size of EBs, histological analysis and monitoring of gene expression by RT-PCR.

5.3 Materials and Methods

5.3.1 Culture of NT-ESCs and F-ESCs

NT-ESCs and F-ESCs used in this study were derived in Chapter 3 and 4. ESC lines at passage number 10, showed highest number of normal chromosome, together with the ability of EBs formation in the preliminary study were chosen. ESCs were cultured on mitomycin C-mitotic inactivated MEF feeder cells in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, USA).) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT, USA), 0.1 mM 2-mercaptoethanol (Invitrogen, Carlsbad, USA), 1% non essential amino acid (Invitrogen, Carlsbad, USA) and 1000 U/ml recombinant murine leukemia inhibitory factor (ESGRO-LIF, CHEMICON). ESCs were incubated at 37 °C and 5% CO_2 in a humidified atmosphere.

5.3.2 Embryoid bodie (EB) formation and *in vitro* differentiation of ESCs

To make feeder-free ESCs before formation of EBs, ESCs were dissociated from the feeder layer, seeded on a culture dish and incubated for 15 min at 37 °C to remove feeder cells (due to most of feeder cells normally attached to the bottom of the culture dish). Then floating ESCs were collected and replated on gelatin-coated dish. Undifferentiated ESCs were cultured in ESCs medium without feeder layer and LIF for 1 wk. To make EBs by hanging drop, drops (20 µl) of 300 cells were placed on the

underside surface of the lid of 100-mm tissue culture dish containing PBS and cultured for 2 days. EBs were then cultured in the suspension in low adhesion culture dishes for certain period of time according to the experimental design.

EBs were cultured in the absence of exogenous differentiation inducer(s). Culture medium consisted of DMEM supplemented with 20% FBS, 0.1 mM non essential amino acid, 0.1 mM 2-mercaptoethanol and 1% penicillin-streptomycin. Culture medium was changed every other day.

5.3.3 Embryoid body characterization and histological Analyses

Gross morphological analysis was performed to determine growth and development. In brief, 10 EBs in each group of EBs were measured every 3-4 days by phase contrast image capturing. According of the three dimension of EBs, the measurement recorded for an individual EBs was the average of the smallest and largest diameter of the EB. The mean diameter of EBs at the specific time points was calculated from the average diameter of 10 EBs. The included EBs for analysis has to meet the criteria; round, cystic and well defined borders.

EBs were collected at weekly interval for histological analysis. They were washed three times with PBS, fixed overnight in 10% neutral-buffer formaline, dehydrated in a series of alcohol gradients, embedded in paraffin and stained the 4 μm of sections with hematoxyline and eosin (H&E). The sections were examined for general histomorphology.

5.3.4 Detection of pluripotency and germ lineage marker expression by EBs

RNA was extracted from NT-ESCs and F-ESCs or EBs collected at days 7, 14, 21, 28 and 35 from each type of ESCs derived EBs according to the manufacturer's instruction. EBs were collected by sedimentation in centrifuge tubes and lysed by TRI reagent. The RNA were suspended in DEPC treated water (0.1% DEPC) and the amount was calculated using a spectrophotometer. The RT-PCRs were made using the 'Titan One Tube RT-PCR Kit' and the protocol of the manufacturer. The samples were amplified in a Perkin Elmer 9600 thermocycler using 40 amplification cycles of 30 sec 95°C denaturation, 60 sec 60°C annealing, 90 sec 72°C elongation. For final extension, one cycle of 10 min at 72°C were used. The PCR products were visualized on 2% agarose gel electrophoresis in 1xTAE buffer

containing 0.5 mg/ml ethidium bromide. Oligonucleotide primers and PCR conditions used for RT-PCR reaction are listed in Table 9.

Table 9 Polymerase chain reaction primers

Gene	Primer
Pou5fl	Forward: GGCGTTCTCTTTGGAAAGGTGTTC
	Reverse: CTCGAACCACATCCTTCTCT
GAPDH	Forward: ACCTCAACTACATGGTCTAC
	Reverse: TTGTCATTGAGAGCAATGCC
Nestin	Forward: GGACAGGACCAAGAGGAACA
	Reverse: TCCCACCTCTGTTGACTTCC
Pax-6	Forward: AGACTTTAACCAAGGGCGGT
	Reverse: TAGCCAGGTTGCGAAGAACT
Flk-1	Forward: CCTGGTCAAACAGCTCATCA
	Reverse: AAGCGTCTGCCTCAATCACT
PECAM	Forward: GTCATGGCCATGGTCGAGTA
	Reverse: CTCCTCGGCATCTTGCTGAA
GATA-4	Forward: CTCCTACTCCAGCCCCTACC
	Reverse: GTGGCATTGCTGGAGTTACC
AFP	Forward: AGTGCGTGACGGAGAAGAAT
	Reverse: TGTCTGGAAGCACTCCTCCT

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5.4 Statistical analysis

The efficiency of EB formation was analyzed by chi-square test, mean of diameter of EBs were analyzed by analysis of variance. The experiments were repeated three times. Statistical significance was determined as $P<0.05$.

5.5 Experimental design

EBs derived from NT-ESCs or F-ESCs were produced and culture in hanging drop for two days. After two days, EBs were cultured in suspension up to 35 days. For measurement of EBs diameters, EBs were collected and measured for the average diameter at day 3, 7, 10, 14, 18, 22, 26 and 28. EBs were collected at weekly interval and subjected to examine histomorphological structure and gene expression by real time RT-PCR. A schematic diagram of experimental design was shown in Fig. 18.

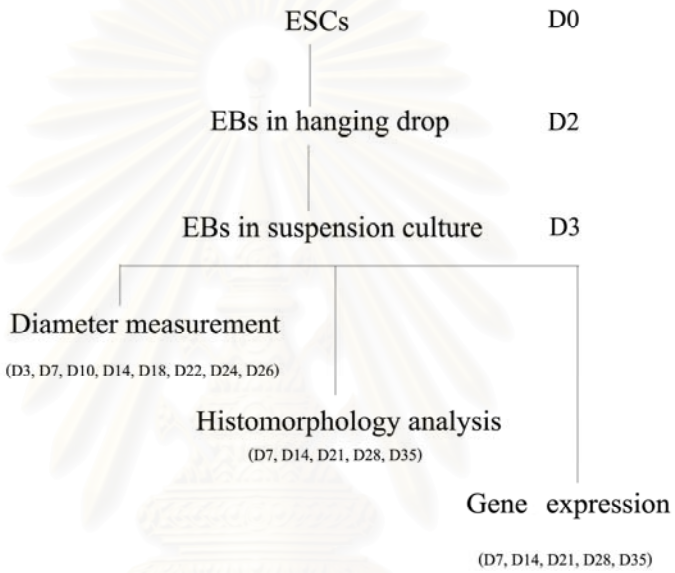

Figure 18 A schematic diagram of experimental design to study in vitro differentiation of ESCs derived from cloned and fertilized blastocysts

5.6 Results

5.6.1 Morphology and size of EBs

Typically, formation of EBs occured in hanging drop within 24 h. The efficiency of EB formation of NT-ESCs and F-ESCs derived EBs was not significantly different [95% (185/193) vs 96.8% (213/220) respectively; ($P > 0.05$)]. After culture in hanging drop for 2 days, the results showed that NT-ESCs and F-ESCs derived EBs formed a similar shape, round and clear edge. After culture for 7-10 days, EBs have developed fluid filled cavity, so called cystic EBs. Cystic EBs have

seen in both NT-ESCs and F-ESCs derived EBs. By day 14, beating areas were found in both NT-ESCs and F-ESCs derived EBs. Cystic EBs remained viable up to 35 days. Growing of EBs derived from NT-ESCs and F-ESCs are shown in Fig. 19.

The mean diameter of EBs derived from different sources of ESCs over a period of 28 days was variable. At day 3, NT-ESCs derived EBs showed no difference of mean diameter to those F-ESCs derived EBs. Interestingly, at day 7 and 11 after continuous culturing in suspension, NT-ESCs derived EBs showed the significantly greater diameter ($P<0.05$) in comparison to F-ESCs derived EBs. From Fig. 21, it appeared that the growth of NT-ESCs derived EBs peaked at day 22 when compared in each time point. Beyond day 22, EBs tended to display budding and/or aggregated morphologies, making them unsuitable for measurement according to the criteria selected. However, budding formation was seen in both NT-ESCs and F-ESCs derived-EBs (Fig 20).

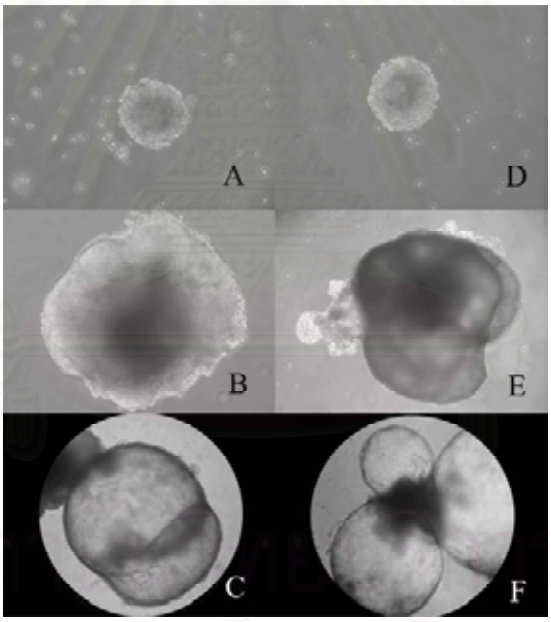

Figure 19 Development of EBs derived from NT-ESCs and F-ESCs

Development in suspension culture of EBs derived from NT-ESCs (A-C) and F-ESCs (D-F). Three days after EB formation, EBs showed simple structure with round shape (A, D; magnification 40x). After 7 days, EBs developed the fluid-filled cavity, “cystic EBs” (B, E; magnification 200x). The primitive yalk-sac-like structures were in EBs after continued culturing in suspension for 35 days (C, F; magnification 200x).

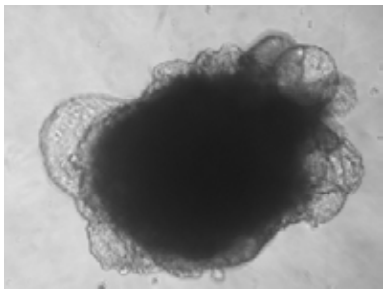

Figure 20 Budding formation of EBs (magnification 200x)

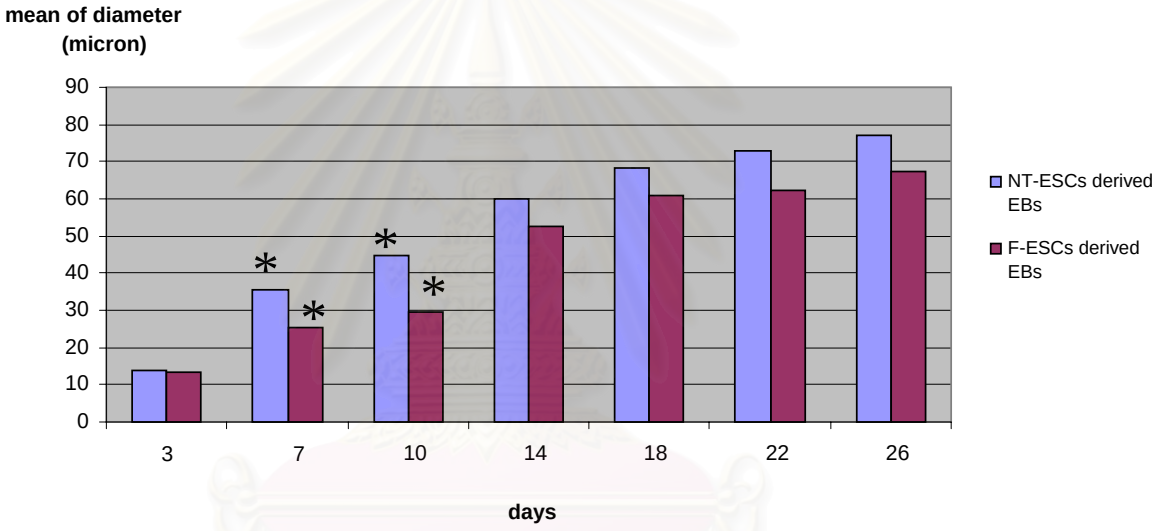

Figure 21 Diameter of NT-ESCs and F-ESCs derived EBs in suspension culture

The images of EBs were captured every 3-4 days and the diameters were measured. The mean of diameter were calculated as the average of the largest and smallest of each EB as in the captured images and compared within the day of measurement. Data are expressed as mean ± SE. For each group n=10. * $P < 0.05$

5.6.2 Histological structure

Histological examinations of EBs using H&E staining, showed the presence of embryonic germ layers at a basic morphological level. At day 14, neural rosettes were observed in NT-ESCs and F-ESCs derived EBs (Fig. 22).

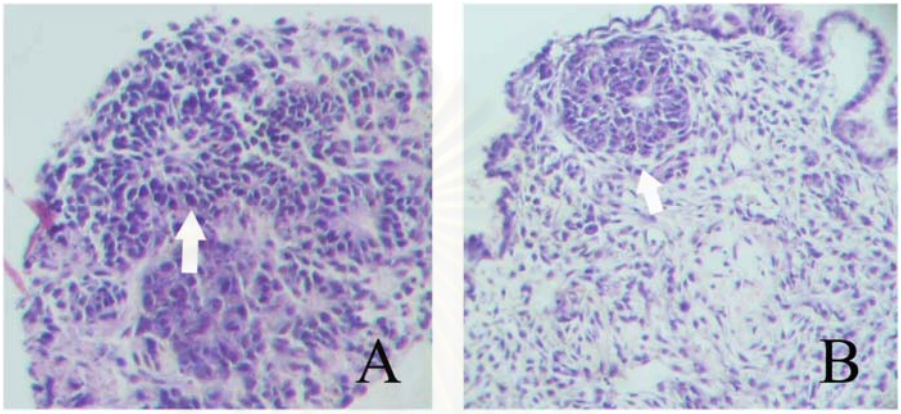

Figure 22 Histological analysis of hematoxylin and eosin-stained EBs (magnification 400x)

EBs derived from F-ESCs (A) and NT-ESCs (B) containing rosette formations resembling that the primitive neural tube as indicated by arrows.

5.6.3 Expression of pluripotency and germ lineage marker by EBs

To determine whether the difference observed in morphology and size of EBs were reflected in the pattern of gene expression, expression of pluripotency and germ lineage genes in developing EBs were monitored using real time RT-PCR. Markers of pluripotency (Pou5f1) and germ lineages, ectoderm (nestin, Pax-6), endoderm (AFP, GATA-4) and mesoderm (Flk-1, PECAM) were included in this analysis. The expression of pluripotent marker and germ lineage markers revealed distinct changes during the process of EB developmet. The comparative gene expression of NT-ESCs and F-ESCs derived EBs was shown in Fig. 23.

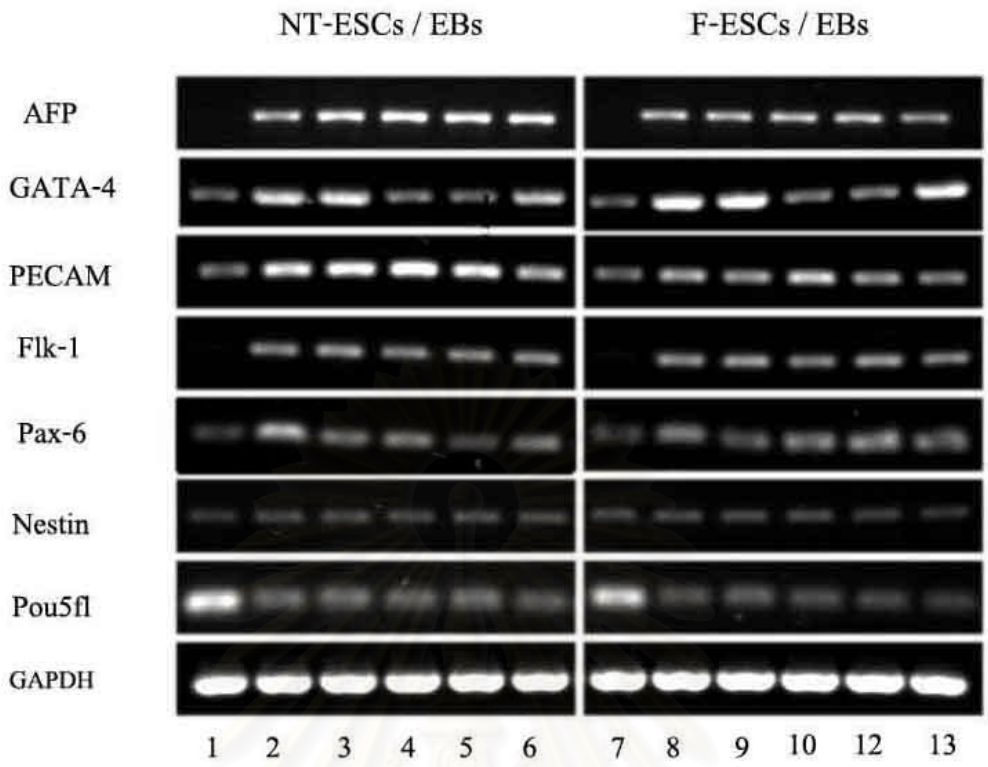

Figure 23 Comparison of mRNA expression of NT-ESCs and F-ESCs derived EBs

mRNA expression analysis of EBs with pluripotent (Pou5fl), ectodermal (Nestin, Pax-6), mesoderm (Flk-1, PECAM) and endoderm (GATA-4, AFP) markers. Lane 1 shows undifferentiated NT-ESCs. Lane 2-6 shows NT-ESCs derived EBs at 1 , 2, 3, 4 and 5 wks respectively. Lane 7 shows undifferentiated F-ESCs. Lane 8-13 shows F-ESCs derived EBs at 1, 2, 3, 4 and 5 wks respectively. GAPDH were used for the quantitation of RNA. Abbreviation: PECAM, platelet and endothelial cell adhesion molecule; AFP, α-fetoprotein

In RT-PCR analysis, mRNA of Pou5fl in EBs progressively decreased with time. However, low-level expression of Pou5f1 persisted in NT-ESCs derived EBs until day 35. The expression of ectoderm marker revealed similar changes during the process of EB development of NT-ESCs and F-ESCs derived EBs. Nestin mRNA, a peptide specific to neural stem cells, were lacking in undifferentiated ESCs but progessively increased at the first week and the expression prolonged for the rest of culture period. The level of Pax-6, another neural stem cell marker, increased at the first week and showed a peak in the fifth week.

The expression of Flk-1, a mesoderm marker, was not detectable in undifferentiated ECSs but detectable in EBs. The highest level of Flk-1 mRNA was detected in the first week of both NT-ESCs and F-ESCs derived EBs and remained strong throughout the rest of culture period. The level of PECAM increased from the first week and reached the peak on the third week and started to decrease a week later.

The expression of GATA-4, marker for endoderm, which expressed in visceral endoderm, showed increasing expression from the first to the second week but slightly decreased from the third to the fourth week, and started to increase again in the fifth week. Difference in gene expression pattern between NT-ESCs and F-ESCs was found in the expression of AFP mRNA, another endoderm marker. AFP was not detectable in undifferentiated NT-ESCs and F-ESCs but expression of AFP in NT-ESCs derived EBs continueously increased while in F-ESCs derived EBs continueously decreased throughout the culture period.

5.7 Discussion

The aim of the present study was to compare the differentiation potential of NT-ESCs and F-ESCs via EB formation, particularly through examination of morphology and gene expression of germ-lineage. This study revealed that in a simple culture condition without any chemical inducer (s), both NT-ESCs and F-ESCs were able to form EBs, the three-dimensional structure, and also differentiated into fully differentiated cells. It has been proved previously that genetic and epigenetic status of NT-ESCs and F-ESCs are equal as they showed similar gene expression profile, DNA methylation as well as germ-line transmission potential (Wakayama et al., 2006; Brambrink et al., 2006). However, this study showed some minor differences in morphology, size of EBs and expression of selected germ lineage genes between EBs derived from NT-ESCs and F-ESCs.

The efficiency of EB formation as well as the uniformity of the size were similar between both types of EBs after culture for 2 days in hanging drop. NT-ESCs and F-ESCs derived EBs showed smooth, spherical aggregrate of uniform size, indicating that NT-ESCs and F-ESCs can differentiate equally by this EB formation method. This uniformity of size observed in EBs has been suggested to be important for subsequent EB differentiation since endogenously produced LIF may exert an autocrine or paracrine function even though exogenous LIF is removed (Ansell and

Hole, 2000). The diameter of EBs from both types of ESCs increases with time of culture period and reaches a peak at Day 22. After that the diameter of both types of EBs seems decreased. It might be related to some cells located inside of EBs which are in apoptotic status and apoptotic process occurs normally during the development of EBs (Choi et al., 2005). This study revealed that NT-ESCs and F-ESCs can form primitive tissues in the simple culture condition. The evidence of this is structures resembling the primitive neural tube which appeared in the 2-week-old EBs different from those reported previously in which similar structures were found in 4-week-old EBs (Choi et al., 2005; Khoo et al., 2005). After the expression of lineage specific markers was analyzed in a time course, it has been found that the expression of Pou5f1, the pluripotent gene, progressively decreased through the culture period in both NT-ESCs and F-ESCs derived EBs. However, the difference of period that EBs can prolong the pluripotency stage was seen in NT-ESCs and F-ESCs derived EBs. Pou5f1 was progressively suppressed in F-ESCs derived EBs while NT-ESCs derived EBs expressed this gene strongly throughout the rest of the culture period, suggesting that F-ESCs derived EBs reached differentiated status earlier than NT-ESCs derived EBs. This prolonged expression of Pou5fl in EBs was also observed in other species (Yamamoto et al., 2007). Among specific markers of germ layers, NT-ESCs and F-ESCs derived EBs displayed a similar pattern of mRNA gene expression in most cases of a marker except AFP. AFP is considered as an endodermal marker initially expressed in the primitive endoderm during early post-implatation stage is maintained in the visceral and parietal endoderm of the yolk sac during gastulation (Choi et al., 2005). The AFP expression in F-ESCs derived EBs progressively decreased, opposite to NT-ESCs derived EBs which was increased throughout the culture period. It has been demonstrated that the expression of AFP in EBs derived from F-ESCs increased between 1 and 3 weeks after suspension culture but started to decrease after 5 weeks (Choi et al., 2005) which is similar the expression pattern of AFP of F-ESCs derived EBs in this experiment. In conclusion, this is the first comparative study of *in vitro* differentiation of EBs derived from NT-ESCs and F-ESCs. The observed differences between F-ESCs and NT-ESCs derived EBs were ESCs type-specific rather than arising from differences in different NT-ESC cell lines, which need to be investigated in the further study.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

Although embryonic stem cells (ESCs) are now commercially available, they can be used only during a limited period of time due to their aging and karyotypic instability after long term culture. Furthermore, surviving ESCs may be damaged or lost during transportation thus establishing new ESC line(s) in the laboratory is still necessary. To derive ESCs from mouse, the selected strains of mouse have to be considered, due to the fact that some strains are still non-permissive for ESC derivation. So far ESCs derived from fertilized blastocysts have been expected to be used for therapeutic application but the problem of immuno-rejection when using these ESCs by the patients is one of the limitations. Lately, patient specific ESCs isolated from nuclear transfer blastocysts became more attractive since these ESCs are derived from the somatic cell (Munsie et al., 2000; Kawase et al., 2000; Wakayama et al., 2001)

ESC derivation is a highly selective process because only the rare cells in embryos are able to proliferate under the tissue culture conditions. Most of inner cell mass (ICM) cells of blastocysts extinguish the expression of Pou5f1, a key pluripotency gene, and cease dividing after the explanting of blastocysts in culture (Buehr et al., 2002). Only a small fraction of the explanted cells are able to maintain Pou5f1 expression and proliferate to give rise to the immortal ESCs. The important point is that, regardless of whether a given ESC line was derived from a fertilized blastocyst of any mouse strains, or cloned blastocysts, the ICM cells had to undergo the stringent selection for *in vitro* survival and proliferation.

In order to develop and establish ESC and NT research in our laboratory, derivation of mouse ESCs from fertilized blastocysts was firstly studied. The knowledge and results from derivation of fertilization-derived ES cells (F-ESCs) were transferred and adapted for derivation of nuclear transfer-derived ES cells (NT-ESCs). Finally, *in vitro* differentiation potential of NT-ESCs in comparison to F-ESCs was investigated via embryoid bodies (EBs) development.

6.1 Method of ESC derivation

The protocol of ESC derivation has been reported since 1981 (Evan and Kaufman, 1981; Martin, 1981) but the standard protocol which repeatable and usually referenced was reported by Robertson (1987). This standard protocol is simple and the success of ESC derivation can be expected. However, this standard protocol has been modified in several ways to yield a higher success rate of ESC derivation and to make it more effective. In this study, the standard protocol was slightly modified by increasing the concentration of LIF in the derivation media. Normally the concentration of LIF at 1,000 IU/ml is able to inhibit the differentiation of ESCs when culture with feeder cells (Evan and Kaufman, 1981; Martin, 1981; Robertson, 1987). LIF was raised to 2,000 IU/ml in the culture medium, during the derivation step and decreased to 1,000 IU/ml when the stable ESC lines were established. The concentration of LIF was increased in order to maintain the pluripotency of ES precursor cells in ICM. Interestingly, this similar modification of LIF concentration in ESC derivation was proved to be suitable for derivation of ESCs from inbred mouse strains (Baharvand and Mattai, 2004). The ability of ES precursor cells in ICM and also ESCs, to maintain their pluripotency does not only depend on LIF. Other unknown growth factors present in culture media whether they were secreted from feeder cells or contained in serum, also affect their pluripotency. It was generally accepted that serum contains some unknown factor(s) which sometime can induce ESCs to differentiate. Thus, using serum replacement instead of serum has been successfully proved to derive ESC lines with high efficiency (Bryja et al., 2006; Cheng et al., 2004). However, by a simply modification of standard protocol, 33 ESC lines derived from fertilized blastocyts and 4 ESC lines derived from cloned blastocysts were established in this present study with an acceptable efficiency.

6.2 Different mouse strains, different stages of blastocysts and different results of ESC derivation

The efficiency of mouse ESC derivation is strain dependent (Kawase et al., 1994; Suzuki et al., 1999). Standard protocol of ESC derivation was not always successfully applied for ESC derivation in all strains. Using *in vivo* produced at expanded blastocysts stage, ESC lines can be established from inbred and hybrid but not outbred strain using standard protocol. Outbred strain has been recognized as a non-permissive strain for ESC derivation and not be able to derived an ESC line in the

previous study (Brook et al., 2003). Surprisingly, outbred ESC lines were able to establish in this study by culturing delayed blastocysts, suggesting that stages of outbred embryos influence the success of ESC derivation. The results from this study confirm the previous information that mouse ESC derivation is strain dependent (Kawase et al., 1994; Suzuki et al., 1999). Furthermore, the results of outbred ESC derivation emphasize that blastocyst stage is important for the success of ESC derivation.

6.3 NT-ESCs and F-ESCs

Cloned blastocysts are absolutely generated in different process from fertilized blastocysts. The somatic cell was reprogrammed by means of the factor(s) inside the cytoplasm of the enucleated oocyte, resulting in the developing embryos. In contrast, fertilized blastocysts were developed via the process of fertilization of sperm and egg. Previous data have shown that embryos developed from somatic donor cells after nuclear transfer exhibited marked differences in gene expression when compared to embryos obtained from fertilized eggs (Smith et al., 2005; Ng and Gurdon, 2005), suggesting that cloned blastocyst retains an epigenetic memory of its donor nucleus (Jaenisch, 2004). Nevertheless, this epigenetic memory of donor nucleus was erased after explantation of these blastocysts in the *in vitro* culture condition for ESC derivation (Jaenisch, 2004).

The efficiency of derivation of ESCs lines from cloned blastocysts is comparable with that obtained from fertilized blastocysts (Wakayama et al. 2005). Furthermore, the transcription profiles of ESCs derived from blastocysts resulting a normal fertilization or NT are identical, and it has been suggested that only nuclei that have undergone appropriate epigenetic reprogramming are capable of generating ESCs from cloned blastocysts (Brambrink et al. 2006). The results in this study was different from the report of Wakayama et al. (2005), cloned blastocysts displayed lower efficiency of giving rise ESC lines compared to fertilized blastocyst. But the result presented here with 14.5% efficiency was comparable to those previous reports (Ju et al., 2007). Even though Pou5f1 positive cells were detected in all examined cloned and fertilized blastocysts, the efficiency of ESC derivation was different. It can be explained by the ability of Pou5f1 positive cells in ICM of cloned blastocysts which had less efficiency to undergo the selection for *in vitro* survival and proliferation compared to those fertilized blastocysts. However, NT-ESCs derived in

this study, were able to maintain their pluripotency as detected by immunostaining of specific murine ESCs markers, expression of pluripotency gene, *in vitro* differentiation and normal chromosome number through progressive passaging.

In the last experiment, differentiation potential of NT-ESCs and F-ESCs via EB development was chosen to study, due to the fact that EBs derived from NT-ESCs and F-ESCs had never been studied comparatively. The measurement of diameter of EBs, as well as the gross morphology through suspension cultures, were applied previously in human and mouse EBs (Khoo et al., 2005; Ezekiel et al., 2007). The results showed that NT-ESCs derived EBs possessed a greater diameter than F-ESCs derived EBs between Day 7 to Day 10. Furthermore, beating EBs which is one of the criteria to specify the pluripotency of ESC lines (Suzuki et al., 1997), were detected in both F-ESCs and NT-ESCs derived EBs at Day 14. Eventually, the difference observed in gross morphology reflected in the pattern of gene expression. RT-PCR, a semi-quantitative analysis of gene expression was selected for this study. It showed that among all the analyzed genes, only the expression pattern of AFP mRNA was expressed differently between NT-ESCs and F-ESCs derived EBs. AFP is the hepatocyte-related gene, not only expressed in the liver, but also in the yolk sac of early embryos (Sellem et al., 1984). This difference may reflected the fact that the epigenetic memory of this NT-ESCs, selected for this study, was not completely erased as NT-ESCs derived EBs could not show similar pattern of gene expression to those F-ESCs derived EBs. With all these results obtained in the comparative differentiation study of NT-ESCs and F-ESCs derived EBs, raised the question whether NT-ESCs are really equal to F-ESCs. However, more investigations in different NT-ESC lines should be performed to confirm this finding.

In conclusion, the effect of mouse strains on the ESC derivation was emphasized in this study and that outbred ESC lines were successfully established from delayed blastocysts. This finding is useful for further development of ESC technology as the selected strain is crucial to the success of the experiment. Besides, the information on the characteristics of blastocysts to ESCs, pluripotentcy as well as differentiation potential of NT-ESCs in comparison to F-ESCs were observed. This will be useful for increasing the efficiency of ESC line derived from cloned blastocysts and provide enough cell lines for further study.

References

Amano, T., Gertsenstein, A., Nagy, A., Kurihara, H. and Suzuki, H. 2006. Nuclear transfer reprogramming does not improve the low developmental potency of embryonic stem cells induced by long-term culture. Reproduction 132: 257-263.

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, 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.

Andrews, P., Matin, M., Bahrami, A., Damjanov, I., Gokhale, P. and Draper, J. 2005. Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. Biochem Soc Trans 33: 1526-1530.

Ansell, J. and Hole, N. 2000. Haematopoietic differentiation of embryonic stem cells. In: DALLAMN, Margaret J and LAMB, Jonathan R. eds. Haematopoietic and Lymphoid cell culture. Cambridge University Press, p. 5-23.

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. Tzukerman, M. 2001. Insulin production by human embryonic stem cells. Diabetes 50: 1691-1697.

Auerbach, W., Dunmore, J.H., Fairchild-Huntress, V., Fang, Q., Auerbach, A.B., Huszar, D. and Joyner, A.L. 2000. Establishment and chimera analysis of 129/SvEv- and C57BL/6- derived mouse embryonic stem cell lines. Biotechniques 29:1024-1028.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Aryes, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meede, H.M., Godke, R.A., Gavin, W.G., Overstom, E.W. and Echelard, Y. 1999. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17: 456-461.

Baharvand, H. and Mattaei, K.L. 2004. Culture condition difference for establishment of new embryonic stem cell lines from C57BL/6 and BALB/c mouse strains. In Vitro Cell Dev Biol Anim 40: 76-81.

Blelloch, R.H., Hochedlinger, K., Yamada, Y., Brennan, C., Kim, M., Mintz, B., Chin, L.and Jaenisch, R. 2004. Nuclear cloning of embryonal carcinoma cells. Proc Natl Acad Sci USA 101:13985–13990.

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.

Brambrink, T., Hochedlinger, K., Bell, G., Rudolf, J. 2006. ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci USA 103: 933-938.

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 compitent embryonic stem cells containing NOD derived genome. Diabetes 52: 205-208.

Brook, F.A. and Gardner, R.L. 1997. The origin and efficient derivation of embryonic stem cells in the mouse. Proc Natl Acad Sci USA 94: 5709-5712.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A. and Vallier, L. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448: 191-195.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I. D. and McKay, R. D. 1999. Embryonic stem cell-derived glial precursors; a source of myelinating transplants. Science 285 (5428): 754-756.

Bryja, V., Bonilla, S., Cajanek, L., Parish, C.L., Schwartz, C.M., Luo, Y., Rao, M.A. and Arenas, E. 2006. An efficiency method for the derivation of mouse embryonic stem cells. Stem Cells 24: 844-849.

Buehr, M., Nichols, J., Stenhouse, F., Mountford, P., Greenhalgh, C.J., Kantachuvesiri, S., Brooker, G., Mullins, J.J. and Smith, A.G. 2002. Rapid loss of Pou5f1 and pluripotency in cultured rodent blastocysts and derivative cell lines. Biol Reprod 68: 222–229.

Buehr, M. and Smith, A. 2003. Genesis of embryonic stem cells. Phil Trans R Soc Lond B 358: 1397-1402.

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.

Chesne, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. and Renard, J.P. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol 20: 366-369.

Choi, D., Lee, H.J., Jee, S., Jin, S., Koo, S.K., Paik, S.S., Jung, S.C., Hwang, S.Y., Lee, K.S. and Oh, B.S. 2005. In vitro different of mouse embryonic stem cells: enrichment of endodermal cells in the embryoid body. Stem Cells 23: 817-827.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., de Leon, F.A.P. and Robl, J.M. 1998a. Cloned transgenic claves produced from non quiescent fetal fibroblast. Science 280: 1256-1258.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A. and Rob, J.M. 1998b. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nature Biotechnol 16: 642-646.

Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J, Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A. and Paylor, R. 1997. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology (Berl) 132: 107–124.

Colman, A and Kind, A. 2000. Therapeutic cloning: concepts and practicalities. Trends Biotechnol 18: 192-196.

Dang, S.M., Kyba, M., Perlingeiro, R., Daley, G.Q. and Zandstra, P.W. 2002. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 78(4): 442–53.

Deacon,T., Dinsmore, J., Costantini, L.C., Ratliff, J. and Isacson, O. 1998. Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation, Exp Neurol 149: 28–41.

Draper, J.S., Moore, H.D., Ruban, L.N., Gokhale, P.J. and Andrew, P.W. 2004. Culture and Characterization of Human Embryonic Stem Cells. Stem Cells and Development. 13(4): 325-336.

Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R. and Jaenisch, R. 2004. Mice cloned from olfactory sensory neurons. Nature 428: 44-49.

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

Ezekiel, U.R., Muthuchamy, M., Ryerse, J.S. and Heuertz, R.M. 2007. Single embryoid body formation in a multi-well plate. Elec J Biotech 10(2): 328-335.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G.Q. 2004. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427: 148–154.

Hamatani, T., Daikoku, T., Wang, H., Matsumoto, H., Carter, M.G., Ko, M.S.H. and Dey, S.K. 2004. Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. Proc Natl Acad Sci USA 101: 10326-10331.

Heindryckx, B., Rybouchkin, A., Van Der Elast, J. And Dhont, M. 2001. Effect of culture media on *in vitro* development of cloned mouse embryos. Cloning 3(2): 41-50.

Hochedlinger, K. and Jaenisch, R. 2003. Nuclear transplantation, embryonic stem cells and the potential for cell therapy. N Engl J Med 349: 275-286.

Itskovitz-Eldor, J., Schuldiner, D., Karsenti, A., Eden, O., Yanuka, M., Amit, M., Sareq, H. and Benvenisty, N. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6: 88-95.

Jaenisch, R. 2004. Human cloning – The science and ethics of nuclear transplantation. N Engl J Med 351: 2787-2791.

Ju, J.Y., Park, C.Y., Gupta, M.K., Uhm, S.J., Paik, E.C., Ryoo, Z.Y., Cho, Y.H., Chung, K.S. and Lee, H.T. 2007. Establishment of stem cell lines from nuclear transferred and parthenogenetically activated mouse oocytes for thereapeutic cloning. Fertil Steril: Nov 1 [Epub ahead of print].

Kahan, B.W., Jacobson, L.M., Hullett, D.A., Ochoada, J.M., Oberley, T.D., Lang, K.M. and Odorico, J.S. 2003. Pancreatic precursors and differentiated islet cell types from murine embryonic stem cells. An in vitro model to study islet differentiation. Diabetes 52: 2016–2024.

Kawase, E., Suemori, H., Takahashi, N., Okazaki, K., Hashimoto, K. and Nakatsuji, N. 1994. Strain difference in establishment of mouse embryonic stem (ES) cell lines. Int J Dev Biol 38: 385-390.

Kawase, E., Yamazaki, Y., Yagi, T., Yanagimachi, R. and Pedersen, R.A. 2000. Mouse embryonic stem (ES) cell lines established from neuronal cell derived cloned blastocyst. Genesis 28: 156-163.

Kim, H.S., Son, H.Y., Kim, S., Lee, G.S., Park, C.H., Kang, S.K., Lee, B.C., Hwang, W.S. and Lee, C.K. 2007. Isolation and initial culture of porcine inner cell masses derived from in vitro produced blastocysts. Zygote 15: 55-63.

Kim, H.S., Oh, S.K., Park, Y.B., Ahn, H.J., Sung, K.C., Kang, M.J., Lee, L.A., Suh, C.S., Kim, S.H., Kim, D.W. and Moon, S.Y. 2005. Methods for derivation of human embryonic stem cells. Stem Cells 23: 1228-1233.

Khoo, M.L.M., McQuade, L.R., Smith, M.S.R., Lees, J.G., Sidhu, K.S. and Tuch, B.E. 2005. Growth and differentiaion of embryoid bodies derived from human embryonic stem cells: effect of glucose and basic fibroblast growth factor. Bio Reprod 73: 1147-1156.

Klug, M.G., Soonpaa, M.H., Koh, G.Y. and Field, L.J. 1996. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 98: 216–224.

Kono, T. 1997. Nuclear reprogramming. Reviews of Reproduction 2: 74-80.

Lanza, R.P., Cibelli, J.B. and West, M.D. 1999. Human therapeutic cloning. Nature Med 5: 975-977.

Laslett, A.L., Filipczyk, A. and Pera, M.F. 2003. Characterization and culture of human embryonic stem cells. Trends Cardio Med 13(7): 295-301.

Leppens, G., Gardner, D.K. and Sakkas, D. 1996. Co-culture of 1-cell outbred mouse embryos on bovine kidney epithelial cells: effect on development, glycolytic activity, inner cell mass: trophectoderm ratios and viability. Hum Reprod 11: 598-603.

Li, T., Wang, S., Xie, Y., Lu, Y., Zhang, X., Wang, L., Yu, S., Wolf, D., Zhou, Q. and Ji, W. 2005. Homologous feeder cells support undifferentiated growth and pluripotency in monkey embryonic stem cells. Stem Cells. 23: 1192-1199.

Liu, J., Schoonjans, L., Tielens, S., Speleman, F., Cornelissen, M., De Sutter, P., Dhont, M. and Van Der Elst, J. 2006. Culturing *in vitro* produced blastocysts in sequential media promotes ES cell derivation. Mol Reprod Dev 73: 1017-1021.

Longo, L., Bygrave, A., Grossveld, F.G. and Pandolfi, P.P. 1997. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimerism. Transgenic Research 6: 321-328.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. And McKay, R. 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292: 1389-1394.

MacLean, H.S. and Evans, M. 1999. Non-surgical method for the induction of delayed implantation and recovery of viable blastocysts in rats and mice by the use of Tamoxifen and Depo-Provera. Mol Reprod Dev 52: 29–32.

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 USA 78: 7634-7638.

McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I. and Choi, D.W. 1995. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 5: 1410-1412.

McWhir, J., Schnieke, A.E and Ansell, R. 1996. Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. Nat Genet 14: 223–226.

Mombaerts, P. 2003. Therapeutic cloning in the mouse. Proc Natl Acad Sci USA 10(1) :11824-11825.

Moritoh, Y., Yamato, E., Yasui, Y., Miyazaki, S. and Miyazaki, J. 2003. Analysis of insulin-producing cells during in vitro differentiation from feeder-free embryonic stem cells. Diabetes 52: 1163-1168.

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

Nadeau, J.H., Balling, R., Barsh, G., Beiser, D., Brown, S.D.M., Bucan, M., Camper, S., Carlson, Copeland N., Eppig, J., Fletcher, C., Frankel, W.N., Ganten, D., Goldowitz, D., Goodnow, C., Guenet, J.L., Hick, G., De Angelis, M.H., Jackson, I., Jacob, H.J., Jenkins, N., Johnson, D., Justice, M., Kay, Steve., Kingsley, D., Lehrach, H., Magnuson, T., Meisler, M., Poustka, A.M., Rinchik, E.M., Rossant, J., Russel, L.B., Schimenti, J., Shiroishi, T., Skarnes, W.C., Soriano, P., Stanford, W., Takahashi, J.S., Wurst, W. and Zimmer, A. 2001. Sequence interpretation: Functional annotation of mouse genome sequenes. Science 291(5507): 1251-1255.

Nagy, A., Gertsenmstein, M., Vintersten, K. and Behringer, R. 2003. Isolation and culture of blastocyst-derived stem cell lines. In: Manipulating the mouse embryo. A laboratory manual, 3rd ed. Cold Spring laboratory Press. pp 359-397.

Ng, R.K. and Gurdon, J.B. 2005. Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer. Proc Natl Acad Sci USA 102(6): 1957-1962.

Ogura, A., Inoue, k., Takano, K., Wakayama, T. and Yanagimachi, R. 2000. Birth of mice after nuclear transfer by electrofusion using tail tip cells. Mol Reprod Dev 57: 55-59.

Park, J.H., Kim, S.J., Oh, E.J., Moon, S.Y., Roh, S.I. and Yoon, H.S. 2003. Establishment and maintenance of human embryonic stem cells on STO, a permanently growing cell line. Bio Reprod 69: 2007-2014.

Pesce, M. and Sholer, H.R. 2001. Pou5f1; Gatekeeper in the beginning of the mammalian development. Stem Cells 19: 271-278.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H.S. 2000. Cloned pig produced by nuclear transfer from adult somatic cells. Nature 407: 86-90.

Rideout, W. M. 3rd ., Wakayama, T., Wutz, A., Eggan K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R. and Jaenisch, R. 2000. Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet 24: 109-110.

Robertson, E.J. 1987. Embryo-derived stem cell lines. In: Teratocarcinomas and Embryonic Stem Cells: a Practical Approach, Oxford: IRL press. pp. 71-112.

Rosner, M.H., Vigano, M.A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W. and Staudt, L.M. 1990. A POU-domain transcription factor in early stem-cells and germ-cells of the mammalian embryo. Nature 345: 686-392.

Rybouchkin, A., Heindryckx, H., Van der Elst, J. and Dhont, M. 2002. Developmental potential of mouse embryonic reconstructed by a conventional technique of nuclear injection. Reproduction 124: 194-207.

Scholer, H.R., Dressler, G.R., Balling, R., Rohdewohld, H. and Gruss, P. 1990. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J 9: 2185-2195.

Schoonjans, L., Kreemers, V., Danloy, S., Moreadith, R.W., Laroche, Y. and Collen, D. 2003. Improved generation of germline-competent embryonic stem cell lines from inbred mouse strain. Stem Cells 21: 90-97.

Sellem, C.H., Frain, M., Erdos, T. and Sala-Trepat, J.M. 1984. Differential expression of albumin and alpha-fetoprotein genes in fetal tissues of mouse and rat. Dev Biol 102: 51-60.

Shamblot, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R. and Gearhart, J.D. 1998. Derivation of pluripotentstem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 95: 13726–13731.

Shi, Y.T., Huang, Y.Z., Tang, F., Chu, J.X. 2006. Mouse embryonic stem cell-derived feeder cells support the growth of their own mouse embryonic stem cells. Cell Biol Intl 30: 1041-1047.

Simpson, E.M., Linder, C.C., Sargent, E.E., Davidsson, M.T., Mobraaten, L.E. Sharp, J.J. 1997. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat Genet 16: 19-27.

Smith, A.G. 1998. Cell therapy: in search of pluripotency. Curr Biol 8: 802-804.

Smith, A.G. 2001. Embryo-derived stem cells: Of mice and men. Annu Rev Cell Dev Biol 17: 435–462.

Smith, L.S., Evert, R.E., Tian, X.C. and Du, F. 2005. Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. Proc Natl Acad Sci USA 102(49): 17582-17587.

Sritanaudomchai, H., Pavasuthipaisit, K., Kitiyanant, Y., Kupradinun, P. and Mitalipov, S. 2007. Characterization and multilineage differentiation of embryonic stem cells derived from a buffalo parthenogenetic embryo. Mol Reprod 74: 1295-1302.

Solter, D. and Knowles, B.B. 1975. Immunosurgery of mouse blastocysts. Proc Natl Acad Sci USA 72: 5099-5102.

Solter, D. and Gearhart, J. 1999. Putting stem cells to work. Science 283: 1468-1470.

Soria, B., Roche, E., Berna, G., et al. 2000. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 49: 157-162.

Strom, S., Inzunza, J., Grinnemo, K.H., Holmberg, K., Matilainen, E., Stromberg, A.N., Blennow, E. and Hovatta, O. 2007. Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines. Hum Reprod 22(12):3051-3058.

Sukoyan, M. A., Kerkis, A. Y., Mello M. R. B., et al. 2002. Establishment of new murine embryonic stem cell lines for the generation of mouse model of human genetic disease. Braz J Med Biol Res 35: 535-542.

Suzuki, H., Kamada, N., Ueda, O., Jishage, K., Kurihara, H., Terauchi, Y., Azuma, S., Kadowaki, T., Kodama, T., Yazaki, Y. and Toyoda, Y. 1997. Germ-line contribution of embryonic stem cells in chimeric mice: influence of karyotype and in vitro differentiation ability. Exp Anim 46: 17-23.

Suzuki, O., Matsuda, J., Takano, K., Yamamoto, Y., Asano, T., Naiki, M. Kusanagi, M. 1999. Effect of genetic background on establishment of mouse embryonic stem cells. Exp Anim 48(3): 213-216.

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.

Tesar, P.J. 2005. Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. Proc Natl Acad Sci USA 7;102(23): 8239-8244.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L. and McKay, R.D. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448: 196-199.

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 USA 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.

Thouas, G.A., Korfiatis, N.A., French, A.J., Jones, G.J. and Trounson, A.T. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod BioMed Online 3(2) No 1: 25-29

Tielens, S., Verhasselt, B., Liu, J., Dhont, M., Van Der Elst, J. and Cornelissen, M. 2006. Generation of embryonic stem cell lines from mouse blastocysts developed in vivo and in vitro: relation to Oct-4 expression. Reproduction 132: 59-66.

Turetsky, T., Aizenman, E., Gil, Y., Weinberg, N., Shufaro, Y., Rever, A., Laufer, N., Simon, A., Abeliovich, D. and Reubinoff, B.E. 2007. Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. Hum Reprod: November 7 [Epub ahead of print].

Wakayama, S., Ohta, H., Kishigama S., Thuan, N.V., Hikichi, T., Mizutani, E., Miyake, M. and Wakayama, T. 2005. Establishment of male and female nuclear transfer embryonic stem cell lines from different mouse strain and tissues. Biol Reprod 72: 932-936.

Wakayama, S., Jakt, M. L., Suzuki, M., Araki, R., Hikichi, T., Kishikami, S., Ohta, H., Thuan, N.V., Mizutani, E., Sakaide, Y., Senda, S., Tanaka, S., Okada, M., Miyake, M., Abe, M., Nishikawa, S-I., Shiota, K. and Wakayama, T. 2006. Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. Stem Cells 24: 2023-2033.

Wakayama, T., Perry, A.C.F., 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, T., Rodriguez, I., Perry, A. C.F., Yanagimachi, R. And Mombaerts, P. 1999. Mice cloned from embryonic stem cells. Proc. Natl. Acad. Sci. USA 96: 14984-14989.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L. And Mombearts, P. 2001. Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292: 740-743.

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(4): 986-993.

Ware, C. B., Akea, L., Sivert, L.A.N., Nelson, A.M., Morton, J.F and Ladiges, W.C. 2003. Utility of a C57BL/6 ES line versus 129 ES lines for targeted mutations in mice. Trans Res 12: 743-746.

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.

Van Soom, A., Vanroose, G. and de Kruif, A. 2001. Blastocyst evaluation by means of ifferential staining: a practical approach. Reprod Domest Anim 36: 29-35.

Yamada, T., Yoshikawa, M., Takaki, M., Torihashi, S., Kato, Y., Nakajima, Y., Ishizaka, S. And Tsunoda, Y. 2002. In vitro functional gut-like organ formation from mouse embryonic stem cells. Stem Cells 20: 41–49.

Yamamoto, M., Tase, N., Okuno, T., Kondo, Y., Akiba, S., Shimozawa, N. And Terao, K. 2007. Monitoring of gene expression in differentation of embryoid bodies from cytomolgus monkey embryonic stem cells in the presence of bisphenol A. J Toxicol Sci 32(3): 301-310.

Yu, Y., Yong, j., Li, X.Y., et al. Qing, T.T., Qin, H., Xiong, X.O., You, J.F., Ding, M.X. and Deng, H.K. 2005. The proteasomal inhobitor MG132 increase the efficiency of mouse embryo production after cloning by electrofusion. Reproduction 130: 553-558.

Yu, Y., Ding, C., Wang, E., Chen, X., Li, X., Zhao, C., Fan, Y., Wang, L., Beaujean, N., Zhou, Q., Jouneau, A. and Ji, W. 2007. Piezo-assisted nuclear transfer affects cloning efficiency and may cause apotosis. Reproduction 133: 947-954.

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