ESTABLISHMENT OF EMBRYONIC STEM CELL LINES FROM TRANSGENIC RHESUS MONKEY BLASTOCYST

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

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การย้ายฝากนิวเคลียสโดยใช้เซลล์ร่างกายในสัตว์ตระกูลลิง เป็นเทคนิคที่เป็นประโยชน์อย่างมากในการทำโคลนนิ่งเพื่อการรักษาโรค การศึกษานี้มีวัตถุประสงค์เพื่อผลิตเซลล์ต้นกำเนิดตัวอ่อนลิงโดยการแยกเซลล์ ICM โดยวิธีกลจากตัวอ่อนปกติ ดัดแปลงพันธุกรรม และโคลนนิ่ง การศึกษาอายุของลิงต่อผลของการเก็บไข่พบว่า ลิงอายุ 5 ถึง 8 ปีจะให้ไข่ระยะ GV และ MII ปริมาณมากกว่าลิงอายุ 9 ถึง 15 ปี ไข่ระยะ MII จะถูกนำมากระตุ้นด้วย 5 μM Ionomycin และเลี้ยงในน้ำยาที่มี 6-DMAP นาน 4 ชั่วโมง (PA-4) หรือ 5 ชั่วโมง (PA-5) เพื่อตรวจสอบวิธีการกระตุ้นที่เหมาะสมต่อการเจริญเติบโตของตัวอ่อน ผลการทดลองพบว่า PA-5 ช่วยสนับสนุนกระบวนการย้อนกลับของเซลล์ร่างกายและการเจริญเติบโตของตัวอ่อนโคลนนิ่งได้ดีกว่า PA-4 ดังนั้นการทดลองต่อมาจึงกระตุ้นตัวอ่อนโคลนนิ่งด้วยวิธี PA-5 การศึกษาประสิทธิภาพของการแยกเซลล์ ICM ด้วยวิธีการตัดเซลล์ตัวอ่อนบางส่วนออกหรือการเลี้ยงตัวอ่อนทั้งใบนั้นได้ทำการศึกษาจากตัวอ่อนดัดแปลงพันธุกรรมที่มีโรค Huntington (ICSI-HD) Alzheimer (ICSI-AD) ตัวอ่อนไม่ดัดแปลงพันธุกรรม (ICSI-WT) ตัวอ่อนโคลนนิ่ง และตัวอ่อนจาก PA-5 เซลล์ต้นกำเนิดจำนวน 12 เซลล์ไลน์ (57.1%) ผลิตจากการตัดเซลล์ตัวอ่อนบางส่วนออก แบ่งเป็น 7 เซลล์ไลน์จาก 14 outgrowth (50%) ที่ผลิตจากเป็นตัวอ่อนที่ถูกดัดแปลงพันธุกรรม (ADrES1, ADrES2, ADrES3, YMES15, HDrES1, HDrES2, HDrES3) 2 เซลล์ไลน์จาก 3 outgrowths (66.7%) ที่ผลิตจากตัวอ่อนไม่ดัดแปลงพันธุกรรม (YRES5, YRES6) 2 เซลล์ไลน์จาก 2 outgrowths (100%) ที่ผลิตจากตัวอ่อน PA (PAES1, PAES2) และ 1 เซลล์ไลน์จาก 2 outgrowths (50%) ที่ผลิตจากตัวอ่อนโคลนนิ่ง (NrES1) ขณะที่มีเพียง 1 เซลล์ไลน์จาก 12 (8.3%) outgrowths ผลิตจากการเลี้ยงตัวอ่อนทั้งใบ (TrES1) ผลการทดลองแสดงให้เห็นว่า แหล่งที่มาของการผลิตตัวอ่อนไม่มีผลกระทบต่อการผลิตเซลล์ต้นกำเนิดตัวอ่อนลิง การแยกเซลล์ ICM โดยการตัดเซลล์ตัวอ่อนบางส่วนออกมีประสิทธิภาพดีในการผลิตเซลล์ต้นกำเนิดตัวอ่อนลิงมากกว่าการผลิตเซลล์ต้นกำเนิดตัวอ่อนโดยวิธีการเลี้ยงตัวอ่อนทั้งใบ รายงานนี้เป็นรายงานแรกที่ผลิตเซลล์ต้นกำเนิดตัวอ่อนลูกผสมลิง (TrES1) ที่มีโรค HD ของคน และ GFP ในเซลล์ต้นกำเนิด และพบการแสดงออกของ ES cell marker การปลูกถ่ายเซลล์ต้นกำเนิด TrES1 ลงในสมองหนู SCID พบว่ามีการเกิดเนื้องอกหลังจากปลูกถ่าย จากการศึกษาการแสดงออกของโรค HD ในเซลล์ต้นกำเนิดตัวอ่อน TrES1 ตลอดช่วงการเหนี่ยวนำให้เป็นเซลล์ประสาทในจานเลี้ยงเซลล์พบว่าการแสดงออกของโรค HD มีการพัฒนาตลอดช่วงการเหนี่ยวนำ มีการสะสมของ oligomeric mutant htt และ intranuclear inclusions

(NIs) เพิ่มขึ้นอย่างรวดเร็ว จากผลการทดลองแสดงว่า TrES1 สามารถใช้เป็นเซลล์ตัวอย่างสำหรับศึกษากระบวนการเกิดโรค HD และสามารถใช้สำหรับการทดสอบยาใหม่ๆ ได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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CHUTI LAOWTAMMATHRON : ESTABLISHMENT OF EMBRYONIC STEM CELL LINES FROM TRANSGENIC RHESUS MONKEY BLASTOCYST.
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EMBRYONIC STEM CELL/SOMATIC NUCLEAR TRANSFER/INNER CELL MASS/HUNTINGTON DISEASE/RHESUS MONKEY

Somatic cell nuclear transfer (SCNT) in non-human primates (NHPs) is a powerful technique for therapeutic cloning. Objective of this study was to establish non human primate embryonic stem cells using mechanical isolation of ICM cells from wild type, transgenic and SCNT embryos. Results of this study indicated that age of oocyte donor affects the number of collected oocytes. Monkeys age of 5 to 8 years provide more number of GV and MII stage oocytes than monkey age of 9 to 15 years. The MII oocytes were activated with 5 μM Ionomycin and subsequently incubated in 6-DMAP for either 4h (PA-4) or 5h (PA-5) to access the activation protocol. The result indicated that PA-5 supports the somatic cell reprogramming and embryo development better than PA-4. Therefore, in subsequences experiments, the cloned embryos were activated by PA-5 protocol. To determine the efficiency of ICM isolation by partial dissected embryo or whole embryo culture of NHPs embryos derived from transgenic Huntington (ICSI-HD), Alzheimer (ICSI-AD), non-transgenic (ICSI-WT), SCNT and PA were used as source of embryo. Twelve nhpESC lines were established from 21 partial dissected embryos (57.1%). Among these, 7 nhpESC lines were established from 14 (50%) outgrowths derived transgenic embryos (ADrES1, ADrES2, ADrES3, YMES15, HDrES1, HDrES2, HDrES3), 2 nhpESC lines from 3 (66.7%) outgrowths derived from ICSI embryos (YRES5, YRES6), 2 nhpESC lines from 2 (100%) outgrowths derived from PA embryos (PAES1, PAES2) and1 nhpESC line from 2 (50%) outgrowths derived from SCNT embryos (NrES1). Only

one nhpESC line out of 12 (8.3%) was established from whole embryo cultured (TrES1). These results demonstrated that the source of embryo had no effect on nhpESC establishment. Mechanical isolation of the ICM by partial dissected embryo proved to be an effective way to derive new nhpESC lines rather than whole embryo cultured. TrES1 is the first reported of hybrid cell line of NHPs carrying human genetic disease, HD, and green fluorescent protein (GFP). The expression of ES cell markers was observed and teratoma formation developed after TrES1 implantation into SCID mice's brain. The progression of HD along *in vitro* neuronal differentiation was examined in the TrES1 cell line. HD cellular pathology developed along the differentiation steps. The accumulation of oligomeric mutant htt and intranuclear inclusions (NIs) dramatically increased along the *in vitro* differentiation to neuron. These results indicated that TrES1 could be use as a model to study the mechanism of HD and drug screening.

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LIST OF ABBREVIATIONS

ES cell	=	Embryonic Stem cell
mES	=	Mouse embryonic stem cell
NHPs	=	Non human primates
hESCs	=	Human embryonic stem cell
nhpESCs	=	Non human primate embryonic stem cells
SCNT	=	Somatic cell nuclear transfer
PA	=	Parthenogenetic activation
TE	=	Trophectoderm
ICM	=	Inner cell mass
MFFs	=	Mouse fetal fibroblasts
HD	=	Huntington disease
htt	=	Huntingtin
AD	=	Alzheimer disease
GFP	=	Green fluorescent protein
Mya	=	Million year ago
ml	=	milliliter
IU	=	international unit
μg	=	microgram
μl	=	microlitre
mM	=	millimolar

CHAPTER I

INTRODUCTION

1.1 Introduction

Stem cell is an immortal pluripotent cell lines capable of self-renewal under suitable conditions (D'Amour and Gage, 2000). They are pluripotent and capable of differentiate to specialized cell types when induced with appropriate stimulants under suitable conditions. Stem cell can be classified into two groups from their origin, adult stem cells and embryonic stem (ES) cells (Thomson et al., 1998). Adult stem cells found among differentiated cells in a tissue or organ that can renew itself and can differentiate to specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Adult stem cells have been described and isolated from various tissues and organs, include blood (heamatopoietic stem cells), bone marrow (mesenchymal stem cells), cord blood (cord blood stem cells), skin and hair (epidermal stem cells) and the latest on the list, amniotic stem cells (Coppi et al., 2007). Unlike ES cells, adult stem cells already commit to some special type of cell such as hematopoietic stem cell can differentiated to many type of blood cells (Majeti et al., 2007), nerve stem cell can only differentiated to various type of brain cells but not blood cells (Kondo and Raft, 2000).

However, ES cells have the ability to develop into any cells in the body when induced by appropriate stimulant (s) and suitable condition (s) (Bongso and Lee,

2005). ES cell may have the potential to treat medical conditions, cell transplantation and tissue regenerative therapies (D'Amour and Gage, 2000). ES cell is a pluripotent cell lines which capable to differentiate into many cell lineages when compare with adult stem cell (Gardner, 2004). ES cell derived from human and nonhuman primate is very useful tool to study therapeutic stem cell and drug recovery (Barberi et al., 2003; Klein et al., 2006).

ES cell could be established by somatic cell nuclear transfer (SCNT) to produced personal ES cells or could be used to develop disease specific stem cell (Byrne et al., 2007). There are many diseases that have high possibility to be cured by therapeutic stem cells such as Huntington, Alzheimer and Parkinson. However, therapeutic stem cell is still not practical to be used because the differentiation to each lineage could not prevent the property of immortal and pluripotentcy which might result to develop to tumor after transplantation. Moreover, the improvement of efficiency of SCNT technique and cell reprogramming after SCNT need to be explored.

Since 1981, most of the ES cell lines were established from ICM cells of blastocyst embryos (Evans and Kaufman., 1981; Martin, 1981). Immunosurgery is the most favorite technique to isolated ICM cells from balstocyst stage embryo because it is the most effective method to isolates the clear ICM cells and labor less (Solter and Knowles, 1975). However, the success rate of ES cell establishment is still low.

Nonhuman primates (NHPs), especially old world macaques, are valuable animal models because of their close relationship to humans and their extensive use in biomedical research (Kumar and Hedges, 1997). So far, number of blastocyst stage embryos from both SCNT and intracytoplasmic sperm injection (ICSI) are still very

low. Therefore, the goal of this study is to establish mechanical ICM isolation technique to establish disease specific, Huntington disease, pluripotent embryonic stem cells.

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CHAPTER II

REVIEW OF LITERATURES

2.1 Research in rhesus monkey (Rhesus macaque; *Macaca mulatta*)

2.1.1 Taxonomy and general information of rhesus monkey

The rhesus macaque (*Macaca mulatta*) is an abundant primate species that diverged from the ancestors of *Homo sapiens* (Kumar and Hedges, 1997). This species is very close to humans and shares a last common ancestor about 25 million years ago (Mya; Figure 2.1.1). Rhesus monkeys are the most widely used nonhuman primate in basic and applied biomedical research because they are genetically and physiologically similar to humans. They have a broad geographic distribution in China, India, Bhutan, Laos, Burma, Nepal, Bangladesh, Thailand, Vietnam, Pakistan and Afghanistan (Kumar and Hedges, 1997; http://pin.primate.wisc.edu/factsheets/entry/rhesus_macaque). The scientific classification of rhesus macaque are shown below;

Kingdom: Animalia

Phylum: Chordata

Class: Mammalia

Order: Primates

Family: Cercopithecidae

Genus: *Macaca*

Species: *M. mulatta*

Rhesus macaques exhibit greater similarity to human physiology and neurobiology. Moreover, rhesus macaques are more sensitive to infectious and metabolic diseases when compared with rodents which the ancestor were separated from humans more than 70 million

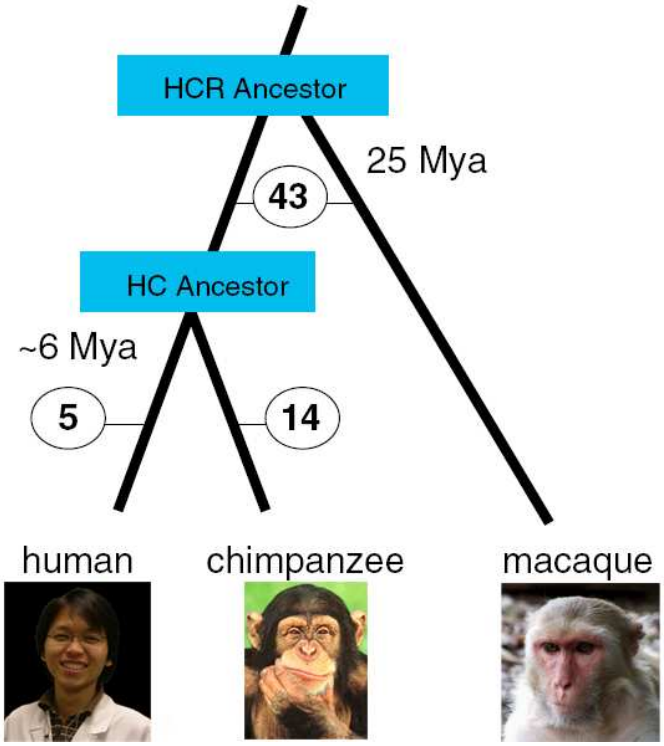

Figure 2.1.1 Evolutionary in the human, chimpanzee and rhesus macaque lineages (lineage-specific breaks). Summary of chromosomal breakpoints are shown on a microscopic scale. The chimpanzee shared a common ancestor with humans approximately 6 Mya. Circled numbers indicate numbers of lineage-specific breaks. Mya: million years ago; HC: human–chimp lineage; HCR: human-chimpanzee-rhesus macaque. (Modified from Gibbs et al., 2007; Chen and Li 2001; Patterson et al., 2006; Hobolth et al., 2007)

years ago (Kumar and Hedges, 1997). The important research in biomedicine in rhesus macaques includes the identification of the rhesus factor (Rh factor) blood groups along with advances in neuroanatomy and neurophysiology (Pennisi, 2007; Gibbs et al., 2007). Rhesus monkeys have been used on vaccine development and used as an animal model for human disease including simian immunodeficiency virus and influenza, Acquired immune deficiency syndrome (AIDS) (Pennisi, 2007; Gibbs et al., 2007).

2.1.2 Production of genetically identical in Rhesus monkey

Since the birth of Dolly (Wilmut et al., 1997), SCNT offspring have been cloned from many species including sheep (Willadsen, 1986; Wilmut et al., 1997), mice (Wakayama et al., 1998), pigs (Onishi et al., 2000; Polejaeva et al., 2000), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999) guar (Lanza et al., 2000; Vogel, 2001), goats (Baguisi et al., 1999), cat (Shin et al., 2002), rat (Zhou et al., 2003), mule (Woods et al., 2003) and horse (Galli et al., 2003). However, the offspring in non human primates (NHPs) has not been reported. The production of NHPs by SCNT is an interesting option for biological researches because of the cloned NHPs could provided identical primates that would reduce the number of animals required for biomedical research and dramatically impact studies pertaining to immune system function, such as development of the human-immunodeficiency-virus vaccine (Mitalipov et al., 2002; Simerly and Navara, 2003; Niu et al., 2008; Zhou et al., 2006).

Genetically identical nonhuman primates could be produce by embryo splitting (Chan et al., 2000) or SCNT (Wilmut et al., 1997; Mitalipov et al., 2002; Simerly and Navara, 2003; Niu et al., 2008). In embryo splitting, embryo are divided at blastocyst stage to generated identical twins. These methods are useful for limited number of embryo and it is inexpensive to produce twinning of nonhuman primates. The advantage of this technique is the offspring are identical in both nuclear and mitochondrial DNA. Disadvantages of this technique include limited number of offspring per embryo. Moreover, phenotype of the offspring is not known until after birth (Simerly and Navara, 2003). SCNT technique can break thought the limited of number offspring. An identical phenotype of the offspring of nuclear donor animal can be obtained. Currently, 2 protocols have been used to produce SCNT either by electro cell fusion (Wilmut *et al*., 1997) or direct injection of nuclear donor cell (Cibelli et al., 1998). For electro cell fusion, an individual donor cell is inserted into perivitelline space of enucleated cytoplasm and the couple is fused with electric pulse. For

the direct injection of nuclear donor cell, the donor nucleus is isolated and directly injected into the cytoplast without fusion by electric pulse. Both approaches require artificial activation to mimic the mechanism after sperm fertilized with egg by stimulate the Ca^{2+} oscillation.

2.1.3 Artificial activation

The first live offspring derived from nuclear transfer in NHPs was reported by using embryonic cells (Meng et al., 1997). There have been many attempts to produce monkey SCNT embryos, however the obtained blastocyst rates are still low (Mitalipov et al., 2002; Simerly and Navara, 2003; Ng et al., 2004; Zhou et al., 2006; Byrne et al., 2007; Yang et al., 2007) and no live offspring has been born. In general, major problem in SCNT are the low efficiency and high incidence of developmental abnormalities in the resultant cloned fetuses or neonates. The failure of embryo development could be related to the timing of genome activation and cleavage (Edwards et al., 1984; Sakkas et al., 1998; Shoukir et al., 1997). In the rhesus monkey, the major embryonic genome activation is thought to occur between the 6 to 8 cell stages (Schramm and Bavister, 1999), which coincides with the timing of nucleologenesis, the development of a functional nucleolus in a new generation. The process of nucleologenesis may be negatively affected by *in vitro* embryo production or nuclear transfer (Maddox-Hyttel et al., 2005). However, it is still unclear how the timing of embryonic genome activation relates to the time course of development in rhesus monkey SCNT embryos.

Reprogramming ability of the oocyte is the most important even needed to be concern. In nuclear transfer, reprogramming of somatic cell back to pluripotentcy stage occurred after chemically activation of the reconstructed egg. Chemical activation is a very crucial step for reprogramming of the differentiated cell back to pluripotent stage. The suitable activation condition could provide higher number of embryos development. Therefore, one of the method that could improve the efficiency of monkey SCNT is the

chemically activation protocol. Many studies have reported that artificial activation treatment is necessary to increase blastocyst formation during *in vitro* culture (Abramcuk et al., 1977; Mitalipov et al., 2001; Roger et al., 2004; Zhou et al., 2006; Okahara-Narita et al., 2007; Niu et al., 2008). Artificial activation of SCNT mimic the mechanism after sperm fertilized with egg by stimulate the Ca^{2+} oscillation. It could also induce development of MII oocytes without fertilization (Mitalipov et al., 2001; Stricker, 1999). During fertilization, sperm stimulates Ca^{2+} oscillation to initiate egg development (Whittingham 1980; Cuthbertson and Cobbold 1985; Stricker, 1999). The targets of calcium-stimulation are maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase. The MPF activity is regulated by the level of cyclin B and phosphorylation states of Cdc2 ($p34^{cdc2}$). MPF has high activity at the metaphase of mitotic cell cycle during nuclear envelop breakdown, chromatin condensation and formation of mitotic spindle (Doree and Galas, 1994). MPF is necessary to be inactivated to allow oocyte release to mitosis. It could be inactivate by ubiquitin-dependent proteolysis of cyclin B and by conversion into inactive pre-MPF by Cdc2 hyperphosphorylation (Solomon et al., 1990). However, MPF activity is quickly restored with chromatin recondensation and re-entry into a metaphase III (MIII) phase arrest (Collas et al. 1995; Susko-Parrish et al. 1994). Additional either inhibition of protein synthesis by cycloheximide (CHX) or protein phosphorylation by 6-dimethylaminopurine (6-DMAP) (Liu et al. 1998; Susko-Parrish et al. 1994) prolong inactivation of the MPF. Therefore, the consequence expose with ionomycin and 6-DMAP, calcium ionophore and DMAP, or ionomycin and CHX, calcium ionophore and CHX could support embryo development in bovine, rabbit and NHP embryo (Liu et al. 1998; Mitalipov et al. 2001; Niu et al., 2008; Susko-Parrish et al. 1994; Tian et al., 2002).

Ionomycin combined with 6-DMAP is the combined reagent that more preferable to activated reconstructed NHPs SCNT (Simerly and Navara, 2003; Simerly et al., 2004; Zhou et al., 2006; Yang et al., 2007; Byrne et al., 2007; Mitalipov et al., 2007; Niu et al., 2008).

Generally, in rhesus monkey oocytes will be activated at 2h after fusion (Mitalipov et al. 2001; Zhou et al., 2006; Byrne et al., 2007; Mitalipov et al., 2007). Recently, the timing of oocyte activation after donor cell injection has been studied by Niu and colleagues (Niu et al., 2008). The timing of oocyte activation after donor cell injection was classified into 3 groups; 4h, 8h and 12h after donor cell injection. The results show that 4h after donor cell injection could provide higher embryo development to blastocyst stage than those from the group of 8h and 12h, respectively. However, in the mouse study, prolonged interval between nuclear injection and oocyte activation increased both pre and post implantation development (Wakayama et al., 1998). It seems that prolonged donor cell to exposure with cytoplasm may facilitate the embryonic genome activation of development in mouse.

2.2 ES cell establishment

In general, ICM from blastocyst embryo is a major source for ES cell line establishment. The blastocyst contains trophectodermal (TE) and inner cell mass (ICM) cells. TE cells would develop to be placenta after the blastocyst implant to endometrium layer of mother uterus. ICM cells would develop to be three embryonic germ layers and yolk sac. The three embryonic germ layers incorporated into a fetus, eventually. In another word, the ICM could be differentiated into three embryonic germ layers of the fetus. Therefore, the ICM is the most wanted source for establishing ES cell. ICM could only be observed in the blastocyst stage embryo. The good quality blastocysts with distinct ICM cells are the most appropriate source for ES cell establishment. ES cell lines have been established from ICM cells of blastocyst embryos since 1981. Until now, there have been several methods of ICM cells isolation developed and some of these methods are still routinely used. Immunosurgery is the most favorite technique of several researchers (Martin, G.R. 1981; Piedrahita et al., 1990; Chen et al., 1999; Anderson et al., 1994; Thomson et al., 1995; Moore and Piedrahita, 1997; Thomson et al., 1998; Reubinoff et al.,

2000; Suemori et al., 2001; Vrana et al., 2003; Cowan et al., 2004; Heins et al., 2004; Ock et al., 2005; Brevini et al., 2005; Shiue et al., 2006; Mitalipov et al., 2006; Mateizel et al., 2006) because it is the most effective method for clear ICM cells isolation and it is also not labor intense (Solter and Knowles, 1975). Unfortunately, it needed to use animal products in the process. Therefore, this is risky point for pathogen contamination especially when using this technique to establish human ES cell lines. Recently, a novel method for ES cell establishment has been introduced by using single blastomeres of the early stage embryos as a source of ES cells (Chung et al., 2006). It has been introduced as another method of choice to step aside from ethical issues, especially on embryo destruction issue. It could also be used as a method for establishing patient specific ES cells. Each procedure has specific characters which needed to be considered.

2.2.1 ICM isolation by Microsurgery

ICM isolation by using microsurgery was first reported by Gardner (1972). The major reason of this study was to investigate the function of TE and ICM cells of mouse blastocyst (Gardner, 1972). The separation of TE and ICM was performed under micromanipulator. The blastocysts were placed in a drop of medium hanging from the coverslip of a chamber filled with heavy liquid paraffin. Then the blastocysts were held by suction pipette against the underside of the coverslip of the hanging drop. A piece of micro-blade was attached and arranged vertically on one manipulator unit. Therefore, the micro-blade surface was parallel with the coverslip of the chamber. Then the micro-blade was slowly moved parallel with ICM cells surface to cut TE cells (Gardner, 1972; Rossant, 1975). Later on, this method has been modified for ICM isolation and used as source of ES cells. The manipulation technique had been changed by using only two fine needles for ICM dissecting under stereomicroscope which is much more practical for less manipulator skill people. It works very well in several *in vivo* produced embryos such as cat (Yu et al., 2008) and human embryo (Kim et al., 2005) because the *in vivo* produced embryos has

more distinct ICM cells than the embryos produced from *in vitro*. Strom and colleagues (2007) reported a new mechanical technique to isolate ICM by a specially made flexible metal needle, made of tungsten with a diameter of 0.125 m. The tip was made thin and sharp using electrolysis. Another blunter needle was used to hold the blastocyst during cutting out the ICM. Both needles were fixed to hand-pieces of pencil-thickness for manual operation under stereomicroscope. The blastocyst was moved to the operation drop then drawn out with the needle so that the blastocyst became attached to the surface of the well. With the blastocyst attached to the plastic, it was possible to make a hole in the zone pellucida with the needle to open up the blastocyst and by two to three cuts removing the ICM from the trophectoderm. The procedure took about 2–3 min/embryo (Strom et al., 2007).

2.2.2 ICM isolation by immunosurgery

ICM isolation by immunosurgery has been reported by Solter and Knowles since 1975. Isolation ICM by this technique can reduce contamination of TE from ICM cell in short time. Immunosurgery worked by the compatibility of antibody to the cell surface antigen of trophoblastic cells of the blastocyst stage embryo. This compatibility causes a selective killing of trophoblastic cells after the complement added into the system. The antibody is usually derived from serum of rabbit anti spleenocytes of the target embryo's species. The blastocyst stage embryos will be incubated with proper concentration of antibody for about 30 minutes (Solter and Knowles, 1975). To discard the unbound antibodies, blastocysts are subsequently washed several times before incubated with suitable concentration of complement for 15-30 minutes. During the incubation with complement, trophoblastic cells swell up and loose their semipermeability and finally lysed. The intensive washed out of the unbound antibody would secured ICM cells because there has no exceed antibody left over to bind the antigen on ICM cells surface after TE cells lyses in complement. Solter and Knowles (1975) suggested that mouse embryo shows

selective uptake of macromolecules from surrounding fluids into cytoplasm of TE and ICM cells. The barrier for non selective uptake of large molecule probably the TE cells themselves and/or tight junction between them. Pilz and colleagues (1970) suggested that the mouse blastocyst is impermeable for molecules with larger then 40A°. This makes non passage of immunoglobulin molecule (antibody) with larger diameter. Therefore, there will be only TE cell lysed on the addition of complement, leaving ICM intact. One of the most advantages of this method is allowing many ICMs to be recovered without the risk of mechanical damage to any of the cells which could found in microsurgical method. Several reports suggested that immunosurgery completely destroy TE cells (Solter and Knowles, 1975; Hadyside and Baton, 1977). To assess the purity of ICM cells after immunosurgery, they studies by the failure detection of fluorescent-conjugate antibody directed again IgG, detect trophoblastic-type outgrowths *in vitro* and found that the dissected ICMs show negative result. Moreover, they found that protein synthetic profile of these ICMs was similar to microsurgically dissected ICMs, and in particular, trophoblast specific spots were absent (Hadyside and Baton, 1977). Moreover, the ICM cells had been transferred to the uterus of the pseudopregnant mice for evaluate the implantation capacity. They only found the ICM derived tissues. This demonstrates the lack of TE cell contamination and functional viability of these ICMs. An important even that causes immunosurgery method is well known was because the first ES cell lines had been established by using this technique in 1981 by Martin (1981). Martin (1981) used immunosurgery as a method for ICM cells isolation before culture in teratomas conditioned medium. Several days after culture, the ICMs show remarkable resemblance pluripotent morphologies as embryonal carcinoma (EC) stem cell line. Then, the pluripotent cell line derived from ICM cells were named as embryonic stem (ES) cell to denote their origin directly from embryos and to separate them from EC cells which derived from teratocarcinomas (Martin, 1981). After the success of first mouse ES cell lines which established by immunosurgery, there are several

attempts to use this technique to establish ES cell from other animal species such as pig (Piedrahita et al., 1990; Chen et al., 1999; Anderson et al., 1994; Shiue et al., 2006; Moore and Piedrahita, 1997; Ock et al., 2005; Brevini et al., 2005), monkey (Thomson et al., 1995; Suemori et al., 2001; Vrana et al., 2003; Mitalipov et al., 2006); human (Thomson et al., 1998; Reubinoff et al., 2000; Cowan et al., 2004; Heins et al., 2004; Mateizel et al., 2006). Currently, according to the number of cited papers found that immunosurgery is one of the most often used methods for establishing ES cells from ICM of blastocyst embryo.

2.2.3 ICM isolation by calcium ionophore A23187

Calcium ionophore A23187 is a monocarboxylic acid antibiotic specific for divalent cations. It could increase intracellular ionized calcium (Reed and Lardy, 1972) and could also include mitogenic effects on lymphocytes (Freedmanet al., 1975; Hesketh et al. 1977), inhibition of morphological changes in cells induced by dibutyryl cyclic AMP (cAMP; Henneberry et al., 1975), release of histamine from mast cells (Foreman et al., 1973), prevention of retinal orientation in developing eyes of *Xenopus laevis* (Jacobson, 1976; Rose and Loewenstein, 1975), parthenogenetic activation of unfertilized eggs such as mouse (Hagemann et al., 1994; Uranga et al., 1996), cat (Grabiec et al., 2007), pig (Wang et al., 1998) and bovine (Liu et al., 1998; Xu and Yang, 2001; Chung et al., 2001; Sedmikova *et al.* 2003). It is also can be used as one of the activation reagents to activate cloned embryo development in several species such as bovine (Milazzotto et al., 2008), buffalo (Saikhun et al. 2003), Human (Heindryckx et al., 2007). For ICM isolation property, Calcium ionophore A23187 has been accidently found during the course of experiments to detect the mitogenic action of A23187 on blastocysts. Surani and colleagues (1978) found that 2 x 10^{-5} M calcium ionophore (A23187) caused selective lysis of trophectoderm cells occurred after approximately 30 min following their swelling and vesiculation but the ICM remained apparently intact (Surani et al., 1978). By using calcium ionophore A23187 to destroy TE cells, the morphologically identical to the lysis of the cell

observed after treatment of blastocyst with antibody and complement similar as immunosurgery method were found. The ICM recovery rate after the late expanded blastocysts treated with ionophore was 100% but the recovery rate was lower when early stage blastocysts were used (75-82%; Surani et al., 1978). The calcium ionophore A23187 had been used in some later reports for ICM isolation in rodent (Harlow and Quinn, 1979; 1980; Piedrahita et al., 1990) and ovine embryos (Piedrahita et al., 1990). The mechanism of TE cells lysis after treated with calcium ionophore A23187 is still not clear. It could be due to an osmotic phenomenon which could be explained by either the suggestion that Na^+ flows into the cells more quickly then the outward flow of potassium (K^+), influx of water then causes swelling and lysis of cells (Green et al., 1959) or the ionophore induced the uncontrolled in flux of Ca^{2+} (Reed and Larde, 1972) followed by swelling and vesiculation after the entry of water into the cells. However, this method is not generally used for ICM cell isolation in present day. This may be because of the uncontrollable osmotic action and mechanism of destroying the TE cells are not clear and, as mentioned above, the calcium ionophore A23187 could be used in several purposes which are possible to generate side effects on success rate of ES cells establishment.

2.2.4 Whole Blatocyst culture

The whole blastocyst and partial blastocyst culture methods seem to be advantageous to produce pathogen-free ES cell lines especially human ES cells, because of the absence of animal products such as antibody and complement used in immunosurgery method. Whole blastocyst culture has been used for ES cell establishment since 1981 (Evans and Kaufman, 1981). Late blastocysts cultured in culture media and let the embryos attach to the dish, after attachment, TE cells grew out and differentiated to giant cells where as ICM cells form egg cylinder shape. The ICM cells can be picked off and dissociated by trypsin treatment and reseeding onto feeder cells. Total of 15 pluripotent cell lines were establish by using this technique (Evans and Kaufman, 1981). This method has been used

in particularly with the animal that has a potential to be used for therapeutic studies such as pig (Evans et al., 1990; Gerfen and Wheeler, 1995; Hochereau-de-Reviers and Perreau, 1993; Kim et al., 2007; Li et al., 2004a; Miyoshi et al., 2000; Piedrahita et al., 1990) and human embryo itself (Heins et al., 2004, Kim et al., 2005). Whole blastocyst culture method is much simpler than other methods because it doesn't need any special chemical reagent or expensive instrument. Whole blastocyst culture method, the zona pellucida should be removed from embryos by using suitable method to allow blastcyst attachment onto the feeder cell. A few days after plating, the attachment of the whole embryo on feeder layer can be seen. The TE cells will be collapse and began to expand where as ICM-like cells formed a dome shape surrounded by differentiated TE cells. When the ICM clump looked big enough, at this point, a finely pulled pipette can use to pick off the ICM clump and transfer to fresh feeder cells. The differentiated TE cells can be left in the plate or discarded after ICM clumps were picked off. This simple procedure, however, runs a much greater risk of TE cell overgrowth than the other methods because the entire TE cells were cultured along with the ICM clump for several days. The ICM cells often will be covered with the differentiated TE cells resulting in unclear observation of ES cell morphologies. The ICM might not grow properly, degenerates or differentiates, eventually (Li et al., 2003).

2.2.5 Partial embryo dissection

To avoid problem of TE overgrowth from whole blastocyst culture, partial blastocyst culture were used in some cases instead of whole blastocyst culture (Kim et al., 2005). This method could provide chances to established ES cell line from expanded blastocyst that exhibited a smaller ICM. The partial blastocyst culture protocol is similar to whole blastocyst culture, except, part of TE cells removed by mechanical technique such as fine needles or glass pipette dissection. The reason why partial embryo dissection not widely used even it can dramatically increase success ES cell establishment rate might be

due to fully skilled personals are needed to control the manipulator to partial dissected the embryos.

2.2.6 Laser dissection

Laser applications have been used in the assisted reproductive technology (ART) for several years, including assisted hatching (Obruca et al., 1994) embryo or polar body biopsy (Veiga et al., 1997; Montag et al., 2000), sperm immobilization (Montag et al., 2000), and ICSI (Rienzi et al., 2001), all of which have resulted in significantly high fertilization rates and pregnancy rate of *in vitro* produced embryos (Obruca et al., 1994; Tanaka et al., 2006). Laser assisted ICM isolation was first reported in mouse (Cortes et al., 2006; Tanaka et al., 2006). The results demonstrated that laser dissection method has no influence on ICM attachment and ES cells derivation when compare with the zona-free whole blastocyst culture and ICMs derived immunosurgery but significantly higher than zona-intact blastocyst culture. Recently, the erbium-yttrium-aluminium-garnet (Er: YAG) laser was used for ICM cells isolate from human embryos (Turetsky et al., 2008). Eight ICMs were isolated from nine hatched blastocysts. They gave rise to three hES cell lines (37.5%; 3/8). This efficiency is similar to the efficiencies reported after isolation of the ICM by immunosurgery (Thomson et al., 1998; Cowan et al., 2004; Ludwig et al., 2006; Mateizel et al., 2006). In general, laser assisted ICM isolation was performed as follows: The zona-intact blastocysts were fixed by two holding pipettes with the ICM being positioned at either 9 o'clock (Tanaka et al., 2006) or 3 o'clock (Turetsky et al., 2008). In mouse embryo, approximately 10 infrared laser pulses at 300 mW × 1 ms (ZILOS-tk™, Hamilton Thorne Research, Beverly, MA USA) were fired to split the blastocyst into two unequal portions – the smaller consisting of ICM, the larger consisting exclusively the trophoblast whereas in human embryo, 20-30 infrared laser pulses at 200 mW x 0.5 ms were used. However, special attention is needed to direct the laser beam far enough from the ICM to prevent heating and damage to the ICM. At any rate, some blastocysts (about

40%) could not undergo successful ICM isolation due to the collapsed of the TE cells during the laser drilling (Turetsky et al., 2008).

2.2.7 Digestive method

Digestive method has also used for ICM cells isolation (Li et al., 2003; 2004b). The enzymatic (Trypsin/EDTA) and Acidic Tyrode's solution were used for TE cell digestion. Pig embryos were used to compare the efficiency of isolation methods among, whole blastocyst culture, immunosurgery and enzymatic method. The procedure of enzymatic method was done by treating the blastocyst stage embryos with either pronase or acidic tyrode's solution to remove the zona pellucida. Then, the zona-free embryos were submerged into a microdrop of 0.25% trypsin–0.04% EDTA solution for several minutes. During the treatment, embryos were observed under stereomicroscope for the dispersion of TE cells. When the TE began to disperse, the blastocysts were transferred to another drop with culture medium and ICM cells were isolated from the dispersed TE cells by the aid of two needles and a pulled mouth pipette. It has to be note that the whole embryo culture method had lower ICM attachment than the ICM isolated by enzymatic method (Li et al, 2003). The success of ICM recovered after enzymatic method was 85% whereas only 40% were obtained from immunosurgery (Li et al., 2004b).

The acidic tyrode's solution is a chemical defined solution wildly used for removal of all or partial zona pellucida of embryos (Cowan et al., 2004; Ellerstrom et al., 2006). It is an effective medium and removes zona pellucida very quickly within a few seconds. Because of the chemically defined solution, acidic tyrode's solution is much more suitable for pathogen-free system than pronase which is a product of bacteria. The acidic tyrode's solution has been used for ICM isolation from human blastocyst (Ellerstrom et al., 2006). The blastocysts were incubated in acidic tyrode's solution with carefully observation until the zona pellucida and TE cells were eliminated. They mentioned that 30-40 seconds is the optimum time to remove both zona pellucida and TE cells. However, this treatment could

not be used to destroy all the TE cells completely without damaging the ICM cells. Therefore, the initial outgrowth from the treated blastocysts composed of heterogenous cells population. However, areas of morphologically distinct ES cells appeared which could be picked off and transferred to fresh plates. They also mention that homogenously expressed Oct4 and morphologically resembled undifferentiated hESCs, with a small cytoplasm-to-nucleus ratio, could be found at passage 5 (Ellerstrom et al., 2006).

2.2.8 ES cell lines derivation from single blastomeres

Since 1981, ES cells have been successfully established from several species in the past decades including mice (Evan and Kaufman, 1981; Wakayama, et al., 2007), monkeys (Suemori, et al., 2001; Thomson et al., 1995), and humans (Baharvand, et al., 2006; Heins, et al., 2006). Although most of the currently available ES cell lines were derived from the ICM cells of a blastocyst stage embryo, It is noted that only a small number of blastomeres from 8-cell (Delhaise et al. 1996) and 16-cell (Eistetter, 1989) mouse embryos were viable for deriving ES cells. Single blastomere biopsy is an alterernative methods of ES cell establishment which is similar technique used in pre-implantation genetic diagnosis (PGD) in IVF clinic (Chung et al., 2006). A single biopsied blastomere would not interference the developmental potential of the biopsied embryos (Chung et al., 2006). There have been several attempts to do single blastomere biopsy after the report of Chung and colleagues (2006) for example, mice (Wakayama et al., 2007; Teramura et al., 2007; Lorthongpanich et al., 2008a,b), monkey (Narita et al., 2008) and human (Klimanskaya et al., 2006; Chung et al., 2008).

ES cells establishment from single blastomere could be performed by 3 different ways;

1. Co-cultured single blastomere with ES supporting cells. This technique has been reported in mouse (Chung et al., 2006) and human embryo (Klimanskaya et al., 2006; Chung et al., 2008). Single blastomeres were biopsied from eight cell stage embryos and

each separated blastomere was aggregated with a small clump (around 100 cells) of GFP-positive ES cells (ES supporting cells) in a 300-mm depression created by pressing a needle into the bottom of a plastic tissue culture plate. After 24-48 h of incubation in ES cell culture medium supplemented with 2,000U/ml mouse leukemia inhibitory factor (LIF) and 50mM MEK1 inhibitor, a GFP negative bud was observed on the side of ES supporting cells. Then the aggregates were transferred to feeder cells and ES culture medium until the GFP negative cells became large enough to subculture. The GFP-negative cells were separated from ES supporting cells by microcapillary under fluorescent microscope. The GFP-negative cells were then expanded and test for ES cell markers. Not only mouse stem cell lines but also extraembryonic (TE) stem cell lines were established by single blastomere culture with ES supporting cells (Chung et al., 2006).

2. *Whole blastocyst derived from single blastomere outgrowth*. This technique has been reported a year later by Wakayama and colleagues (2007). They cultured the separated blastomeres individually in 96 well plates precoated with feeder cells in ES culture medium with 20% Knockout Serum Replacement (KSR) and 0.1mg/ml adrenocorticotropic hormone (ACTH; fragments 1–24) instead of fetal calf serum (FCS). During the culture, the blastomeres could divide and develop to blastocyst. After 10 days or more, proliferation outgrowths were dissociated and replated to expand until stable ES cell line grew out. According to this technique, they could produce several ES cell lines from single blastomeres derived from two-cell (establishment rate, 50%–69%), early four-cell (28%–40%), late four-cell (22%), and eight-cell (14%–16%) stage embryos (Wakayama et al., 2007).

3. *Immunosurgery of blastocyst derived from single blastomere.* Since the single blastomeres derived blastocysts contain small amount of ICM cells (Lorthongpanich et al., 2008a). Therefore, there are not many attempts to use immunosurgery with single blastomere derived embryo. However, there was a report successfully use immunosurgery

with blastocysts derived from single blastomeres of two-cell stage embryos which containing of prominent ICM cells (Teramura et al., 2007).

2.3 Non human primate embryonic stem cells (nhpESCs)

ES cell lines from preimplantation embryos has been reported in many mammals including mouse (Evans and Kaufman, 1981), sheep (Handyside et al., 1987; Tsuchiya et al., 1994; Dattena et al., 2006), mink (Sukoyan et al., 1992), cattle (Saito et al., 1992; Cherny et al., 1994; Stice et al., 1996), equine (Saito et al., 2002), rabbit (Neimann & Strelchenko, 1994), pig (Gerfen & Wheeler, 1995; Shim et al., 1997; Chen et al., 1999), rhesus monkey (Thomson et al., 1995; Byrne et al., 2007), baboon (Simerly et al., 2009) and human (Thomson et al., 1998; Shamblott et al., 1998).

The establishment of pluripotent stem cell lines has given rise to a powerful tool for *in vitro* research of developmental processes at both cellular and organismal levels. ES cells also offer tremendous potential for clinical application which served as an unlimited source of cells for transplantation and tissue regeneration therapies. Isolation of the original cell source to establish ES cell lines from mouse (Evans and Kaufman, 1981) were similar to the reported derived from nonhuman primate (Thomson et al., 1995) and human embryos (Thomson et al., 1998; Shamblott et al., 1998; Reubinoff et al., 2000). Three primary sources of human tissue have been demonstrated to give rise to pluripotent cell lines. Embryonic stem (ES) cells were derived from the inner cell mass (ICM) of blastocyst-stage embryos (Thomson et al., 1998; Reubinoff et al., 2000), embryonic germ (EG) cells (Shamblott et al., 1998) from the primordial germ cells of the embryonic gonad, and embryonic carcinoma (EC) cells from teratocarcinoma tissue (Andrews et al., 1984). However, most of the monkey ES cells lines were established by ICM isolation from blastocyst stage embryos (Thomson et al., 1995; Mitalipov et al., 2006; Byrne et al., 2007).

ES cell lines from different species were claimed to exhibit different properties from murine ES cells such as morphology of undifferentiated colonies and the expression profile of stem cell markers. Among the complications in assessing cell line in undifferentiated ES colonies of different species, the morphologically appearance of ES colonies vary in different animal species such as mouse ES undifferentiated colonies exhibit colonies with clear edge, dome shape and high nuclear – cytoplasmic ratio while the undifferentiated primate ES cells appeared as flat colonies with high nuclear – cytoplasmic ratio (Thomson et al., 1995). Moreover, the primate ES cells are differ from the murine ES cells in the expression pattern of stem cell markers and cytokine factors that are important for maintaining the undifferentiated stage (Table 2.1).

2.3.1 Maintaining of primate ES cells in their undifferentiated state

- **Basic fibroblast growth factor (bFGF or FGF2)**

Mouse ES cells (mESC) can be maintained in an undifferentiated state by adding leukemia inhibitory factor (LIF) into the culture medium. LIF binds a heterodimer of LIF receptor and gp130 that activate JAK/STAT3 is sufficient to maintain undifferentiated stage of mouse ES cells culture in serum (Smith et al., 1988; Williams et al., 1988). However, additional of LIF to culture medium (Thomson et al., 1998) or activation of STAT3 pathway (Humphrey et al., 2004) does not maintain undifferentiated stage of human ES cells (hESC). Therefore, LIF does not support undifferentiated stage of hESC and the JAK/STAT3 pathway does not need to become activated to maintain hESC (Thomson et al., 1998) and non human embryonic stem cell (nhpESC) (Thomson et al., 1995).

Fibroblast growth factors (FGFs) constitute a large family of signaling polypeptides that are expressed in various cell types from early embryos to adults. The first discovery of FGFs was reported since 1974 (Gospodarowitz, 1974). During embryonic development, FGFs have several biological processes functions including cell proliferation, differentiation, and migration (Bottcher and Niehrs, 2005). In mammalian embryos, the

members of FGF family are expressed in many cell types. However, the timing of expression are varies. In addition, several FGFs members are present in embryonic tissues but the others FGFs member including basic fibroblast growth factor (bFGF) are expressed in both embryonic and adult tissues. In adult organism, FGFs represent important homeostatic factors and play a role in response to injury and tissue repair (Cutroneo, 2003).

Table 2.1 Comparision of murine, monkey and human ES cell

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

(http://stemcells.nih.gov/info/scireport/appendixC.asp)

Undifferentiated hESCs possess functional FGF signaling pathway that can basically operate in two different ways: 1) Autocrine via low molecular-mass bFGF and FGFRs; 2) Intracrine through translocation of high molecular-mass bFGF into the nucleus. Autocrine FGF signaling pathway is unconditionally vital for proliferation of hESCs in the undifferentiated state have been propose (Dvorak and Hampl, 2005). Even several reports have showed that bFGF could support undifferentiated stage of hESC and nhpESC but the exact signaling mechanism remain unknown.

To test whether autocrine FGF signaling is important for growth of undifferentiated hESCs cells, they were treated with the pharmacological inhibitor of FGF receptor tyrosine kinases, SU5402, which specifically interacts with intracellular catalytic domain of fibroblast growth factor receptors (FGFRs) (Mohammadi et al., 1997). After 2 days of continuous exposure to SU5402, cells in the centers of some colonies start to differentiate. After further culture in the ES medium added with SU5402 ES cell morphology was changed and lost of ES markers expression of undifferentiated cells such Oct-4 and SSEA-4. Moreover, decreased of phosphorylation of mitogen-activated protein kinase kinase (MEK1/2) and its substrate ERK1/2 were both observed in cells maintained in medium with the FGF inhibitor. Importantly, the occurrence of differentiated cells was accompanied by up-regulation of cyclin-dependent kinase inhibitor p27, a common event that characterizes differentiation of cells of early embryonic origin, and by a slower proliferation (Bryja et al., 2004). Moreover, differentiation process was manifested by up-regulation of TROMA-1, a marker for primitive endoderm, and of nestin, a marker for developing neuroepithelium and for epithelial precursors in the embryonic pancreas (Bryja et al., 2004).

In primate, bFGF have better effect than other growth factors to promote hESC self-renewal (Amit et al., 2000). Additional of bFGF at low concentration (4 ng/ml) in culture medium with serum replacement could support undifferentiate proliferation of both hESC (Thomson et al., 1998; Shamblott et al., 1998; Amit et al., 2000; Xu et al., 2001) and

nhpESC (Thomson et al., 1995; Mitalipov et al., 2006; Byrne et al., 2007) on feeder layer. It has been reported that only bFGF alone is sufficient to maintain hESC self-renewal in the absence of feeder cells, however some region still show background of differentiation (Xu et al., 2005; Levenstein et al., 2006). The comparative study of bFGF concentration at 4, 24, 40, 80, 100 and 250 ng/ml in uncondition medium without feeder shows that hESC with 100 ng/ml bFGF proliferate close to hESC growth on feeder layer with 4 ng/ml and low background of differentiated hESC was observed (Levenstein et al., 2006).

Adding bFGF into culture medium may stimulate hESCs only *via* their high-affinity receptors. In contrast, bFGF produced endogenously by hESCs may function in two ways depending on the presence or absence of the nuclear localization sequence: high molecular-mass isoforms can be directly targeted to the nucleus and operate independently of cell surface receptors in an intracrine manner; whereas the low molecular mass isoform may be exported from the cells and act via FGFRs as an autocrine or paracrine factor. The stimulation of hESCs by bFGF at the concentration that is routinely used for undifferentiated hESC culture leads to tyrosine phosphorylation of various proteins and to activate extracellular signal regulated kinases, ERK1/2, in particular. However, undifferentiated hESCs maintained for several days in bFGF-free medium possess an unexpectedly high basal level of phosphorylation of ERK1/2, which contrasts with the undetectable ERK1/2 phosphorylation typically demonstrated by mESCs. The constitutive activation of ERK1/2 could be caused, at least in part, by unusually high expression of exportable and nuclear isoforms of bFGF in hESCs conferring a reduced dependency of ERK activity on extracellular signals (Dvorak and Hampl, 2005).

- **Bone morphogenetics proteins (BMPs) enhance ES cell differentiation**

In serum-free medium of mouse ES cells, adding of BMP4 and LIF are sufficient to support the growth of mouse ES cells (Ying et al., 2003). Moreover, BMP could synergize with LIF to maintain self-renewal of mouse ES cells by induced expression of Id gene

(Ying et al., 2003). However, when BMP4 is added to hESCs in serum free medium, it could not support undifferentiated proliferation and rapid differentiation occured (Xu et al., 2002). Xu and colleague report that trophoblast differentiation was promoted by adding bFGF and BMPs in condition medium. Moreover, blocking BMP2 activity in serum by BMP antagonist noggin does not maintain hESCs in undifferentiation stage but enhance hESCs differentiation to neuron lineage by inhibit non-neuron differentiation (Pera et al., 2004). However, adding of noggin combined with bFGF at extremely high concentration (40 ng/ml) supports the undifferentiated proliferation of hESCs in the absence of fibroblast feeder cells. As hESCs cultured in fibroblast- unconditioned medium show high levels of BMP signaling activity compared to hESCs maintained in fibroblast-conditioned medium, this effect was explained in a way that BMP antagonist noggin synergizes with bFGF to repress trophoblast-inducing BMP signaling and thus sustains undifferentiated growth of hESCs (Ying et al., 2003).

2.4 Huntington's disease

2.4.1 What is Huntington's disease (HD)?

In 1872, Dr. George Huntington reported about the disease that he call "Huntington's chorea". The patient with Huntington's chorea has uncontrollable of body movement, which include continues and irregular twisting and jerking movements which is why he named this disease as "Chorea". The repeated sequence CAG (cytosine-adenine-guanine) in the gene encoding the Huntington protein, resulting in long stretches of the amino acid glutamine (Q) with in the protein has been identify as a cause of Huntington's disease (Nance, 1996). Huntington is a rare, progressive and fatal autosomal dominant neurodegenerative disorder, typically of adult inset. It is also an inherited and age related disease. HD is caused by the degeneration of cells or neuron located in striatum region deep within the brain that is part of structure call basal ganglia (Figure 2.4.1). In normal people,

neurons in striatum work to shut off excitatory signals from the motor cortex, the part of the brain that dictates movement. The damages in striatum, therefore, resulted in uncontrolled body movement (Mattson, 2002). HD can be separated into two groups according to number of repeated CAG triplets sequence, wild type and mutation. In wild type, the repeated sequences are between 6-39 triplets whereas the mutation expressed more than 39 triplets repeat (Li et al., 1999). The most thorough study of the neuropathology of HD classified the neuropathological changes into five grades: grade 0, in which HD brains show no gross or generalized microscopic abnormalities consistent with HD, despite premotem symptomatology and positive family history, progressing to grade 4, in which the most extreme atrophy is observed (Vaonsattel et al., 1985). All grades exhibit 30% areal reductions in cerebral cortex, white matter, hippocampus, amygdale and thalamus (de la Monte et al., 1988).

As mentioned, HD is a rare disease; Global incidence varies, from 3 to 7 per 100,000 people of Western European descent, down to 1 per 1,000,000 of Asian and African descent. However, this is severe disease because it is inherited which cause suffering to everyone in the HD patient's family. The age of onset varies markedly, typically occurred between ages 35-50 but varying from early childhood – 80 years old. HD symptom is insistently progressive with death occurring about 15-20 years after the disease onset. Death usually occurs by pneumonia, malnutrition or heart failure. (http://www.scq.ubc.ca/huntington-disease-overview-of-a-genetic-neurodegenerative-disorder/).

2.4.2 The importance of CAG repeats.

Cause of HD is the expansion of a CAG repeat sequence in the first exon of a gene of chromosome 4p16.3, which encodes the protein huntingtin (Davies and Ramsden, 2001). The CAG repeats have different levels which reflect to the early or late onset of Huntington's symptom such as

- Between 6 - 27 repeats is the normal range for the number of polyQ.

- Between 29-35 repeats will not develop HD themselves, but a small percentage of their children may develop it. However, patient with CAG repeats between 30-34 needs specific laboratory and clinic evaluations to interpret other repeat levels such as between, indicating those who will not develop the disease but transmit it for even generations before it develops into disease.

- Between 35 - 39 repeats: may not develop it but will definitely have it develop in their immediate children.

- 40 repeats or more repeated CAG segments will definitely develop Huntington disease.

- Greater than 55 will even have disease onset in childhood such as patients with CAG repeats between 93 – 130 repeats show disease onset at around 3-10 years old (Davies and Ramsden, 2001; Nahhas et al., 2005; Ribai et al., 2007; Yoon et al., 2003).

The first HD animal model, R6/2, was established by introducing a transgene consisting of exon 1 of the HD gene with 141-150 CAG repeats (Mangiatini et al., 1996). These mice develop a progressive neurological phenotype with onset at around 8 weeks after being transgenic. The phenotype includes motor symptoms such as a resting tremor, abrupt shuddering movements, stereotypic grooming movements and epileptic. In additional, they found that the mice have brain weight loss starts after 4 weeks and body weight decrease begins at 8 weeks, however, the selective neurodegeneration is not found until 14 weeks in R6/2 mice (Mangiatini et al., 1996). Kovtun and colleagues (2000) found that the size of the CAG repeat was influenced by the sex of the offspring from identical father, whereas the opposite was seen in females which are similar to human. Moreover, the juvenile onset HD patients (patient who younger than 20 years old) seem more often to have inherited their disorder from the paternal than from the maternal (Ribai et al., 2007; Ridley et al., 1988). Even thought, the expanded CAG repeat is transmitted predominantly

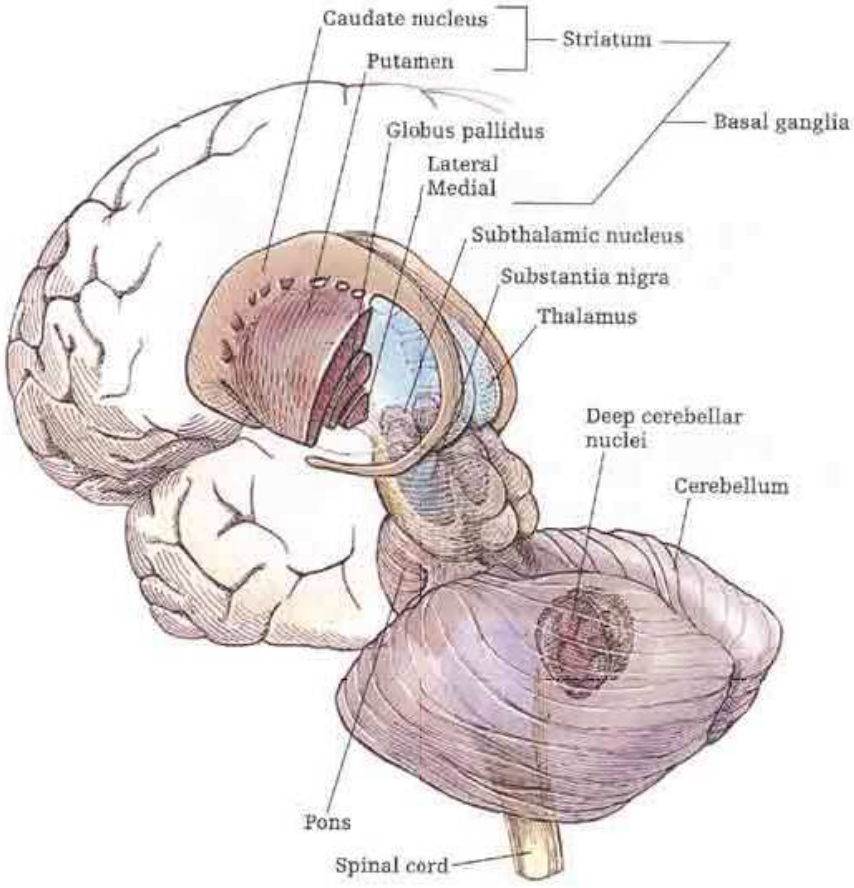

Figure 2.4.1 Location of striatum area in human brain. HD is characterized by a striking specificity of neuron loss. The most sensitive regioun is the striatum with about 57% loss of cross sectional area from the caudate nucleus and about 65% loss of putamen in typical postmortem samples.

Picture from http://epistemic-forms.com/L-basal-ganglia.jpeg

through the male germ line in humans and transgenic mice but sometime the very large expansions can also occur through the maternal lineage (Nahhas et al., 2005). Paulsen and colleagues (2006) studied the brain structure of 24 preclinical HD patients as measured by brain MRI and compared them to 24 healthy control subjects matched by age and gender. Preclinical HD individuals had substantial morphologic differences throughout the cerebrum compared to controls (Figure 2.4.2). The volume of cerebral cortex was significantly increased in preclinical HD, whereas basal ganglion and cerebral white matter

volumes were substantially decreased. Although decreased volumes of the striatum and cerebral white matter could represent early degenerative changes, the finding of an enlarged cortex suggested that developmental pathology occurs in HD (Paulsen et al., 2006).

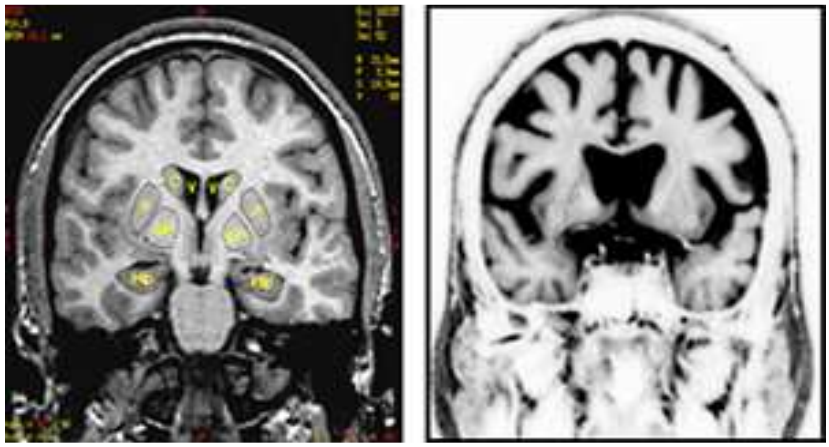

Figure 2.4.2 Brain scans show the dramatic difference between a healthy individual (left), and the effects of Huntington's disease (right). (http://discoverysedge.mayo.edu/de07-2-neuro-mcmurray/index.cfm)

2.4.3. Role of Huntingtin protein in HD

Huntingtin (htt) is the protein coded by the gene huntingtin (MacDonald et al., 1993). It is variable in its structure as there are many polymorphisms of the gene which can lead to variable numbers of glutamine residues present in the protein. In its wild-type (normal) form, it contains 6-39 glutamine residues, however, in individuals affected by Huntington's disease contains between 39-180 glutamine residues. Normally, huntingtin is a cytoplasmic protein expressed at high levels in the striatal neurons sensitive to degeneration in HD and at low level or undetectable in neuron resistant to degeneration (Gutekunst et al., 1999). Huntingtin has a predicted mass of ~350 kDa, however, this varies dependent on the number of glutamine residues in the protein. Normal huntingtin is generally accepted to be 3,144 amino acids in size (Davies and Ramsden, 2001). Nasir and colleagues (1995)

reported that the huntington gene is essential for postimplantation development and that it may play an important role in normal functioning of the basal ganglia in mouse and absence of huntingtin is lethal in mice. The huntingtin protein is highly expressed in neurons and testes in humans and rodents (Cattaneo et al., 2005). From immunohistochemistry, electron microscopy, subcellular fractionation studies of the molecule has found that Huntingtin is primarily associated with vesicles and microtubules (DiFiglia et al., 1995; Hoffner et al., 2002). These appear to indicate a functional role in cytoskeletal anchoring or transport of mitochondria. The htt protein is involved in vesicle trafficking as it interacts with HIT1, a clathrin binding protein, to mediate endocytosis (Figure 2.4.3), the absorption of materials into a cell (Mattson, 2002; Velier et al., 1998; Waelter et al., 2001). In patients with Huntington's disease, the htt protein has an abnormally long tract of glutamine (Q) amino acids. This mutant protein may lead to abnormal endocytosis and secretion in neurons. In addition, it causes striatal neurons to die by the process of apoptosis (Mattson et al., 2002). Gervais and colleagues (2002) show that the long tract of glutamines weakens htt's interaction with Hip1, which is then free to bind the protein Hippi, and so to activate apoptosis through caspase-8 and caspase-3. Caspase-3 also cleaves htt, producing fragments that clump together and form inclusions in the neuron and its nucleus (Gervais et al., 2002). Therefore, neurons communication by secreting neurotransmitters would not occur smoothly since the vesicles could not be formed properly (Mangiatini et al., 1996).

2.4.4. Inclusion body

HD is characterized by the aggregation of htt into microscopic intranuclear aggragates (INs) called inclusion bodies (IBs) and by the death of striatal and cortical neurons. Intranuclear aggregates formed by expanded polyglutamine proteins have also been reported in the brains of patients with HD or other glutamine repeat disorders (Becher et al., 1998; DiFiglia et al., 1997; Paulson et al., 1997; Ross, 1997). These studies clearly indicate that expanded polyglutamine tract can cause huntingtin and other proteins to form

aggregates in the nucleus. However, the relationship between htt deposition and neurodegeneration is controversial. Sometimes IBs formation has been associated with neurodegeneration (Becher et al., 1998; Davies et al., 1997; DiFiglia et al., 1997; Ordway et al., 1997), at other times, there was no or negative correlation (Klement et al., 1998; Saudou et al., 1998; Taylor et al., 2003). In 2004, Arrasate and colleagues reported that the IBs formation reduces levels of mutant huntingtin and risk of neuronal death. They suggested that the IBs does not trigger the neuronal cell death since several death cells of HD without IBs formation were found (Arrasate et al., 2004). Thus, inclusion body formation can function only as a coping response to toxic mutant huntingtin.

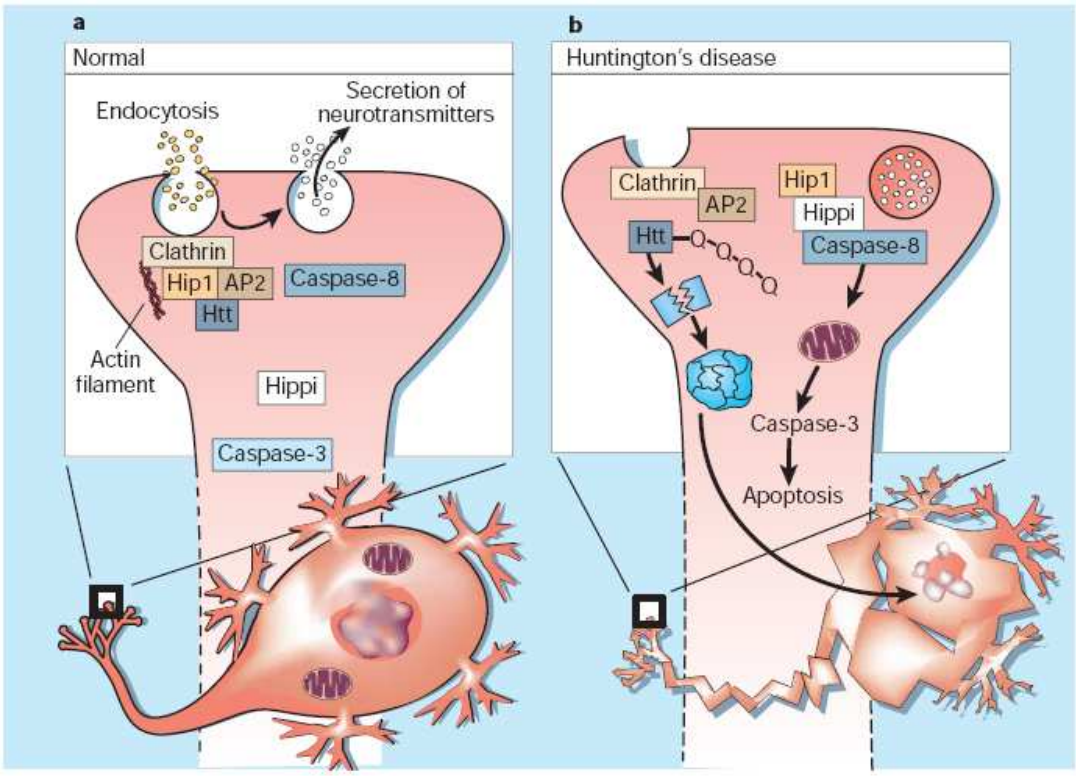

Figure 2.4.3 Possible functions of the normal and mutant huntingtin protein. In normal nerve cells, huntingtin (htt) can form a complex with the proteins Hip1, clathrin and AP2. Clathrin and AP2 (and so perhaps htt) are involved in endocytosis - the internalization of sections of the plasma membrane - which is crucial in allowing neurons to communicate by secreting neurotransmitters

(a). In patient with HD, the long tract of glutamines weakens htt's interaction with Hip1, which is then free to bind the protein Hippi, and so to activate apoptosis through caspase-8 and caspase-3. Caspase-3 also cleaves htt, producing fragments that clump together and form inclusions in the neuron and its nucleus (b) (Mattson, 2002).

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CHAPTER III

DEVELOPMENTAL POTENTIAL OF RHESUS MONKEY EMBRYOS DERIVED FROM SOMATIC CELL NUCLEAR TRANSFER

3.1 Abstract

Somatic cell nuclear transfer (SCNT) in non-human primates (NHPs) is a powerful animal model for therapeutic cloning. This study shows that age of oocyte donor affects number of collected oocytes. Monkeys age of 5 to 8 years provide more number of GV and MII stage oocytes than monkey of 9 to 15 years (GV: 31/171, 18.1% vs. 13/95, 13.7%; MII: 93/171, 54.4% vs. 51/95, 53.7%, respectively). The MII oocytes were activated with 5 μM Ionomycin and subsequence incubated in 6-DMAP for either 4h (PA-4) or 5h (PA-5) to access the activation protocol. The result indicated that PA-5 could better support the somatic reprogramming and embryo development than PA-4. Therefore, in the subsequences experiments, the cloned embryos were activated by PA-5 protocol. SCNT embryo reached compacted morula and blastocyst stage with significantly lower rates (9/21, 42.9% and 5/21, 23.8%, respectively) than PA-5 (15/18; 83.3% and 12/18; 66.7%, respectively). Blastocyst rate of PA-5 (12/18; 66.7%) was significantly higher than those from PA-4 (9/19, 47.4%) but was not different from ICSI (12/20; 60.0%) group. The data showed that activation of monkey oocytes in 5 μM Ionomycin for 5 min followed by 5h in 6-DMAP could provide better blastocyst rate, which has also demonstrated in monkey SCNT derived embryos.

3.2 Introduction

Many animal species have been cloned since the first SCNT, Dolly, was born (Wilmut et al., 1997). The first cloned NHPs was reported by using embryonic cells to produced live offspring (Meng et al., 1997). Somatic Cells Nuclear Transfer (SCNT) in rhesus monkey could be used as a model of human genetics disorders. If somatic cell nuclear transfer efficient in non-human primates it would support studies of therapeutic cloning. There have been many attempts to produce monkey SCNT embryos, however the obtained blastocyst rates are still low (Mitalipov et al., 2002; Simerly and Navara, 2003; Ng et al., 2004; Zhou et al., 2006; Byrne et al., 2007; Yang et al., 2007). Only one report had recently demonstrated that two monkey ES cell lines could be established from 20 SCNT produced blastocysts (Byrne et al., 2007). However, no success report of producing live cloned NHPs has been achieved by SCNT.

Until now, many investigators are investigating the strategies to increase the efficiency of SCNT and decrease the incident of developmental abnormalities in cloned animal (Mitalipov et al., 2002; Zhou et al., 2006; Niu et al., 2008). Reprogramming ability of the oocyte is the most important factor needed to be concern. In nuclear transfer, reprogramming of somatic cell back to pluripotentcy stage occurred after chemically activation of the reconstructed egg. Chemical activation is very crucial for reprogramming of the differentiated cell back to pluripotent stage. The suitable activation condition could provide higher number of embryos development. Therefore, one of the method that could improve the efficiency of monkey SCNT is the chemically activation protocol. Many studies have reported that artificial activation treatment is necessary to increase blastocyst formation during *in vitro* culture (Abramcuk et al., 1977; Mitalipov et al., 2001; Roger et al., 2004; Zhou et al., 2006; Okahara-Narita et al., 2007; Niu et al., 2008). Artificial activation of SCNT mimic the mechanism after sperm fertilized with egg by stimulate the Ca^{2+} oscillation. It also could induce development of MII oocytes without fertilization

(Mitalipov et al., 2001; Stricker, 1999). During fertilization, sperm stimulates Ca^{2+} oscillation to initiate egg development (Whittingham 1980; Cuthbertson and Cobbold 1985; Stricker, 1999). The targets of calcium-stimulation are maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase. The MPF activity is regulated by the level of cyclin B and phosphorylation states of Cdc2 ($p34^{cdc2}$). MPF was high activity at metaphase of mitotic cell cycle during nuclear envelop breakdown, chromatin condensation and formation of mitotic spindle (Doree and Galas, 1994). MPF is necessary to be inactivate to allow oocyte release to mitosis. It could be inactivate by ubiquitin-dependent proteolysis of cyclin B and by conversion into inactive pre-MPF by Cdc2 hyperphosphorylation (Solomon et al., 1990). However, MPF activity is quickly restored with chromatin recondensation and re-entry into a metaphase III (MIII) phase arrest (Collas et al. 1995; Susko-Parrish et al. 1994). Additional either inhibition of protein synthesis by cycloheximide (CHX) or protein phosphorylation by 6-dimethylaminopurine (6-DMAP) (Liu et al. 1998; Susko-Parrish et al. 1994) prolong inactivation of the MPF. Therefore, the consequence expose with ionomycin and 6-DMAP, calcium ionophore and 6-DMAP, or ionomycin and CHX, calcium ionophore and CHX could support embryo development in bovine, rabbit and NHP embryo (Liu et al. 1998; Mitalipov et al. 2001; Niu et al., 2008; Susko-Parrish et al. 1994; Tian et al., 2002). As the above reviews demonstrated that the suitable activation protocol could improve the efficiency of somatic cell reprogramming and embryo development in nuclear transfer derived embryo, this study aimed to develop the optimal activation protocol for SCNT of rhesus monkey.

3.3 Materials and Methods

3.3.1 Establishment of rhesus monkey fibroblasts cell line

Fibroblasts were generated from skin tissue from miscarried transgenic (rHD-5) with GFP and human huntingtin (htt) gene (Figure 5.1). The skin tissue were washed

several time in Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS) that supplemented with 100 U/ml Penicillin/ 100 ug/ml Streptomycin (Invitrogen). Skin tissues were cut into small pieces by scissors in 0.05% trypsin/EDTA (Invitrogen) and digested at 37˚C for 10-15 minutes. Then, the supernatant of digested tissue were transfer to tissue culture dish in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone), 2mM L-Glutamine (Invitrogen) and 100 U/ml Penicillin/ 100 ug/ml Streptomycin (Invitrogen). Fibroblast-like morphologies were passage when they reach to 95% confluence. Cells were frozen in DMEM with 10% FBS and 10% dimethylsulphoxide (DMSO; Sigma) and stored in liquid nitrogen until used.

3.3.2 Rhesus monkey ovarian stimulation and oocytes collection

Cycling female monkeys were superovulated by subcutaneous injection once daily with gonadotropin-releasing hormone (GnRH) antagonist, Antide (Serono Inc.) and twice daily with recombinant human follicle stimulating hormone (r-hFSH; Serono Inc.) for 8–9 consecutive days. On the last couple day of r-hFSH, animals were subcutaneous injection twice daily with recombinant human luteinizing hormone (r-hLH; Serono Inc.). Ovarian development was examined on day 7 of hormone stimulation by ultrasonography. If follicles diameter larger than 3-4 mm, animals were subcutaneous injection by recombinant human chorionic gonadotrophin (r-hCG; Serono Inc.).

Cumulus-oocyte complexes (COCs) were collected around 37 hrs after r-hCG injection by using laparoscopic follicular aspiration. The suction needle was connected with continuous vacuum and punched into abdominal cavity. Follicular fluid with COC diluted immediately with Tyrode's Lactate-Pyruvate-HEPES medium (TALP-HEPES) (Bavister *et al.*, 1983). COCs were stripped of cumulus cells by pipetting after exposure to 0.1% hyaluronidase to allow the classification of nuclear maturity as GV (existence of germinal vesicle), MI (disappearance of GV), MII (extrusion of the first polar body). Oocytes were cultured in *in vitro* maturation medium consisting of Connaught Medical Research

Laboratories medium (CMRL-1066) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 40 μg/ml sodium pyruvate, 150 μg/ml glutamine, 550 μg/ml calcium lactate, 100 ng/ml estradiol 17β and 3 μg/ml progesterone, at 37°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 24h. Numbers of each stage of oocyte after collection from monkey age from 5 to 15 year old were recorded.

3.3.3 Somatic cell nuclear transfer

Oocytes with an extruding of the first polar body (1st PB, Figure 3.1a) were placed in TALP-HEPES supplemented with 5 μg/ml of cytochalasin B for 15 minutes. The zona pellucida above the 1st PB was dissected by glass needle (Figure 3.1b, c). The 1st PB and small amount of cytoplasm (about 10%) underneath the 1st PB were squeezed out (Figure 3.1d). The success enucleation was confirmed by staining the first polar body and squeezed out cytoplasm with 5 μg/ml of Hoechst 33342 (Figure 3.1e-g). The enucleated oocytes were washed 5 times in TALP-HEPES and kept in this media until donor cell injection. An individual fibroblast cell diameter 14-16 μm was inserted into perivitelline space of enucleated oocyte (Figure 3.2a, b). The donor cell and cytoplasm were fused by fusion electrodes (Figure 3.2c) in manitol fusion medium with two direct currents, 30 volts 30 μsec generated by electro cell fusion machine (Nepa Gene Company, Japan). The reconstructed embryos were cultured in HECM 9 supplemented with 50 nM trichostatin A (TSA) for 10-12 hours.

3.3.4 Pathenogenetic activation (PA) and embryo culture

Two hours after fusion, the reconstructed embryos were activated by 5 μM ionomycin (Io) in TALP-HEPES medium for 5 minute and subsequence incubated in 2 mM 6-DMAP in HECM-9 for 4 hrs (PA-4) or 5 hrs (PA-5) in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37˚C. The reconstructed embryos were then cultured in HECM-9 for 8 days in the same condition. Embryo development rates of PA-4 and PA-5 were daily recorded. The better treatment was chosen to activate the reconstructed embryos

in nuclear transfer experiment.

3.3.5 Semen preparation and intracytoplasmic sperm injection (ICSI)

Semen was collected by penile electroejaculation. Semen was kept at room temperature for 10 minutes, and then washed three times with TALP-HEPES. The sperm concentration and motility were evaluated and recorded. The sperm was then incubated in TALP-HEPES medium at room temperature for at least 2 hours before a single spermatozoon was expelled into ooplasm. Oocytes with a first polar body were selected for ICSI. Sperm were individually immobilized by scoring the midpiece. A single sperm was aspirated from the sperm droplet and moved to a droplet containing oocytes. An oocyte was captured with the holding pipette and immobilized with its polar body at either the 6 or 12 O'clock position, and a spermatozoon was injected into the cytoplasm. Injected oocytes were transferred to HECM-9 (Zheng *et al.*, 2001), and cultured in 4 well dish cover with mineral oil at 37°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 8 days.

3.3.6. Statistical analysis

Statistical analysis of embryos development was analyzed between each treatment group using general linear model (GLM). This test was used to analyze significant differences between NT, parthenogenetic and ICSI embryos development. The analysis was performed using the Statistical Analysis System Software (SAS, version9.0; SAS, Cary, NC, USA) and value of $P<0.05$ was considered as significantly difference.

3.4 Results

3.4.1 Age of oocyte donor effects number of recovered oocytes.

Oocytes were collected from 5 to 15 years old monkeys. Total ten monkeys were classified into two groups depending on age of the monkey. The monkey from 5 to 8 years old were classified into group one whereas monkeys age older than 8 years old

were classified into group two. Total 171 oocytes were collected from 4 monkeys in the first group (Table 3.1). The average number of collected oocytes per monkey was 42.7 (Table 3.2). Only 95 oocytes were collected from 6 monkeys in group two (Table 3.1). The average number of collected oocytes per monkey was 13.7 which lower than monkeys in group one (Table 3.2). However, the propotion of oocytes reached GV, MI and MII stage was not significantly affect by age of monkey. The results suggesting that younger monkey could slightly produce more oocytes than elder monkey.

Table 3.1 Number of recovered oocytes after hormone stimulation.

Monkey Age	**No. Monkey**	**No. Collected oocyte**	**GV (%)**	**MI (%)**	**MII (%)**
Group 1 (< 8 Years old)	4	171	31 (18.1)	47 (27.5)	93 (54.3)
Group 2 (> 8 Years old)	6	95	13 (13.7)	31 (32.6)	51 (53.7)
Total	**10**	**266**	**44 (16.5)**	**78 (29.3)**	**144 (54.1)**

Average number of recovered oocytes per monkey were shown in Table 2 indicated that group one monkey could produce 7.7 GV stage oocyte/monkey which is higher than group two, 2.2 oocytes/monkey. Moreover, group one monkey also produced more MI stage oocytes (11.8 oocytes/monkey) than group two monkeys (5.2 oocytes/monkey). Group I monkey (23.2 oocyte/monkey) also provided more MII oocytes than group two monkey (8.5 oocytes/monkey). However, the ratio of MII stage from both groups was not significantly difference. These results suggesting that the effect of superstimulation hormones oocytes donor become older.

Table 3.2 The average number of recovered oocyte per monkey after superstimulation.

Monkey Age	Average No. oocyte collection	GV (%)	MI (%)	MII (%)
< 8 Years old	42.8	7.7 (18.1)	11.8 (27.5)	23.2 (54.4)
> 8 Years old	15.8	2.2 (13.7)	5.2 (32.7)	8.5 (53.7)

3.4.2 Effect of activation protocol on monkey parthenogenetic embryos development

In the early stage of this experiment, MII oocytes were used for parthenogenetic activation to test the suitable activation protocol which will be applied to reconstructed embryos later on. The results showed that the cleavage rates of PA-4 and PA-5 derived embryos were not significant different (95.0% and 94.7%, respectively). The development of embryos to 8 cell stage in PA-5 was significantly higher than those in PA-4 (94.4% and 78.9%, respectively; Table 3.3). Moreover, the PA-5 produced embryos could reach blastocyst with significantly higher rate than PA-4 derived embryos (75.0% and 47.4%, respectively). These results clearly demonstrated that PA-5 is more efficient than PA-4 in term of embryo development to blastocyst. Therefore, the PA-5 protocol was used as a protocol for activation of monkey reconstructed embryos.

3.4.3 Monkey SCNT derived embryos development

The MII oocytes were used as recipient cytoplasm for monkey SCNT embryo production. After fusion, the reconstructed embryos were kept in TALP-HEPES medium for 2h prior to activation with ionomycin and 6-DMAP for 5h. Prior to this experiment, some reconstructed embryos were kept in TALP-HEPES medium for 1h before activation,

Table 3.3 The effect of activation protocol on monkey parthenogenetic embryos development.

Treatment	No. embryo	Cleavage (%)	No (%) embryos developed to			
			8-C	Mor	ComMor	Blast
PA-4	20	19 (95.0)	15[a] (78.9)	15 (78.9)	12 (63.1)	9[a] (47.4)
PA-5	19	18 (94.7)	17[b] (94.4)	16 (88.9)	15 (83.3)	12[b] (66.7)

[a,b] indicate significant difference between column at P<0.05

Abbreviation 8-C: eight cell stage embryo,

Mor: morular stage embryo,

ComMor: compacted morular stage embryo.

Blast: blastocyst

Table 3.4 Monkey SCNT derived embryos development

Treatment	No. embryo	Cleavage (%)	No (%) embryos developed to			
			8-C	Mor	ComMor	Blast
SCNT 2-5	23	21[a] (91.3)	18[a] (85.7)	13[b] (61.9)	9[b] (42.9)	5[b] (23.8)
PA-5	19	18[a] (94.7)	17[a] (94.4)	16[a] (88.9)	15[a] (83.3)	12[a] (66.7)
ICSI	20	20[a] (100.0)	19[a] (95.0)	14[ab] (70.0)	14[a] (70.0)	12[a] (60.0)

[a,b] indicate significant difference between column at P<0.05

however these embryo did not develop to balstocyst stage (data not show). The SCNT, PA-5 and ICSI derived embryos development rates (Figure 3.3) were shown in Table 3.4. After *in vitro* culture, cleavage rates of each treatment were not significantly different. Embryos developed to 8 cell stage of PA-5 (17/18, 94.4%) and ICSI (19/20, 95.0%) was higher than those from SCNT group (18/21, 85.7%). Additionally, PA-5 (15/18; 83%) and ICSI (14/20; 70%) derived compact morulae were significantly higher than those generated by SCNT (9/21; 42.9%). Furthermore, blastocyst rate from ICSI (12/20; 60%) and PA-5 (12/18; 66.7%) were significantly higher than those from SCNT (5/21; 23.8%).

3.5 Discussion

The results of this experiment suggesting that group one monkey (< 8 years old) produced higher number of oocytes than group two (>8 years old). However, proportion of GV, MI and MII was not different between the two groups of monkey. The PA embryos were initially produced in this experiment to test the activation protocols. The results showed that incubation of the monkey MII oocytes with ionomycin and 6-DMAP for 5h (PA-5) could significantly increase the embryo development to blastocyst when compare with 4h activation. Then the 5h activation protocol was applied to reconstructed embryos in later experiment. The SCNT, PA and ICSI derived embryos could develop to blastocyst with different rates. The SCNT and PA-5 embryos development rate from cleavage to morula stage were not significant different from those ICSI derived embryos. The blastocyst from SCNT (23.8%) were significantly lower than embryo from PA-5 (66.7%) and ICSI derived blastocysts (60.0%).

Many factors affect the success of animal cloning procedure such as activation protocols (Shin et al., 2001; Akagi et al., 2003; Choi et al., 2004;), ages of recipient oocytes (Takano et al., 1993; Tanaka and Kanagawa, 1997; Shen et al., 2008), and activation agents (Tanaka and Kanagawa, 1997). Several reports indicated that aged oocytes have higher

activation rates but lower developmental potential than those younger oocytes (Presicce and Yang, 1994; Yang et al., 1993; Takano et al., 1993; Tanaka and Kanagawa, 1997). Study in human also found that age of woman affects the number of retrieved oocytes (Cohen et al., 1999; Ng et al., 2003; Wong et al., 1996; Zhen et al., 2008). In human younger donor oocyte provides more oocytes per retrieval than older woman (Wong et al., 1996; Ng et al., 2003). Number oocytes retrieval decrease in ageing woman is probably due to the lower number of primordial follicles (Block, 1952; Ng et al., 2003). Normally, the cyclic development of follicles is controlled by a sequence of hypothalamic-pituitary-ovarian interactions and angiogenesis is an important component of both the follicular and luteal phase of an ovarian cycle. The study of aged donor oocyte found that FSH level increased in early follicular phase with advancing age by a reduced inhibin mediated feedback towards the pituitary gland (Abulafia and Sherer, 2000). The results of this study also show that there was correlation between aged of oocytes donor and number of retrieval oocytes in rhesus monkey.

Donor nuclear exposure to the egg cytoplasm is critical to the reprogramming processes. In bovine, sheep and mice, the relationships between the timing of nuclear exposure to cytoplasm and the outcome of embryos development *in vitro* have been studied. The studies suggested that delayed activation could improve developmental rates of cloned embryos (Campbell et al. 1996; Stice et al. 1996; Wakayama et al. 1998; Wells et al. 1999; Shin et al., 2001; Akagi et al., 2003; Choi et al., 2004). However, in monkey SCNT experiment, Niu and colleagues (2008) reported that delayed activation treatment after fusion for 8 and 12h did not improve the formation of blastocyst (Niu et al., 2008). The reason of Niu's result different from other reports (Abramcuk et al., 1977; Mitalipov et al., 2001; Roger et al., 2004; Zhou et al., 2006; Okahara-Narita et al., 2007) might be that the period of delayed, 8 and 12h, is not appropriate period of time.

In our current study, we have shown that monkey donor nucleus needed to be

exposed to the cytoplasm of recipient cytoplasm for 2h prior to activation. According to the 2h exposure, the monkey SCNT derived embryos could develop to blastocysts stage (23.8%) where as the activation of the reconstructed embryos at 1h after fusion could not reach blastocyst stage (data not show). The results demonstrated that prolonged exposure of donor nucleus with cytoplasm could improve the developmental rate of monkey SCNT embryo, and 2h after fusion is better than 1h to start chemical activation treatment.

3.6 Conclusion

The results of this experiment suggesting that younger monkey (<8 years old) provided more oocytes than older monkey (>8 years old). However, portion of GV, MI and MII were not different with ages of monkey. The PA embryos were initially produced in this experiment to test the activation protocol. The results showed that incubation of the monkey MII oocytes with ionomycin and 6-DMAP for 5h (PA-5) could significantly improve the embryo development to blastocyst when compare with 4h activation. Then the 5h activation protocol was applied to reconstructed embryos in nuclear transfer experiments. The SCNT 2-5, PA and ICSI derived embryos could develop to blastocyst with different developmental rates. The SCNT 2-5 and PA-5 embryos developmental rate from cleavage to morula stage were not significant different from those ICSI control derived embryos. The SCNT 2-5 embryos could develop to blastocyst (23.8%) which is significantly lower than the PA-5 (66.7%) and ICSI (60.0%).

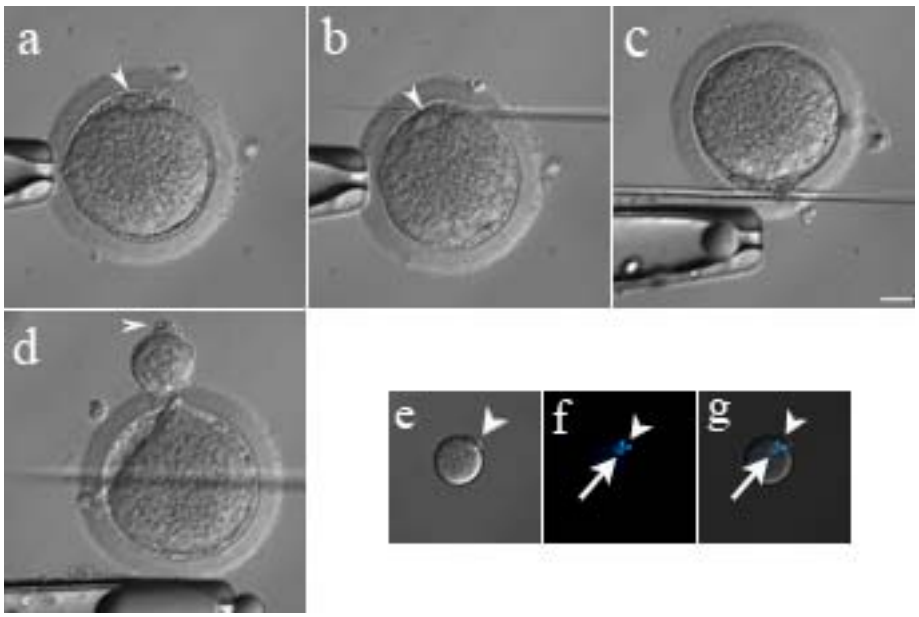

Figure 3.1 Enucleation of MII oocytes. The first polar body of MII oocyte was adjusted to 12 o'clock position (a). The zona pellucida above the first polar body was cut with glass needle (b). Holding pipette was used to help hold the oocyte during cutting zona pellucida (c). Approximately 10% of cytoplast underlying the first polar body was squeeze out of the zona pellucida (d). Confirmation of removal of the metaphase plate by epifluorescent microscopy (e, f, g), merged view (g) hoechst staining (f) and bright field (e). Arrow head showed first polar body. Arrow showed metaphase plate of oocyte. Scale bar: 20 μm.

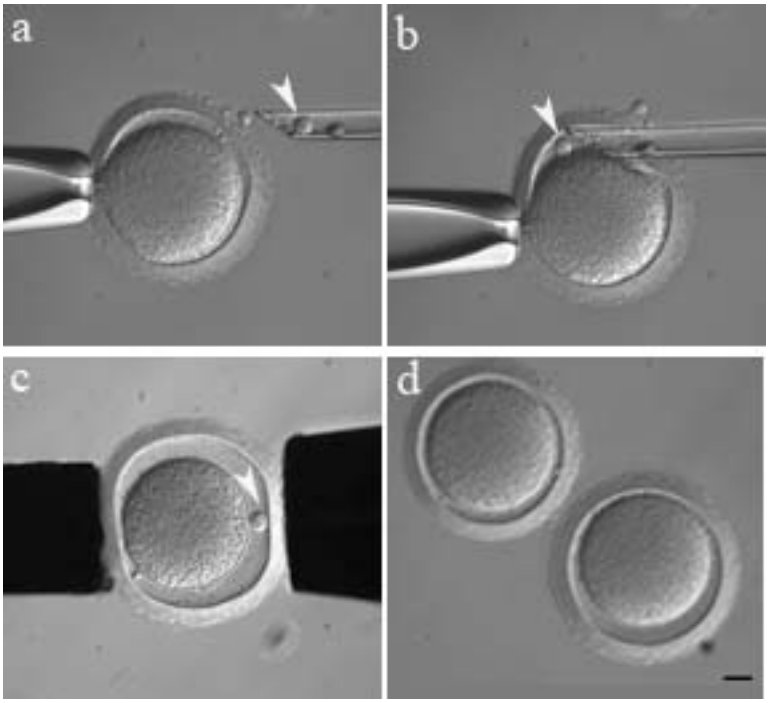

Figure 3.2 Monkey SCNT was performed by micromanipulator using donor cell from miscarried transgenic HD monkey at four months of gestation. Donor cell diameter 14-16 μm was selected and sucked into injecting pipette (a). Each individual donor cell was inserted into perivitelline space of enucleated oocyte (b). Somatic cell-cytoplast complexes (SCCCs) were fused by fusion electrode (c). One hour after fusion, donor cell already fused with oocyte cytoplasm (d). Arrow head showed the position of donor cell. Scale bar: 20 μm.

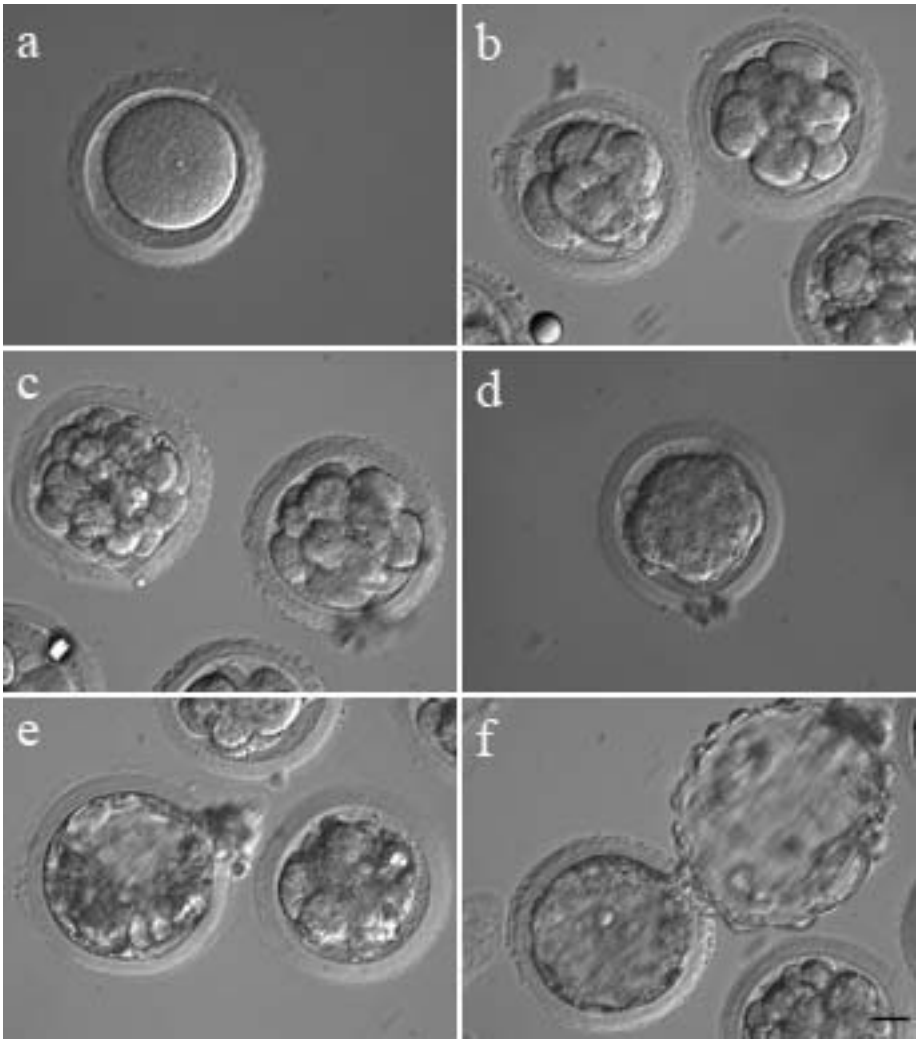

Figure 3.3 The monkey SCNT embryos at 1 cell (a), 8 cell (b), morula (c), compacted morula (d), blastocyst (e) and hatching blastocyst stage (f). Scale bar: 20 μm.

3.7 References

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CHAPTER IV

ESTABLISHMENT OF RHESUS MONKEY ES CELLS FROM WHOLE AND PARTIAL DISSECTED EMBRYOS CULTURE

4.1 Abstract

Transgenic Huntington (ICSI-HD), Alzheimer (ICSI-AD), non-transgenic (ICSI-WT), SCNT and PA derived rhesus monkey embryos were used to establish nhpESC lines. Expanded and hatching blastocysts derived from those embryos were used as whole and partial dissected embryos culture. In this experiment, twelve nhpESC lines were established from 21 partial dissected embryos (57.1%). Among these, 7 nhpESC lines were established from 14 (50%) outgrowths derived transgenic embryos, 2 nhpESC lines from 3 (66.7%) outgrowths derived from ICSI embryos, 2 nhpESC lines from 2 (100%) outgrowths derived from PA embryos and 1 nhpESC line from 2 (50%) outgrowths derived from SCNT embryos. However, only one out of 12 (8.3%) outgrowths could develop with distinctive morphologies of monkey ES cells. These results demonstrated that the source of embryo production had no effect on nhpESC establishment. Mechanical isolation of the ICM by partial dissected embryo proved to be an effective way to derive new nhpESC lines rather than whole embryo culture. Moreover, the partial dissection technique is also benefit in avoiding pathogen contamination.

4.2 Introduction

The blastocyst contains trophectodermal (TE) and inner cell mass (ICM) cells.

TE cells will develop to be placenta after the blastocyst implant to endometrium layer of the mother uterus. ICM cells will develop to be three embryonic germ layers and yolk sac. The three embryonic germ layers incorporated into a fetus, eventually. In another word, the ICM could be differentiated into three embryonic germ layers of the fetus. Therefore, the ICM is the most wanted source for ES cells establishment. ICM could only be observed at blastocyst stage embryo. Good quality blastocysts with distinct ICM cells are the most appropriated for ES cell establishment. Almost all of the ES cell lines established from ICM cells of blastocyst embryos since 1981 (Evans and Kaufman., 1981; Martin, 1981). Immunosurgery is one of the most favorite technique for ES cell establishment in mouse (Evans and Kaufman, 1981; Martin, 1981), sheep (Handyside et al., 1987; Tsuchiya et al., 1994; Dattena et al., 2006), mink (Sukoyan et al., 1992), cattle (Saito et al., 1992), rabbit (Neimann & Strelchenko, 1994), pig (Gerfen & Wheeler, 1995; Shim et al., 1997; Moore and Piedrahita, 1997; Chen et al., 1999; Ock et al., 2005; Brevini et al., 2005; Shiue et al., 2006), rhesus monkey (Thomson et al., 1995; Mitalipov et al., 2006; Byrne et al., 2007), cynomolgus monkey (Suemori et al., 2001; Vrana et al., 2003), baboon (Simerly et al., 2009) and human (Thomson et al., 1998; Shamblott et al., 1998; Reubinoff et al., 2000; Cowan et al., 2004; Mateizel et al., 2006). Immunosurgery is the most effective technique for ICM cells isolation and labor less (Solter and Knowles, 1975). However, this technique needs to use animal products in the process. Therefore, this is a risky point for pathogen contamination especially when this technique is used to establish human ES cell lines. The whole embryo culture and partial dissected embryo culture technique seem to be advantageous to produce pathogen-free ES cell lines especially human ES cells, because of the absence of animal products such as antibody and complement which used in immunosurgery method. Whole blastocyst culture has been used for ES cell establishment since 1981 (Evans and Kaufman, 1981). This technique has been used in particularly with the animals that have a potential to be used for therapeutic studies such as pig (Piedrahita et

al., 1990; Evans et al., 1990; Hochereau-de-Reviers and Perreau, 1993; Gerfen and Wheeler, 1995; Miyoshi et al., 2000; Li et al., 2004; Kim et al., 2007) and human embryo itself (Heins et al., 2004, Kim et al., 2005).

The objectives of this study were to establish the mechanical ICM isolation technique and to determine the effects of source of embryos on nhpESCs establishment. Here, we introduce a new ICM isolation technique which is suitable to use as a tool for monkey ES cell lines establishment by using partial dissection embryo technique. The partial dissection technique presented here should provide as a useful tool toward the establishment of ES cell lines.

4.3 Materials and methods

4.3.1 Establishment of rhesus monkey fibroblast cell line

Monkey fibroblasts cell preparation using the same protocol as in chapter 3.

4.3.2 Rhesus monkey ovarian stimulation and oocytes collection

Cycling female monkeys were superovulated and collected using the same protocol as in chapter 3.

4.3.3 Somatic cell nuclear transfer

Monkey SCNT were using the same protocol as in chapter 3.

4.3.4 Parthenogenetic activation (PA) and embryo culture

Two hours after fusion, the reconstructed embryos were activated by 5 µM Ionomycin (Io) in TALP-HEPES medium for 5 minute and subsequence incubated in 2 mM 6-Dimethylaminopurine (6-DMAP) in HECM-9 for 5 h in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37˚C. The reconstructed embryos were culture in HECM-9 for 8 days in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37˚C. Embryos developments were daily recorded.

4.3.5 Semen preparation and intracytoplasmic sperm injection (ICSI)

Monkey semen preparation and ICSI were using the same protocol as in chapter 3.

4.3.6 ICSI produced transgenic embryos

Lentiviruses carrying foreign gene under the control of human polyubiquitin-C promoter were microinjected into the perivitelline space of monkey metaphase-II-arrested oocytes followed by ICSI and *in vitro* culture. In this experiment, there were two kinds of transgenic embryos produced. First, the oocytes transfected by lentivirus vector carrying GFP and htt gene with 84 CAG repeats (htt-84Q; ICSI-HD) (Yang et al., 2008). Second, oocytes transfected by using lentivirus vector carrying Tau and Amyloid Precursor Protein (APP) gene (ICSI-AD).

4.3.7 Feeder cells preparation

Pregnant mouse at day 13 post coitum were sacrifice by cervical dislocation. The uterine horns were dissected out from the placenta and each embryo were separated. Head and internal organs were remove out and washed several times in PBS to remove blood. The fetus tissues were minced to be small pieces and suspended in Trypsin/EDTA at 37˚C with gentle shaking for 15 min. The Trypsin/EDTA were neutralized with culture medium and transfer to a 50 ml conical tube. The tissues were left to settle down to the bottom of the tube in a few minutes and the supernatant were carefully collected and subjected to low speed centrifugation for 5 min. The cell pellet were then resuspend and culture in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 200 mM L-glutamine (Invitrogen) and 1x Penicillin/Steptomycin (Invitrogen) and plated out in the culture flasks and cultured at 37˚C under humidified atmosphere of 5% CO_2 in air. The medium was changed on the following day to get rid of debris and death cell. At sub-conference, mouse fetal fibroblast cells (MFFs) were trypsinized and cultured. MFFs were frozen at passage 1 or 2 of cell culture. The MFFs were inactivated with 5 µg/ml mitomycin C (Sigma) for two hours followed by a thorough wash before plating.

4.3.8 Blastocyst derivation

Rhesus monkey embryos at expanded and hatching blastocysts stage were selected for ES cell establishment by whole embryo culture or partial dissected embryo. Blastocysts from this experiment were derived from 5 sources; ICSI-WT, ICSI-HD, ICSI-AD, SCNT and PA.

4.3.9 ES cell establishment from partial dissected embryo technique

Expanded blastocysts were selected for partial dissection. The embryos were washed several times in TL-HEPES (Bavister et al., 1983) and placed in a micro-drop of TL-HEPES. Adjust position of ICM region at the position 12 O'clock (Figure 4.1a, b). Fined glass needle was pressed on top of embryo under ICM region (Figure 4.1b). Holding pipette was placed underneath the fined pipette then the holding pipette was moved back and forth several times to tear off the TE cells and ZP (Figure 4.1c, d). The partial embryo with ICM region was washed several times in ES medium before plated on feeder layer. On day 3 after plating, the attachment of the partial dissected embryo to the feeder layer was recorded. Half of the ES medium was replaced by fresh medium every other day. The outgrowth morphology was daily observed for 10 to 14 days before mechanically sub-culture.

4.3.10 ES cell establishment by whole embryo culture

Zona pellucida (ZP) of blastocysts was removed before plated on feeder layer. An individual blastocyst was cultured on freshly prepared MFFs feeder layer in monkey ES medium composed of knockout-Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen), 1 mM glutamine, 1% non-essential amino acids and 4 ng/ml of human basic fibroblast growth factor (bFGF; Chemicon, Inc.) at 37˚C under humidified atmosphere of 5% CO_2 in air for 10-14 days. Half volume of the ES medium was changed every other day. On day 2 after plating, the attachment of the whole embryo to the feeder layer was recorded. The outgrowth

morphology was daily observed before mechanically sub-culture.

4.3.11 Establishment and maintenance of monkey ES cell

Outgrowths from both whole and partial dissected embryo that showed ES cells morphology were mechanically sub-cultured. Only the distinctive ES cells morphology were mechanically dissociated into small pieces and transferred to freshly prepared MFFs feeder layer which pre-incubated with ES medium. Two days after sub-culture, ES cells morphology were observed. The embryonic outgrowths were daily monitored for about two weeks. Only the colonies that exhibited distinct monkey ES-like cells morphology were selected for further sub-culture.

4.3.12 Embryoid body formation

ES cells colonies were mechanically detached from feeder cells and carefully transferred into 35 mm non attachable dish containing ES cells culture medium. Half of the medium was replaced every other day. The detached ES colonies were cultured for 7 days.

4.3.13 Immunocytochemistry for ES cells markers

ES medium was removed and the ES cells were washed several times in PBS without Ca^{2+} and Mg^{2+}. ES cells were fixed with 4% paraformaldehyde solution at room temperature for 30 min and washed several times with PBS. Alkaline phosphatase staining was done by using an alkaline phosphatase staining kit (Vector® Blue Alkaline Phosphatase Substrate Kit III; SK-5300). For cell membrane markers, SSEA-3, TRA-1-60 and TRA-1-81, the ES cells and embryoid bodies were blocked in PBS with 4% goat serum at room temperature for 1 h. Then, sample were subsequently incubated with the following primary antibodies: rat monoclonal antibody anti-SSEA-3 (1:200 dilution, Chemicon, MAB 4303), mouse monoclonal antibody anti-TRA-60 (1:200 dilution, Chemicon, MAB 4360), mouse monoclonal antibody anti-TRA-81 (1:200 dilution, Chemicon, MAB 4381). For nuclear markers, ES cells were permeabilized by 0.2% Triton-X and 0.1% Tween 20 for 1 h. Then incubated with primary nuclear antibody as mouse monoclonal antibody anti-Oct3/4 (1:200

dilution, Santa cruz biotechnology, inc., SC-5279), Mouse anti Alpha-Fetoprotein (1:200 dilution, Sigma, A8452), Goat anti Vimentin (1:20 dilution, Sigma, A4630). The ES cells were incubated with primary antibody overnight at 4˚C. After washed out the exceed primary antibody, ES cells were incubated with appropriate secondary antibody conjugated with either Alexa Flour 488 (1:1000 dilution, Invitrogen) or Alexa flour 594 (1:1000 dilution, Invitrogen) for 1 h. ES cells were co-stained with Hoechst 33342 for 5 min and then examined under fluorescence microscopy.

4.3.14 PCR analysis

Single ES cell colony was collected for genomic DNA extraction. Genomic DNA was extracted by using K-buffer. DNA quality was examined by BioPhotometer (Eppendorf). Amyloid Precursor Protein (APP) gene was detected by Ubiquitin-F1 forward primer (5'-GAGGCGTCAGTTTCTTTGGTC-3') and APP-R1 reverse primer (5'-GCAAACATCCATCCTCTCCTG-3'). The expect PCR product size is 678 bp after amplification of genomic DNA from single ES cell colony from transgene APP ES cell. PCR was accomplished with first 94˚C for 3 min then 35 cycles of 94˚C for 30 sec, 62˚C for 30 sec and 72˚C for 40 sec. The last cycle was followed with 72˚C for 5 min. The Human-Tau gene in APP nhpES cell was detected by Ubiquitin-F1 forward primer (5'-GAGGCGTCAGTTTCTTTGGTC-3') and hTau-R1 reverse primer (5'-TCTTTCCTGTCCCCCAACCC-3'). The expected PCR product size is 322 bp. The Ubiquitin-F1 forward primer (5'GAGGCGTCAGTTTCTTTGGTC-3') and hHD-Exon1-R reverse primer (5'-TGAGGAAGCTGAGGAGGCGG-3') were used to detect htt gene. The expect PCR product size is 586 bp. PCR was amplified with first 96˚C for 5 min then 35 cycles of 96˚C for 45 sec, 63˚C for 45 sec and 72˚C for 1 min. The last cycle was followed with 72˚C for 5 min. GFP gene was detected by FUGW-EGFP-F1 forward primer (5'-TTCAAGGACGACGGCAACTAC-3') and FUGW-EGFP-R1 reverse primer (5'-TAGTGGTTGTCGGGCAGCAG-3'). The expected PCR product size is 302 bp. PCR was

amplified with first 94˚C for 3 min then 35 cycles of 94˚C for 25 sec, 62˚C for 25 sec and 72˚C for 40 sec. The last cycle was followed with 72˚C for 5 min.

4.3.15 Statistical analysis

The analysis was performed by using the Statistical Analysis System Software (SAS, version9.0; SAS, Cary, NC, USA) and value of $P<0.05$ was considered as significantly difference.

4.4 Results

4.4.1 Outgrowths of ES like cells from partial embryo dissection

Partial dissected embryos with ICM and small amount of TE cells were cultured on MFFs feeder layer. On day 3 to 4 after cultured ICM and TE outgrowths were started to spread on the feeder layer (Figure 4.2). TE cells outgrowth started to growth on feeder layer faster than ICM outgrowth. However, after 7 days of cultured, ICM outgrowth expanded dramatically faster than the TE cell outgrowth. Overgrowth of TE cell tends to inhibit ICM outgrowth proliferation. Partial dissected embryo technique could reduce the overgrowth of TE cell because most of the TE cells were discarded by the fined glass pipette before plating on the feeder layer. However, from this experiment 2 out of 7 had the TE cells overgrown and finally these two outgrowths were not able to develop to be ES cells. Total 30 partial dissected embryos were cultured (Table 4.1), only 23 (23/30; 76.7%) embryos attached to the feeder layer. However, 21 out of 23 embryos (21/23; 91.3%) could further proliferate. After 10 to 14 days of cultured, ICM outgrowths were mechanically sub-cultured. The ICM outgrowths were cut into small clumps of cells using fined glass pipette and transferred to freshly prepared feeder layer pre incubated with ES medium. After several sub-culture, 12 ICM outgrowths (12/23; 57.1%) showed ES like cells morphology and no TE cells were found.

4.4.2 Outgrowths of ES like cells from whole embryo culture

The expanded blastocyst were removed ZP and then cultured on feeder layer in ES medium. Embryos were attached onto feeder layer 2 to 3 days after cultured (Figure 4.3). Twelve embryos were plated onto the feeder layer and all of them were able to attach and grow out (Table 4.1). Both ICM and TE outgrowths continued to grow through day 7 of *in vitro* culture, but only one out of 12 colonies (1/12; 8.3%) shows a very clear ICM outgrowth which was able to be passage. During day 10 to 14 after culture, the outgrowth was mechanically divided into small clump of cells. ES like cells were established from only one out of 12 outgrowths. Even though, the whole embryo culture technique exhibits the overgrowth of TE cells higher than the partial dissected embryo technique in the initial period of culture (about 7 days). But, after a certain point of time when the ICM outgrowth is big enough, the size of ICM outgrowth dramatically proliferated faster than the TE cells (Figure 4.3e, f, g).

Table 4.1 Establishment of ES cells from partial embryo dissection and whole embryo culture.

Type of embryo	No. culture	No. (%) attached on feeder layer	No. (%) Outgrowth	Passage number	
				1-5 (%)	> 5 (%)
Partial dissected embryo	30	23[b] (76.7)	21[b] (91.3)	12[a] (57.1)	12[a] (57.1)
Whole embryo	12	12[a] (100)	12[a] (100)	1[b] (8.3)	1[b] (8.3)

[ab] Values with different superscripts in each column are significantly different ($p < 0.05$).

4.4.3 Partial dissected embryo method improved ES cell establishment rate of NHP embryos derived from ICSI, SCNT and PA

Expanded blastocysts were produced by parthenogenetic activation (PA; 5 embryos), somatic cell nuclear transfer (SCNT; 2 embryos), tetraploid (2 embryos), ICSI wild type (ICSI-WT, 4 embryos), transgenic htt and GFP gene derived ICSI (ICSI-HD, 8 embryos) and transgenic Tau and APP gene derived ICSI (ICSI-AD, 9 embryos). ES cell establishment rate of partial dissected embryos were shown in Table 4.2. Four PA embryos attached onto feeder layer (4/5; 80%) among those two outgrowths were observed (2/4; 50%). Those two outgrowths proliferated and showed clear ICM outgrowths (2/2; 100%). After mechanical sub-cultured, both outgrowths showed distinct ES like cells morphology. Both cell lines were confirmed for their ES cells properties by ES cells markers expression analysis. The results showed that both ES cells lines expressed all common ES cells markers including Oct3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 4.4.1). After EBs formation, the EBs were stained with endodermal marker (AFP) and mesodermal marker (vimentin; Figure 4.4.2) to confirm the *in vitro* differentiation ability. The nhpESC lines derived from PA were named as PAES1 and PAES2.

During enucleation procedure a slit had been made at the zona pellucida. Therefore, SCNT embryos, at blastocyst stage, normally started the hatching blastocyst stage earlier than PA and ICSI derived embryos. Late hatching blastocyst stages of SCNT embryos were selected for partial dissected embryo. After partial dissection, two embryos were plated onto feeder layer, two outgrowths were found (2/2; 100%). However, only one outgrowth showed clear ICM outgrowth morphology (1/2; 50%). The expression of common ES cell markers, Oct-3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 4.5.1) were observed after immunostaining. The expression of AFP and vimentin (Figure 4.5.2) were also detected after EBs formation. The ES cell line derived SCNT was named NrES1. GFP gene was found in this cell line because donor cell was transfected with GFP gene (Figure 4.5.3).

The derivation of ES cells line from tetraploid embryos was not achieved in this experiment. After two ICMs derived from partial dissected embryos were plated onto feeder layer, both ICMs were not able to attach onto the feeder layer. On the other hands, two ES cell lines derived from ICSI wild type (ICSI-WT) were successfully established. Four expanded blastocysts produced by ICSI method were used for partial dissected embryo. The result showed that 3 out of 4 embryos (75%) were able to attach onto the feeder layer and further proliferated (3/3; 100%). However, the two outgrowths of ICM were observed (2/3; 66.7%) whereas another one ICM showed only TE outgrowth morphology. After mechanical sub-cultured, two ES cell lines were established and both cell lines expressed of Oct3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 4.6.1). The expression of AFP and vimentin (Figure 4.6.2) were detected after immunostaining of EBs. Both ES cell lines from ICSI were named as YRES5 and YRES6.

After partial dissection had been performed on eight embryos derived from ICSI-HD transgenic embryos. Six out of eight embryos (75%) were able to attach onto the feeder layer. From these, three outgrowths were observed and after several sub-culture, all three ES cell lines expressed common ES cell markers and also AFP and vimentin (Figure 4.7.1) were expressed after EBs formation. The successes of transfection with htt gene by lentivirus vector were verified by PCR (Figure 4.7.2). Three transgenic ES cell lines were named as HDrES1, HDrES2, HDrES3, respectively.

After partial dissection has been performed on nine embryos from ICSI-AD derived transgenic embryos, eight embryos (8/9; 88.9%) were able to attach and further proliferate on the feeder layer. Among these, four out of eight ICMs (50%) showed clear ICM outgrowth morphology and after several sub-culture, all ES cell lines expressed Oct3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 4.8.1) and also AFP and vimentin (Figure 4.8.2) were expressed after EBs formation. Transgenic AD-ES cell lines were named as ADrES1, ADrES2, ADrES3 and YMRS15, respectively. The success of double transfection with

Table 4.2 ES cell established from partial embryo dissection using different source of embryo.

Source of Embryo	No. culture	No. (%) attached on feeder	No. (%) outgrowth	Passage number	
				1-5 (%)	> 5 (%)
PA	5	4 (80)	2 (50)	2 (100)	2 (100)
SCNT (Diploid)	2	2 (100)	2 (100)	1 (50)	1 (50)
ICSI-WT (Wild type)	4	3 (75)	3 (100)	2 (66.7)	2 (66.7)
ICSI-HD (Transgenic)	8	6 (75)	6 (100)	3 (50)	3 (50)
ICSI-AD (Transgenic)	9	8 (88.9)	8 (100)	4 (50)	4 (50)

PA: Parthenogenetic activation SCNT: Somatic cell nuclear transfer

HD: Huntington's disease AD: Alzheimer's disease

ICSI: Intracytoplasmic sperm injection

APP and Tau gene by lentivirus vector were confirmed by PCR. The PCR products showed that ADrES-1, ADrES-2 and ADrES3 carried both the APP and the Tau genes whereas YMES15 carried only the Tau gene (Figure 4.8.3).

4.4.4 Whole embryo cultured technique effects the success rate of ES cell establishment

Success rate of ES cell established by whole embryos cultured technique was are

shown in Table 4.3. Twelve expanded blastocysts were produced by 4 different techniques, PA (2 embryos), SCNT (6 embryos), tetraploid (1 embryo) and ICSI (3 embryos). The zona pellucida free blastocysts from all groups were cultured onto the feeder layer. The result showed that the embryos from every group were able to attach onto the feeder cells and the embryos flatten down onto the feeder layer. At the beginning of cultured, TE outgrowths proliferated faster than the ICM cells outgrowth. Therefore, there was only TE outgrowth found in all groups. However, ICM outgrowth was found in tetraploid derived embryos (1/1; 100%). After common ES cell markers characterization indicated that ES like cell showed positive to all markers, Oct3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 4.9.1). AFP and vimentin (Figure 4.9.2) expression were found after EBs formation. Tetraploid ES cell line was named as TrES-1. To confirm the successful of nuclear transfer,

Table 4.3 ES cell established from whole embryo cultured method using different source of embryo.

Source of embryos	**No. culture**	**No. (%) attached on feeder**	**No. (%) outgrowth**	**Passage number**	
				1-5 (%)	**> 5 (%)**
PA	2	2 (100)	2 (100)	0	0
SCNT	6	6 (100)	6 (100)	0	0
Tetraploid	1	1 (100)	1 (100)	1 (100)	1 (100)
ICSI-WT	3	3 (100)	3 (100)	0	0

the present of htt gene (Figure 4.9.3) was verified from both TrES1 (ES cell line) and rHD-5 (fibroblast cells donor).

The identical htt gene was confirmed by using HD specific primers. PCR results showed that TrES1 had the same PCR product as skin fibroblasts derived from transgenic htt and GFP gene rhesus monkey, rHD-5 (Figure 4.9.3).

4.5 Discussion

This study introduced the efficient technique to establish ES cell lines from transgenic NHPs embryos produced by SCNT and ICSI methods. The comparisons of two methods, partial dissected embryo and whole embryo cultured, have been done. The results found that partial dissected embryo technique is more efficient than the whole embryo cultured technique. In this study, twelve ES cell lines were established by partial dissected embryo technique (Table 4.2) whereas only one ES cell line was established by whole embryo cultured technique (Table 4.3).

After Solter and Knowles (1975) established immunosurgery technique for ICM cells isolation from mouse blastocyst, this technique has been used by several research groups in many different animal species including primate (Martin, 1981; Piedrahita et al., 1990; Chen et al., 1999; Anderson et al., 1994; Thomson et al., 1995, 1998; Moore and Piedrahita, 1997; Byrne et al., 2007). However, immunosurgery is not suitable to apply with low quality embryos (Kim et al., 2005; Byrne et al., 2007). In human ES cell establishment, the possibility that human ES cells could be contaminated with animal pathogens during the antibody and complement treatment is also an issue concerned by many researchers. To overcome the limitations, whole embryo culture or partial dissected embryo culture technique was explore.

In the beginning of this experiment, whole blastocysts cultured were used because of the simplicity of the technique. Whole embryo cultured does not need any special

chemical reagent, technique or expensive instrument. However, the most critical point of the whole embryos cultured technique is the positions of ICM cells during attachment onto the feeder layer. Since ICM cells were often covered with differentiated TE cells resulting in unclear observation of ES cell morphology. The ICM cells might not be able to growth properly and become degenerate or differentiate, eventually (Li et al., 2003). This study found that the embryos float in the ES medium for 2-3 days before settle down to the feeder layer. Therefore, the position of ICM cells was randomly attached on the feeder layer. Moreover, TE overgrowth seems to limit ICM proliferation if the position of ICM cells located at the center of the outgrowth. TrES-1 derived whole embryo cultured in Figure 4.3b was clear evidence that position of ICM in outgrowth is the key point of success in ES cell establishment. ICM cells of TrES1 were located at the edged of outgrowth (Figure 4.3b) and ICM outgrowth was clearly observed. However, if the position of ICM was located deep inside or underneath the TE cells outgrowth, the ICM outgrowth might be limited. However, peeling out of TE cell overgrowth after plating by sharp pulled pasteur pipette able to enhance the efficiency of ICM outgrowth (Kwon et al., 2009).

Partial dissected embryo might accelerate establishment of embryonic stem cell lines from NHPs and other mammals. Especially with the blastocyst containing small or unclear ICM clump. The expanded blastocyst that exhibited small clump of ICM cells has higher risk to lose of their ICM during immunosurgery. Partial dissected embryo technique seem to be a better alternative choice to used in case of the expanded blastocyst has a small clump of ICM cells. The partial dissected embryo technique, unlike the whole embryo cultured technique, TE overgrowth by virtue of surgical isolation of the region in which the ICM is located. This surgical procedure was determined to significantly reduce the risk of the TE overgrowth, which tends to inhibit the growth of the ICM. Partial dissected embryo reduced the TE contamination and allow the ICM to get better attachment directly to the feeder layer. The data from Table 4.1 showed that half of the outgrowths from the partial

dissected embryo cultured contains ICM outgrowth after plating on feeder layer and all become ES cell lines.

The data from Table 4.1 and 4.2 showed that some partial dissected embryos were not able to attach on feeder layer (7/30; 23.3%) and some partial embryos degenerated soon after attached on feeder cell (2/23; 8.7%). The quality of blastocysts affect the partial dissected embryo attachment and proliferation on the feeder layer (Kim et al., 2005). Blastocyst embryos derived from SCNT normally have lower quality than IVF derived embryos (Byrne et al., 2007). Blastocysts derived from tetraploid embryos could not attach on the feeder layer may be due to the embryo quality and small distinct of ICM clump.

In this study, using partial dissected embryo technique, we successfully established seven transgenic nhpESC cell lines (ADrES1, ADrES2, ADrES3, YMES15, HDrES1, HDrES2, HDrES3) derived from transgenic monkey embryos, two nhpESC cell lines from ICSI derived embryos, two nhpESC cell lines from parthenogenetic derived embryos, one nhpESC cell line from SCNT derived embryo and only one transgenic nhpESC cell line established from whole embryo cultured technique. In this study, we did not find the effect of source of embryos on nhpESC cell establishment.

The result of this study clearly demonstrated that ICM cell clump with less TE cells has higher chance to develop to ES cell line. The partial dissected embryo technique is more effective than whole embryo culture because the contamination from TE cell of partial dissected embryo was reduced by mechanical tear out of the TE cells by fine needles dissection (Figure 4.1). However, partial dissected embryo is not recommended for blastocyst that has very small clump of ICM cells, but whole embryo cultured is suggested because it could apply to embryos which unable to clearly visualize the ICM clump. However, the number of TE cells contamination after partial dissected embryos is the critical point of this technique because TE overgrowth tends to inhibit of ICM outgrowth (Lee et al., 2003; Kim et al., 2005).

In our study, we have shown that ES cell establishment technique affects on the nhpESC cell establishment rate. According to nhpESC cell lines establishment, the results clearly demonstrated that partial dissected embryo dramatically enhanced nhpESC cell lines establishment when compare to the whole embryo culture.

4.6 Conclusion

The results of this experiment suggesting that ICM isolation by partial dissected embryo improved the efficiency of nhpESC establishment. Twelve nhpES cell lines were established by partial dissected embryo technique, Seven nhpESC cell lines were established from transgenic embryos (ADrES1, ADrES2, ADrES3, YMES15, HDrES1, HDrES2, HDrES3), two nhpESC cell lines from ICSI derived embryos (YRES5, YRES6), two nhpESC cell lines from parthenogenetic derived embryos (PAES1, PAES2) and one nhpESC cell line from SCNT derived embryo (NrES1) whereas only one nhpESC line was established from whole embryo culture (TrES1). The results demonstrated that embryo production techniques do not affect the ES cell establishment. Moreover, partial dissected embryo could enhance the success rate of ES cell establishment. The results of this experiment clearly demonstrated that technique of ICM cell derivation is crucial for ES cell establishment.

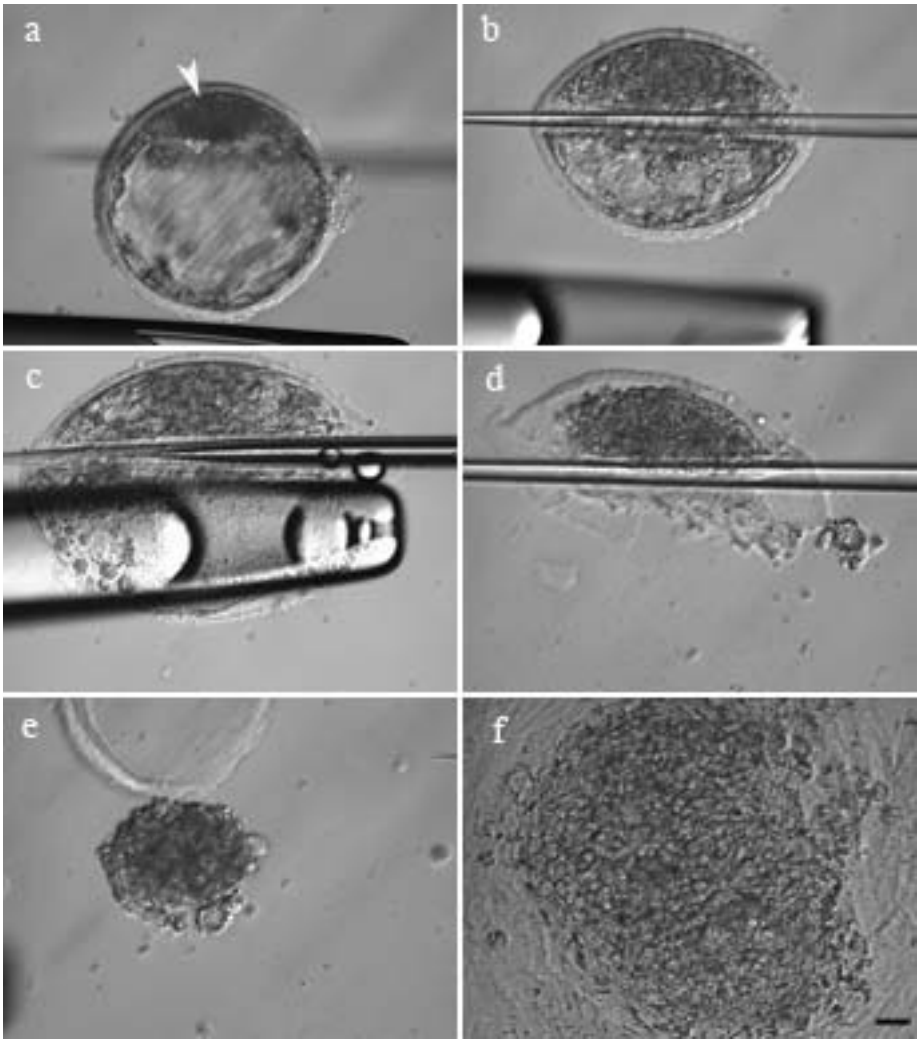

Figure 4.1 Mechanical partial dissected embryos technique. ICM region (arrow head) was arranged to 12 O'clock position (a). Fine glass needle was placed on top of the embryo (b), The TE cells were removed using a holding pipette directly pressed onto the embryo under the ICM cell region and rub the TE cells regions several times to tear off the TE cells (c). Remove the remainder of zona pellucida out and plate the ICM clump on the feeder layer (d and e). After partial dissected embryo, small amount of TE cells remain surrounding the ICM clump (e). ICM outgrowth morphology on day 4 after plated on the feeder layer (f). Scale bar: 20 μm.

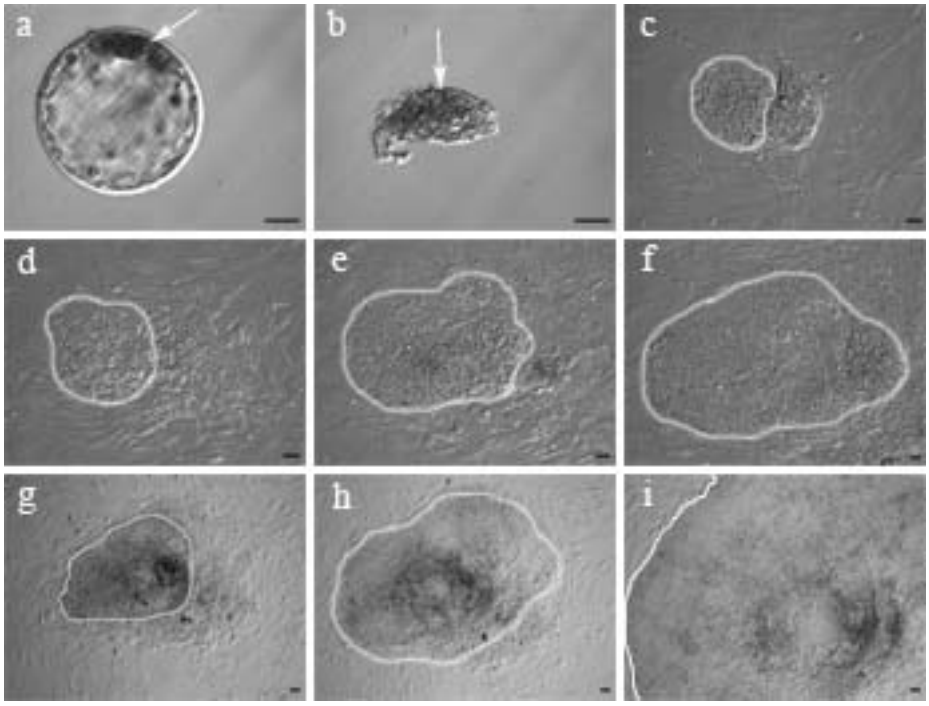

Figure 4.2 The progress of ES cell outgrowth derived from partial dissected embryo of ADrES-1 ES cell line. Expanded blastocyst with a clear ICM clump (arrow) was selected (a). White circle line indicated area of ICM out growth. The ICM clump after partial dissected embryo (b). Day 4 of culture, ICM and TE cells attached and spread out (c). Day 5, ICM clump was tightly observed and formed a circular-like shape (d). Day 6, the ICM outgrowth proliferates faster than the TE cells (e). Day 7, distinctive area of ICM outgrowth was clearly observed (f). Day 8 (g), 9 (h) and 11 (i) showed the edged of ES like cells expanded faster more than two folds in everyday. Scale bar: 20 μm.

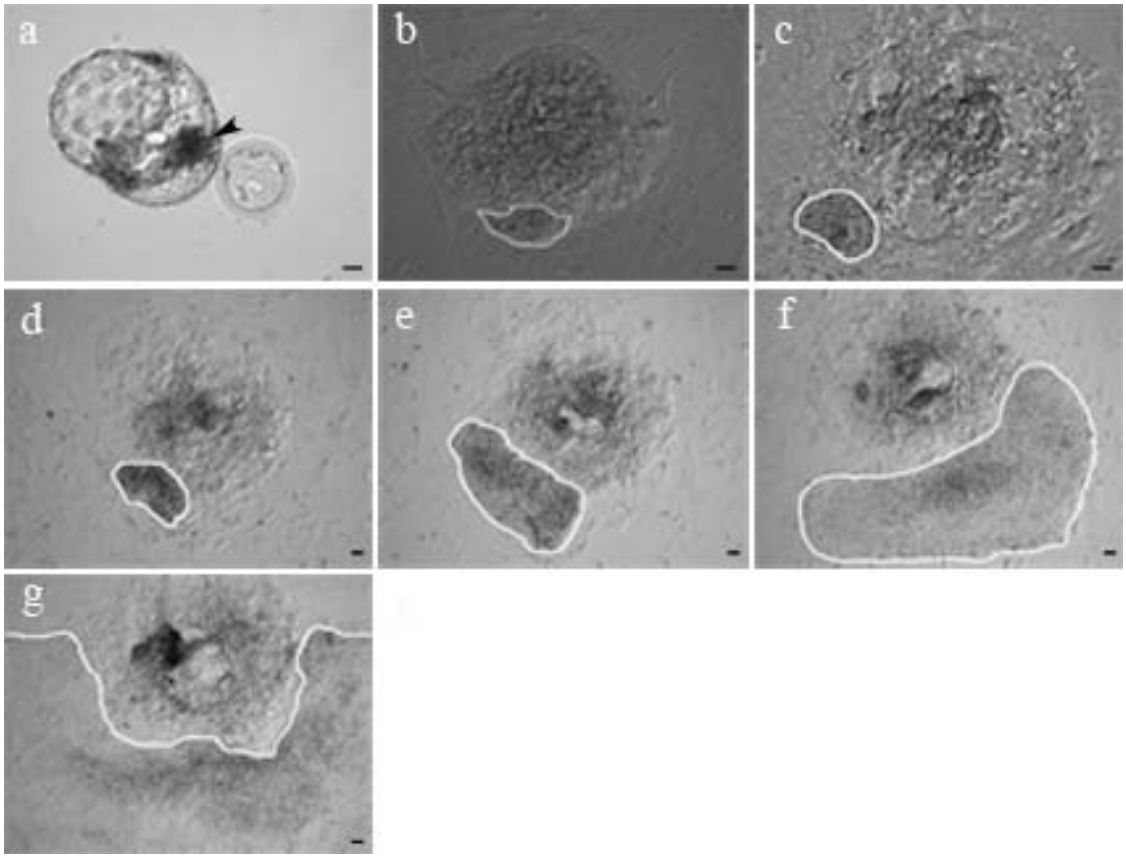

Figure 4.3 The progress of ES cell outgrowth derived from whole embryo culture technique (TrES-1). Expanded blastocyst with a clear ICM clump (arrow head) was selected to culture on the feeder layer (a). White circle line indicated the area of ICM outgrowth. On day 2 of culture, embryo attached and spread on feeder layer (b). ICM clump was tightly observed and formed a circular-like shape on day 4 (c). Day 6, the ICM clump was clearly observed (d). Day 8, ICM outgrowth proliferates faster than TE cells (e). ICM outgrowth on day 9 (f) and day 12 (g) were expanded bigger than two times in everyday. Scale bar: 20 μm.

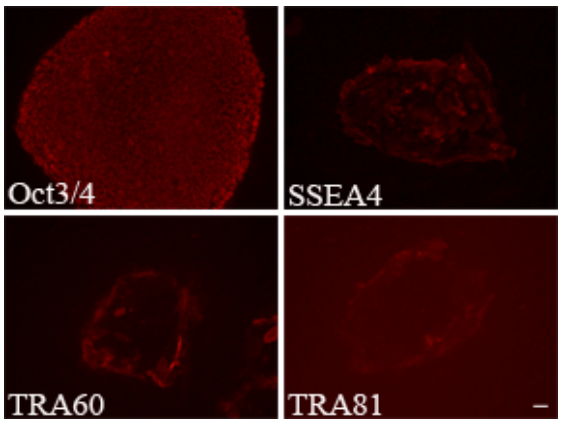

Figure 4.4.1 The expression of Oct3/4, SSEA4, TRA60 and TRA81 of PAES cell line. Scale bar: 20 µm.

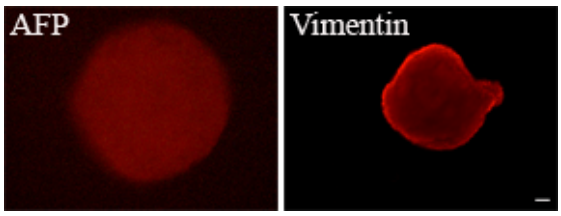

Figure 4.4.2 The expression of AFP and vimentin after embryoid body formation of PAES cell line. Scale bar: 20 µm.

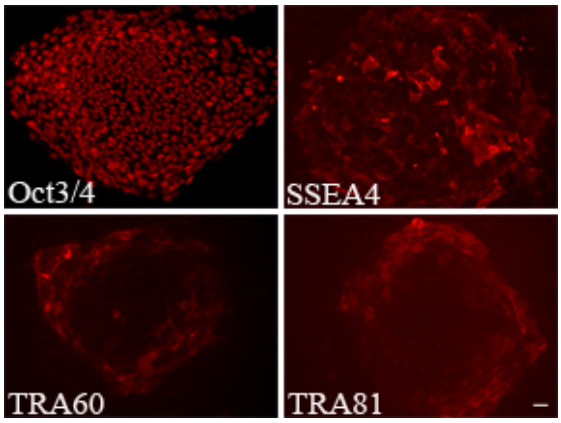

Figure 4.5.1 The expression of Oct3/4, SSEA4, TRA60 and TRA81 of NrES1. Scale bar: 10 µm.

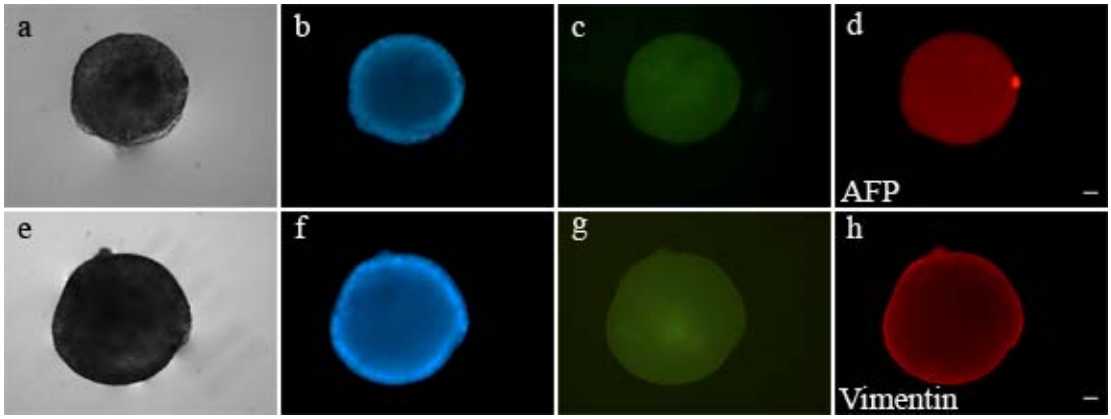

Figure 4.5.2 The expression of AFP (Top panel) and vimentin (Bottle panel) from NrES1 after embryoid body formation. GFP expression were detected under epifluorescence (c, g). Scale bar: 20 μm.

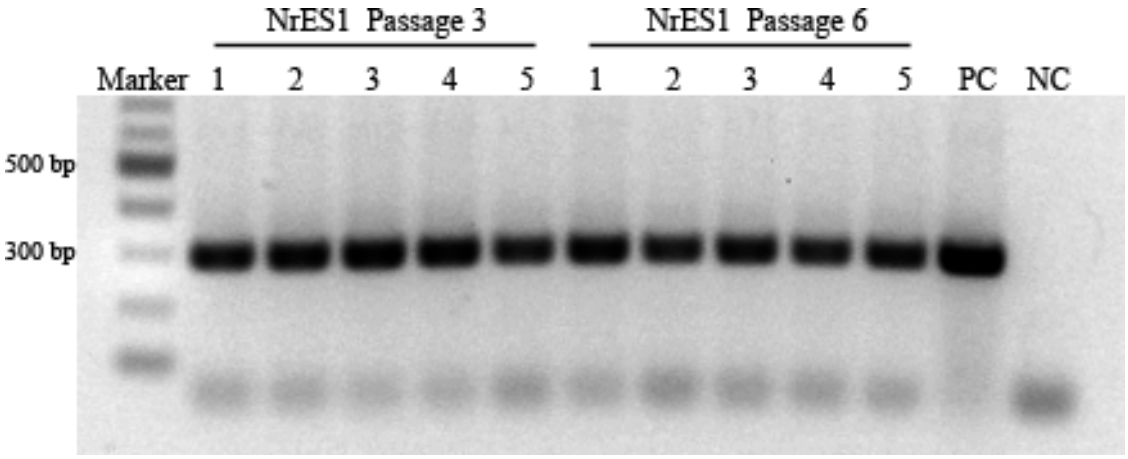

Figure 4.5.3 PCR results of nhpESC NrES1 cells using GFP primer derived. The PCR product of GFP is 302 bp. Positive control (PC) is transgenic monkey skin fibroblast as a donor cell for SCNT. Negative control (NC), No template.

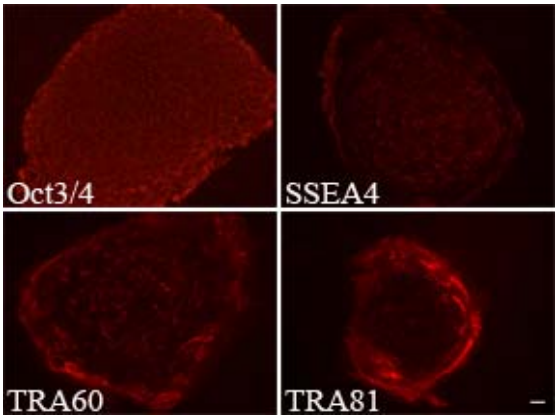

Figure 4.6.1 The expression of Oct3/4, SSEA4, TRA60 and TRA81 of YRES. Scale bar: 20 μm.

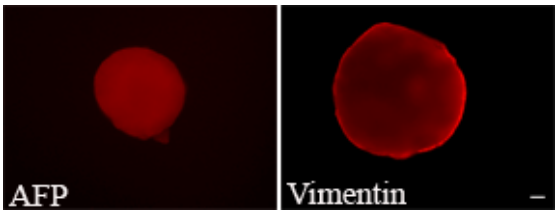

Figure 4.6.2 The expression of AFP and vimentin after embryoid body formation of YRES cell line. Scale bar: 20 μm.

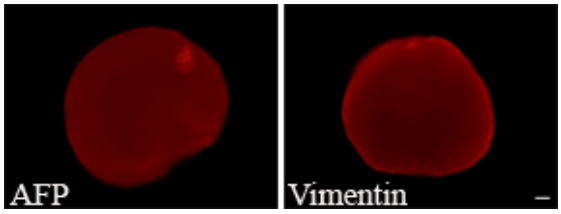

Figure 4.7.1 The expression of AFP and vimentin after embryoid body formation of HDrES cell line. Scale bar: 20 μm.

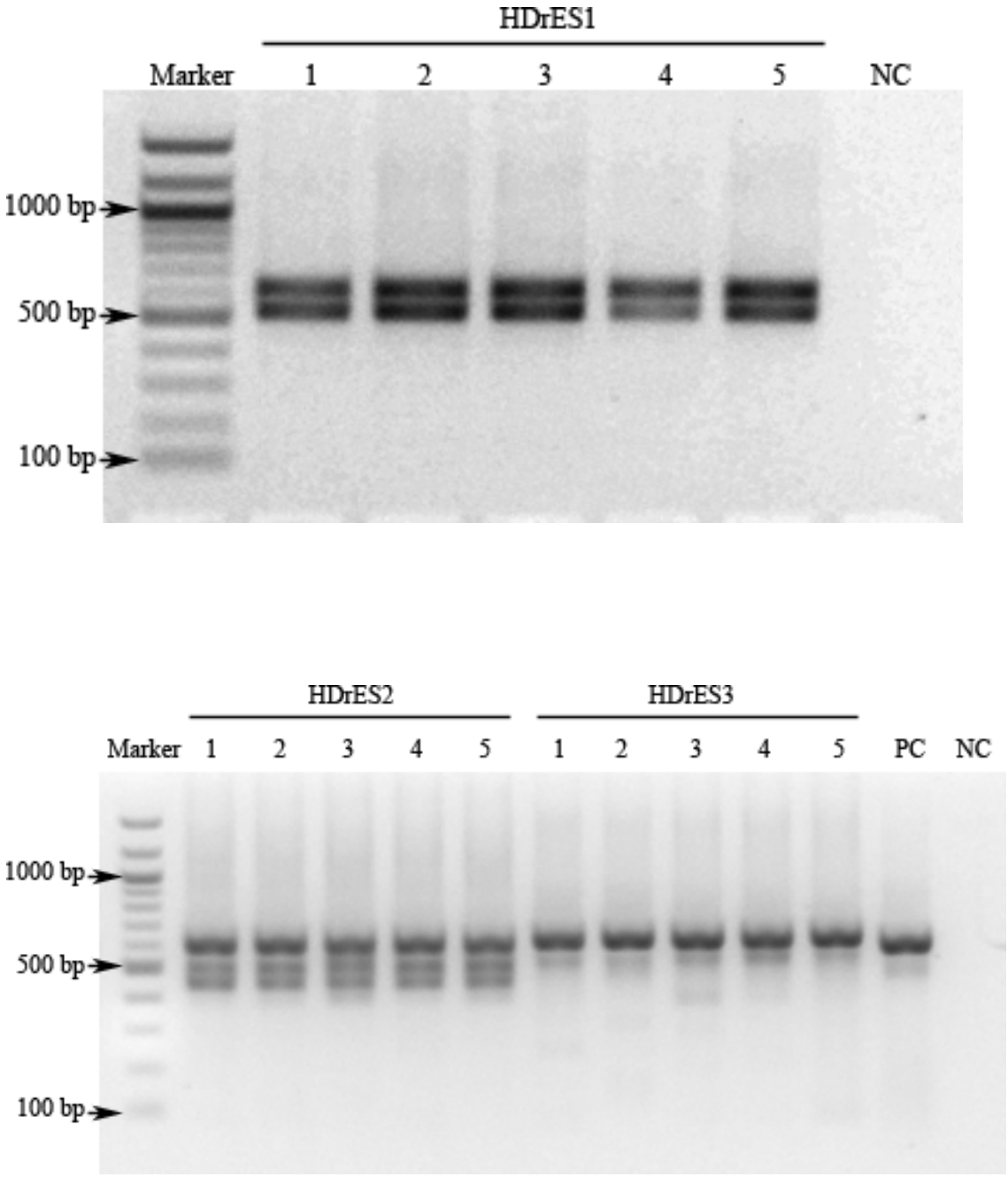

Figure 4.7.2 PCR results of transgenic htt gene from nhpESC HDrES1, HDrES2 and HDrES3. The PCR product of htt gene is 586 bp. Top panel showed the PCR result of HDrES1, with 2 difference poly Q repeat of 87Q and 57Q. The lower panel showed the PCR product of HDrES2 and HDrES3. HDrES2 had 3 difference poly Q repeat of 32Q, 37Q and 79Q. HDrES3 had only 1 poly Q repeat of 83Q.

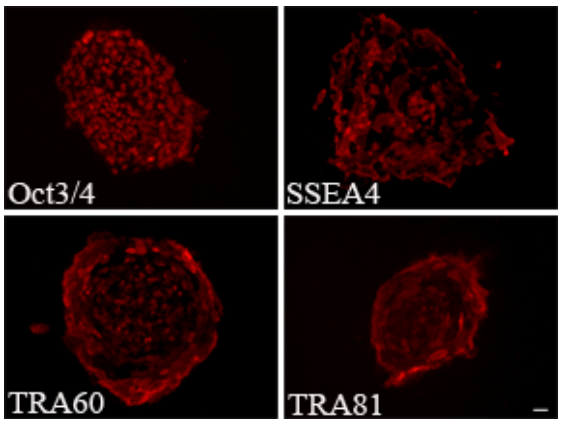

Figure 4.8.1 The expression of Oct3/4, SSEA4, TRA60 and TRA81 of ADrES. Scale bar: 20 μm.

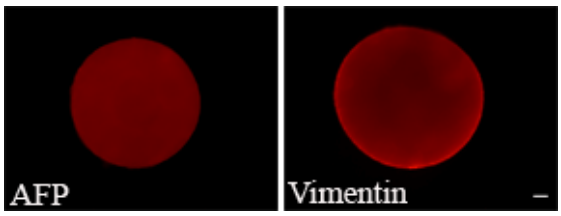

Figure 4.8.2 The expression of AFP and vimentin after embryoid body formation of ADrES cell line. Scale bar: 20 μm.

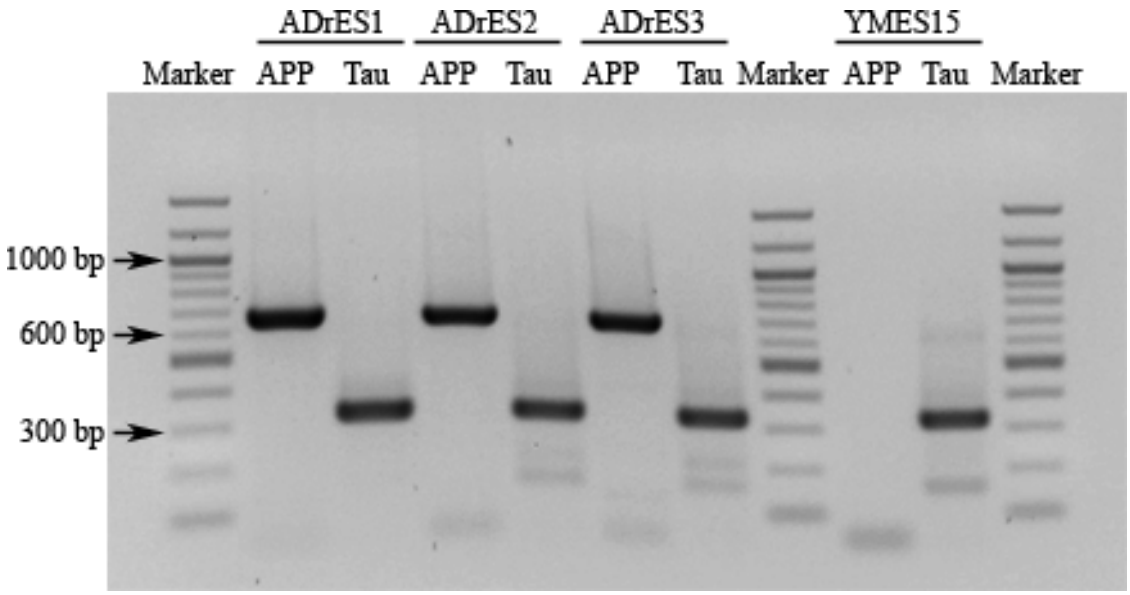

Figure 4.8.3 PCR results of transgenic APP and Tau gene of nhpESC ADrES1,

ADrES2 and ADrES3. PCR product of APP and Tau gene is 678 bp and 322 bp, respectively. Transgene APP by lentivius vector was missing in YMES15. Therefore, only *Tau* gene was detected.

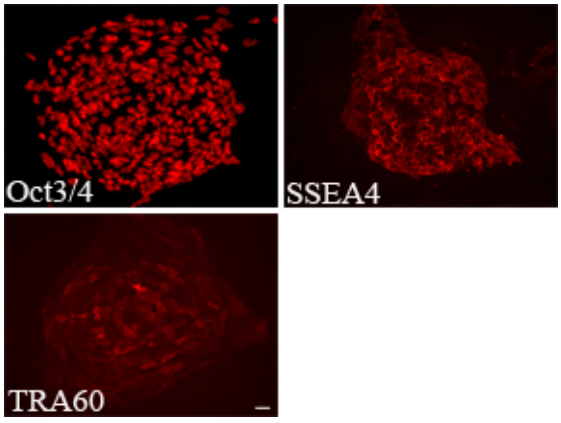

Figure 4.9.1 The expression of Oct3/4, SSEA4 and TRA60 of TrES1. Scale bar: 20 μm.

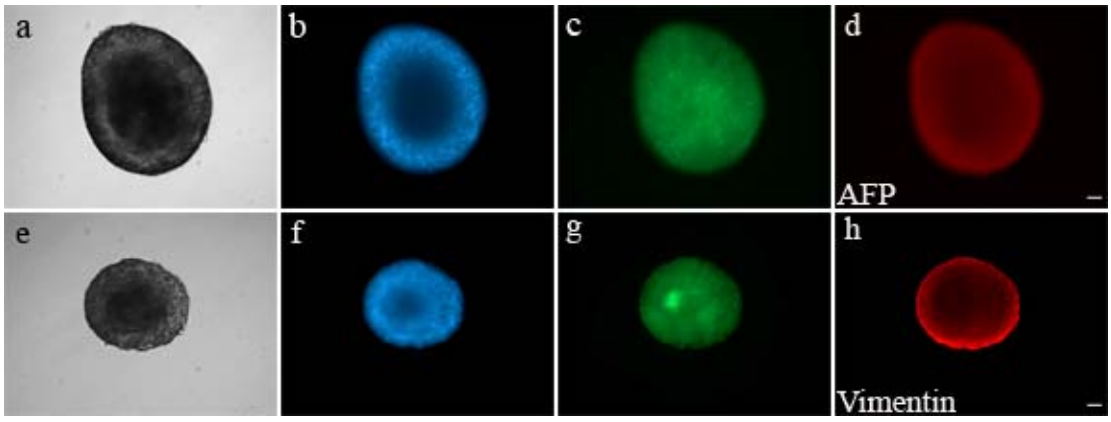

Figure 4.9.2 The expression of AFP (Top panel) and vimentin (Bottle panel) from TrES1 after embryoid body formation. GFP expression were detected under epifluorescence (c, g). Scale bar: 20 μm.

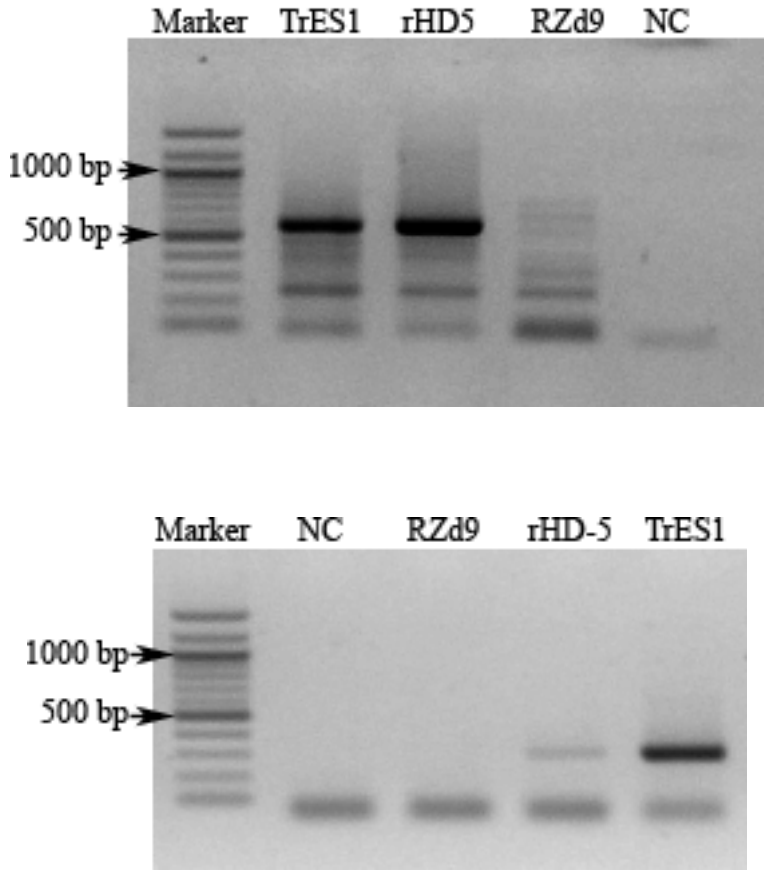

Figure 4.9.3 PCR results of transgenic htt and GFP gene of nhpESC TrES1. Top panel showed the PCR product of htt gene. PCR product of htt gene is 586 bp. The lower panel showed the PCR product of GFP gene. PCR product of GFP is 302 bp. Tetraploid nhESC produced by using skin fibroblast from transgenic rhesus monkey (rHD-5) fused with MII oocytes. RZd9: wild type male rhesus monkey. NC: No template negative control.

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CHAPTER V

HUNTINGTON'S DISEASE MONKEY HYBRID STEM CELL MODEL

5.1 Abstract

This study is the first reported of hybrid cell line of non human primate (NHPs) that carries with human genetic disease, Huntington disease (HD). The hybrid cell line, TrES1, established by fused skin fibroblast derived from transgenic HD and GFP monkey to metaphase II oocyte. Pluripotent HD hybrid cell line was established by whole embryo culture on feeder layer. Tetraploid HD at blastocyst stage was plated on feeder layer. TrES1 expressed both mutant htt and green fluorescent protein (GFP). The expression of ES cell markers was observed and teratoma formation developed after implant TrES1 into brain of SCID mice. Neuronal markers were observed during neuron differentiation and HD cellular pathology was developing along differentiation step. The accumulation of oligomeric mutant htt and intranuclear inclusions (NIs) was dramatically increased along *in vitro* differentiation to neuron. Thus, the hybrid cell line could be use as a model to study the mechanism of HD and drug screening.

5.2 Introduction

Huntington's disease is autosomal dominant neurodegenerative disorder, typically of adult inset. It is also an inherited and age related disease. The age of onset varies markedly, typically occurred between ages 35-50 but varying from early childhood to 80 years old. HD symptom is insistently progressive with death occurring about 15-20 years after the disease onset. HD is caused by the degeneration of cells or neuron

located in striatum region deep within the brain that is part of structure call basal ganglia. In normal people, neurons in striatum work to shut off excitatory signals from the motor cortex, the part of the brain that dictates movement. The damages in striatum, therefore, resulted in uncontrolled body movement (Mattson, 2002). Cause of HD is the expansion of a CAG encoded the polyglutamine (polyQ) repeat sequence in the first exon of a gene of chromosome 4p16.3, which encodes the protein huntingtin (Davies et al., 2001). The protein is highly expressed in neurons and testes in humans and rodents (Cattaneo and Zuccato, 2005). Normally, huntingtin is a cytoplasmic protein expressed at high levels in the striatal neurons sensitive to degeneration in HD and at low level or undetectable in neuron resistant to degeneration (Gutekunst et al., 1999).

HD gene is essential for postimplantation development and that it may play an important role in normal functioning of the basal ganglia in mouse and absence of huntingtin is lethal in mice (Nasir et al., 1995). HD is characterized by the aggregation of htt into microscopic intranuclear aggregates called intranuclear inclusion (NIs) or inclusion bodies (IBs) and by the death of striatal and cortical neurons. Intranuclear aggregates formed by expanded polyglutamine proteins have also been reported in the brains of patients with HD or other glutamine repeat disorders (Becher et al., 1998; DiFiglia et al., 1997; Paulson et al., 1997; Ross, 1997). Transgenic htt gene pluripotent stem cell can be used to study the impact of cell type and neuron specific degeneration from HD pathogenesis by differentiates into different cell type. Human HD-ES (hHD-ES) cell lines were established by using hHD embryos (Mateizel et al., 2006) or induced pluripotent fibroblast cell from HD patient to stem cell (Park et al., 2008). Unfortunately, the pathological of HD was not clearly to show the impact of HD after *in vitro* culture. Until now, no report the good hHD-ES cell line that can be used as the model of HD pathological after differentiation ES cell to neuron cell during *in vitro* culture. The recent reported of transgenic HD monkey suggest that N-terminal fragments of htt and expanded polyQ can

accelerate the onset of HD in higher primates with the development of distinctive neuropathological and cognitive behavioral characteristics, while the severity of HD is also related to the dosage of mutant htt (Yang et al., 2008).

In this study, HD monkey hybrid cell line "TrES1" was established by fusion of transgenic HD monkey fibroblast cell into monkey oocyte. This cell line dramatically demonstrates the progressive and severity development of HD pathology during *in vitro* culture.

5.3 Materials and methods

5.3.1 Establishment of rhesus monkey fibroblast cell line

Fibroblasts were generated from skin tissue from miscarried transgenic (rHD-5) with GFP and htt gene (Figure 5.1). The skin tissue was washed several times in DPBS that supplemented with 100 U/ml Penicillin/ 100 ug/ml Streptomycin. Skin tissues were cut into small pieces by scissors in 0.05% trypsin/EDTA and digested at 37˚C for 10-15 minutes. Then, the supernatant of digested tissue were transfer to tissue culture dish in DMEM medium supplemented with 10% heat inactivated FBS 2mM L-Glutamine and 100 U/ml Penicillin/ 100 ug/ml Streptomycin. Fibroblast-like morphologies were passage when they reach to 95% confluence. Cells were frozen in DMEM with 10% FBS and 10% DMSO and stored in liquid nitrogen until used.

5.3.2 Rhesus monkey ovarian stimulation and oocytes collection

Cycling female monkeys were superovulated and collected using the same protocol as in chapter 3.

5.3.3 Production of transgenic HD monkey tetraploid embryos

Oocytes with the 1st PB were placed in TL-HEPES supplemented with 5µg/ml of cytochalasin B for 15 minutes. A small slit was made at the zona pellucida above the 1st PB and the 1st PB was then gently squeezed out by applying pressure on the oocyte (Figure

5.2). The oocytes were washed five times in TL-HEPES. An individual fibroblast was placed into the PVS of the PB free oocyte (Figure 5.3). The couplet was fused by electrofusion using fusion electrodes in 0.3M Manitol fusion medium with two direct currents, 30 volts 30 μsec. The reconstructed embryos were cultured in medium supplemented with 50 nM TSA for 10-12 hours. Two hours after fusion, the reconstructed embryos were activated by 5 μM ionomycin in TL-HEPES for 5 minutes and then incubated in 2mM 6-Dimethylaminopurine (6-DMAP; Sigma) in HECM-9 (Zheng et al., 2001) for 5 hours at 37˚C with 5% CO_2, 5% O_2, 90% N_2. The reconstructed embryos were further cultured in HECM-9 medium for 8 days with 10% FBS added on Day two of culture.

5.3.4 Feeder cells preparation

Feeder cell preparation was using the same protocol as in chapter 4.

5.3.5 Establishment of Huntington's monkey ES cells from tetraploid blastocyst by whole embryo culture

Zona pellucida of blastocysts was removed before plated on feeder layer. An individual blastocyst was cultured on freshly prepared of mitomycin C treated MFFs feeder layer in monkey ES medium composed of KO-DMEM supplemented with 20% KSR, 1 mM glutamine, 1% non-essential amino acids and supplemented with 4 ng/ml of bFGF at 37˚C under humidified atmosphere 5% CO_2 in air for 10-14 days. Half of the ES medium was replaced every other day. On day 2 after plating, the attachment of the whole embryo onto feeder layer was observed. The outgrowth morphology was daily observed. The outgrowths were cultured for 10 to 14 days before mechanically sub-cultured.

5.3.6 Transgenic status of the HD monkey ES cells

For detecting the htt-84Q gene, ubiquitin forward primer (5'-GAGGCGTCAGTTTCTTTGGTC-3') and htt-84Q-R reverse primer (5'-GCTGGGTCAC TCTGTCTCTG-3') were used to yield an 818 bp of expected size PCR product after

amplification of genomic DNA from the HD monkey tissues. Genomic DNA (100 ng) from different tissues were subjected to PCR for 35 cycles at 96°C for 5 min, 96°C for 45 sec, 62°C for 45 sec, and 72°C for 150 sec, followed by 72°C for 7 mins. To determine the numbers of CAG repeats in HD monkeys, the PCR products were sequenced using HD exon 1-F primer (5'-GGCGACCCTGGAAAAGCTGA-3'). For GFP gene, GFP-F forward primer (5'-TTCAAGGACGACGGCAACTAC-3') and GFP-R reverse primer (5'-TAGTGGTTGTCGGGCAGCAG-3') were used for amplification for 35 cycles at 94°C for 5 min, at 94°C for 30 sec, 64°C for 30 sec, and 72°C for 20 sec, followed by 72°C for 5 min, which yielded a product of 302 bp. DNA from WT-monkeys were used as the negative control, and plasmid htt-84Q and GFP were used as the positive controls.

5.3.7 Genotyping

Genotyping was executed using a panel of 13 microsatellites that are highly polymorphic in rhesus macaques and possessing high levels of heterozygosity in other rhesus macaque populations (Rogers et al., 2005; 2006). Primers for each microsatellite were obtained with one of the standard Applied Biosytems (AB) 5-dye labels. Amplification reactions were performed on AB 9700 thermal cyclers using $MgCl_2$ concentrations of either 1.5 mM or 2.0 mM. Electrophoresis was carried out using an AB 3730 genetic analyzer, with all subsequent genotyping analysis using Genemapper 4.0. All genotyping was performed blind, with a positive and negative control included for each reaction.

5.3.8 Mitochondria inheritance analysis

Sequencing primers were designed in primer 3 (http://frodo.wi.mit. edu) in order to amplify two regions of rhesus mitochondrial DNA (*Macaca mulatta* NCBI reference sequence NC_005943). PCRs were performed using standard amplification reactions on AB 9700 thermal cyclers using 2.0 mM $MgCl_2$ concentration. PCR products were checked for expected size by electrophoresis on agarose gels. Shrimp alkaline phosphatase and

Exonuclease I were added to remove single strand DNA. Sequencing reactions were done using AB Big Dye terminator on a 9700 thermal cycler. The reaction was purified with an EDTA/EtOH protocol, and sequencing reactions were performed on an AB 3730 genetic analyzer. Subsequent analysis was done using SeqScape genetic software. Positive and negative controls were sequenced along with experimental samples for each region.

5.3.9 Immunocytochemistry for ES cell markers

ES medium was removed out and ES cells were washed several times with PBS without Ca^{2+} and Mg^{2+}. ES cells were fixed in 4% paraformaldehyde solution at room temperature for 30 min and washed several times with PBS. Alkaline phosphatase staining was done by using an alkaline phosphatase staining kit (Vector® Blue Alkaline Phosphatase Substrate Kit III; SK-5300). For cell surface markers such as SSEA-3, TRA-1-60, TRA-1-81 staining, the ES cells colony and embryoid bodies were blocked in PBS with 4% goat serum at room temperature for 1h whereas ES cells were permeabilized with 0.2% Triton-X and 0.1% Tween 20 for the Oct3/4 nuclear marker staining, Then, samples were subsequently incubated with the primary antibody: SSEA-3 (1:200; rat monoclonal), TRA-60 (1:200; mouse monoclonal), TRA-81 (1:200; mouse monoclonal), Oct3/4 (1:200; mouse monoclonal), Alpha-Fetoprotein (1:200; mouse monoclonal), Vimentin (1:20; Goat monoclonal), Nestin (1:250; rabbit polyclonal; Chemicon, AB5922), glial fibrillary acidic protein (GFAP; 1:100; rabbit monoclonal; Sigma, G9269), Beta-III tubulin (1:250; mouse monoclonal; Chemicon, MAB1637). Cells sample were incubated with primary antibody overnight at 4˚C. After washing, cells were incubated with the appropriate secondary antibody conjugated with either Alexa Flour 488 (1:1000) or Alexa flour 594 (1:1000) for 1 h. ES cells were co-stained with Hoechst 33342 (5 µg/ml) for 5 min and then examined under fluorescence microscopy.

5.3.10 Immunocytochemistry of mutant htt.

ES cells were fixed in 4% paraformaldehyde solution at room temperature for 30

min and washed several times with PBS. ES cells were permeabilized by 0.2% Triton-X and 0.1% Tween 20 and subsequently incubated with the primary antibody mEM48 (1:50 dilution) overnight at 4˚C. The first antibody was intensively washed out with PBS before incubated with secondary antibody conjugated with Alexa Red (Molecular Probe). DNA was co-stained with Hoechst 33342. After staining, the specimen was covered with PBS before examined with an epifluorescent microscope.

5.3.11 Cytogenetic analysis

TrES1 was passaged into a T-25 flask at passage 25 and treated with KaryoMax® colcemid (Invitrogen) for 20 minutes to block cells at metaphase. Cells were dislodged with 0.05% Trypsin-EDTA, centrifuged and gently resuspended in hypotonic 0.075 M KCL solution for 20 minutes. Following centrifugation the cells were fixed three times in a 3:1 ratio of methanol to glacial acetic acid. The cell pellet was resuspended in 1 ml of fixative and stored at 4°C. For GTL-Banding, the fixed cell suspension was dropped on wet slides, air dried, and baked at 90°C for 1 hour. Slides were immersed in 0.5x Trypsin-EDTA (Invitrogen) with two drops of 67 mM Na_2HPO_4 for 20 to 30 sec, rinsed in distilled water and stained with Leishman Stain (Sigma) for 90 sec. Twenty metaphases were analyzed for numerical and structural chromosome abnormalities using an Olympus BX-40 microscope. Images were captured and at least two cells were karyotyped using the CytoVysion® digital imaging system (Applied Imaging).

5.3.12 *In vitro* differentiation to neuronal lineage

ES cell colony were mechanical detached from feeder cell and carefully transferred into 35 mm suspension culture dish that contain ES cell culture medium, half of the medium was changed every other day. For the formation of EBs ES cell were cultured in non attachable culture dish for 7 days. EBs were then allowed to attach onto a gelatin coated plate and cultured in N1 medium for 7 days, N2 medium for 14 days and N3 medium for 7 days to allow differentiation into neuronal cell types. A 4-weeks extended culture at N3

stage was added to enhance maturation of neurons and mimic post-mitotic neuronal cell types in the brain. The N1 medium was composed of KO-DMEM supplemented with minimum essential amino acid, 200 mM of L-glutamine and N2 supplement (Invitrogen). The N2 medium was composed of N1 medium supplemented with 20 ng/mL bFGF. The N3 medium was composed of KO-DMEM supplemented with 1% FBS and B27 supplement (Invitrogen). NPCs were stained with nestin, whereas successful differentiation of neuronal cell types was confirmed by the expression of neuron specific βIII tubulin and MAP2 (Kuo et al., 2003).

5.3.13 Quantitative RT-PCR (Q-PCR)

The total RNA of cell samples was extracted using RNeasy Mini Kit (Qiagen). RNA quality was determined by BioPhotometer (Eppendorf). Reverse transcription was performed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and the resulted cDNA was used for Q-PCR. 2x Power SYBR® Green PCR Master Mix (Applied Biosystems) was mixed with specific primers and cDNA, and first subjected to the iQ5 real-time PCR detection system (Bio-Rad) for 50 cycles at 96°C for 12 min; then at 96°C for 15 sec, 60°C for 30 sec for 50 cycles. The specific primers for mutant HTT specific primers were: HD Exon 1-F: ATGGCGACCCTGGAAAAGCT and HD Exon 1-R: TGCTGCTGGAAGGACTTGAG. The specific primer for 18S: 18S F: CGGCTACCACAT CCAAGGAA and 18S R: CCTGTATTGTTATTTTTCGTCACTACCT.

5.3.13 Western Blot Analysis

Total proteins were extracted from TrES1 cells, and their concentration was determined by Bradford assay (Pierce). Equal amounts (20-30 μg) of protein extract with loading dye were boiled prior to loading into four to 15% gradient polyacrylamide gel (Bio-Rad). Following electrophoresis, proteins were transferred onto a PVDF membrane (Bio-Rad) using Bio-Rad's transblot followed by blocking in 5% skim milk for two hours. The membrane was then incubated with primary antibodies, mouse mEM48 (1:50 dilution), and

γ-tubulin (Sigma; 1:2000 dilution), followed by secondary antibody conjugated with peroxidase (Jackson ImmunoResearch laboratories) for detecting proteins with an Amersham ECL kit (Perkin Elmer).

5.3.14 *In vivo* differentiation of TrES1 and formation of teratoma in SCID mice

Undifferentiated of TrES-1 were collected by mechanical dissociation. Neuron progenitor cells (NPCs) of TrES1 were collected at N2 stage by using 0.05% trypsin/EDTA (invitrogen) to dissociate NPCs to single cells. Then $1x10^5$ of undifferentiated cells and NPCs of TrES cells were resuspensed in 50 µl of DPBS and stereotaxically injected into the striatum of SCID mice. Animals were euthanized during 4 to 10 weeks. Animal brain was recovered for immunohistological analysis. Brains were fixed in 4% paraformaldehyde overnight and transferred to 30% sucrose at 4°C. After post fixed, brain was embedded and cut to 50 µm sections. The sections were incubated with blocking solution for 1h at room temperature, and incubated with primary monoclonal antibody, mEM48 (1:50), at 4°C over night. After washing, cell was incubated with secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:1000 dilution, Chemicon) for 1h and co-stained DNA with Hoechst33342 for 5 min. Then epifluorescent images were examined by confocal microscopy.

5.4 Results

5.4.1 Establishment of transgenic stem cell line from tetraploid embryo

The miscarried monkey was confirmed transgenicity of the mutant htt (Figure 5.1c) and GFP gene by PCR and Southern blot analysis (Data not shown). A total of 72 CAG repeats was confirmed in the transgenic mutant htt gene. Two HD monkey hybrid blastocysts were placed onto feeder cells (Figure 5.4a). Hybrid blastocysts started to attach onto the feeder cells on day 2 after culture (Figure 5.4b). The outgrowths were forming after attachment onto the feeder cells (Figure 5.4c-g). From days 10 to 14, the outgrowth ES like

cells were identified based on the distinctive ES cells morphology. After mechanical dissociation, ES like cells colonies were found and ES cells morphology also showed large nucleus and a high nuclear to cytoplasmic ratio (Figure 5.4h). After cytogenetic analysis, the TrES1 was found to be a tetraploid hybrid cell line with three "X" chromosomes and one "Y" chromosome (Figure 5.5). A set of "XY" chromosome was derived from skin fibroblast of the male donor cell HD monkey while a set of "XX" chromosome was derived from the recipient monkey oocyte. However, the mutant htt and GFP gene of hybrid cell line can only be derived from the HD monkey skin fibroblast. Genetic identification of hybrid cell line of TrES1 was performed by microsatellite analysis of mitochondrial DNA comparison of HD monkey, monkey oocytes donor and TrES1. All alleles of HD monkey skin fibroblast and the lymphocyte of oocyte donor were also presented in TrES1 (Table 5.1). The result of genetic analysis demonstrated that TrES1 was a tetraploid hybrid cell line that contained both source of genetic material from HD monkey and oocyte donor. Partial sequences of mitochondrial DNA were compared in TrES1, skin fibroblast from HD monkey and lymphocyte of the oocyte donor. Sixteen rhesus macaque specific single nucleotide polymorphisms (SNPs) were analyzed. After SNPs analysis, all sixteen macaque SNPs of TrES1 matched with the oocyte donor (Table 5.2). The mitochondrial DNA analysis showed that mitochondrial DNA of TrES1 mainly derived from oocyte donor.

5.4.2 Monkey transgenic Embryonic Stem cell characteristic and pluripotency

The characteristic of TrES1 was determined using immunostaining. The common ES cells markers of nonhuman primate were examined. The immunostaining indicated TrES1 cells expressed AP, Oct3/4, and SSEA-4 and TRA-1-60 (Figure 5.6). The pluripotency of TrES1 were determined by *in vitro* differentiation. Neuron differentiation by step-wise protocol was used in this study. The immunostaining was performed during neuron differentiation at different stages of neuron differentiation using specific marker

(Kuo et al., 2003). At N2 stage, nestin expression was observed in neural progenitor cells (NPCs; Figure 5.7). Matured neurons were found after induction in N3 stage for one week (N3-1w). Extend culture in N3 stage for 4 weeks was performed to mimic mature neuron in adult brains to determine the htt progression (N3-4w). Neuronal specific βIII tubulin and GFAP were detected by immunostaining at N3-1 and N3-4w (Figure 5.7).

5.4.3 Expression of mutant htt in TrES1 derived neuronal differentiation

Mutant htt expression were found in all cell types, however the expression level of mutant htt is highly expressed in brain tissue. It also showed the NIs in brain tissue (Yang et al., 2008). After quantitative real time PCR (qRT-PCR) analysis the expression of mutant htt in undifferentiated TrES1 and wild type monkey ES cell show similar expression profile at differentiation stage (Figure 5.8A). The expression level of mutant htt significantly increase in TrES1 at N2, N3-1w and N3-4w when compare with undifferentiated TrES1 and wild type monkey ES cells. However, the expression of mutant htt of TrES1 show no significant different at N2, N3-1w and N3-4w. Western blot analysis was performed. The mutant htt was not detected in undifferentiated TrES-1 nor wild type ES cell monkey at any stage. However, mutant htt was observed in N2, N3-1w and N3-4w stage of TrES1 (Figure 5.8B). The results clearly showed that mutant htt in TrES1 gradually increased during *in vitro* differentiation to neural cells (Figure 5.8B). Moreover, numbers of positive cells to HD antibody of TrES1 after immunostaning significantly increased during *in vitro* differentiation to neuron cells at N2, N3-1w and N3-4w stage, respectively (Figure 5.8C).

To identify expression of mutant htt and NIs during *in vitro* differentiation of TrES1, immunostaning by mEM48 antibody were performed at every stages of differentiation. The expression of mutant htt and NIs were not detected after immunostaining in the undifferentiated TrES1 whereas the number of cells which positive to mEM48 was significantly higher in N3-4w (32.2%; 132 ± 42.5; n = 1484) than N3-1wk

(8.4%; 30.3 ± 19.4; n = 1078) and N2 (0.26%; 2 ± 0; n = 1271), respectively (Figure 5.8C). The NIs positive cells were separated into 3 groups by degree of NIs, 1 to 5, 6 to 10 and more than 10 of NIs (Figure 5.8D). At N3-4w, Degree of TrES1 express mutant htt in soluble form and presenting 1 to 5 of NIs was significantly higher when compare to N3-1w and N2, respectively. This expression of mutant htt of TrES1 show similar profile with qRT-PCR and Western blot analysis.

5.4.4 *In vivo* differentiation of TrES1 in the striatum of SCID mice

The implantations of undifferentiated TrES1 and TrES1 at N2 stage into the striatum of the contralateral hemisphere of severe compromised immune deficient (SCID) mice were performed. Animals were euthanized at 4 to 10 weeks post-implantation. Then brains were recovered for histological study and immunohistochemistry using different antibodies to determine neural differentiation and the expression of mutant htt. After histological study, the undifferentiated TrES1 were form to formed teratoma in the hemisphere. Teratoma in the brain had differentiated into endoderm (gut-like epithelium), mesoderm (muscle) and ectoderm (neural tissue) as show in figure 5.9A. Teratoma formation was not observed in hemisphere after implantation with TrES1 at N2 stage. The expressions of GFP expression and neuronal markers were found in the both hemisphere of SCID mice (Figure 5.10). However, only some region of teratoma after implantation of undifferentiated TrES1 differentiated into neuronal cell. On the other hand, implantation TrES1 at N2 stage show all area of cell graft differentiated to neuronal cells and show mature neurons expression after immunostaining by MAP2 (Figure 5.10). The intensity of mEM48 staining was observed in hemispheres implanted with TrES1 after N2 stage while in the contralateral hemisphere show less intensity of mEM48 staining (Figure 5.9B). All SCID mice implanted withundifferentiated TrES1 and N2 stage show similar results as above.

5.5 Discussion

A hybrid cell line, TrES1, were established. It shows pluripotentcy and able to differentiated to neuron cell and other cell type after implantation of the undifferentiated TrES1 into hemisphere of SCID mice. TrES1 was established by fusing skin fibroblast of HD monkey with monkey oocytes. After *in vitro* differentiation of TrES1 to neuron, the expression of mutant htt gradually increased from NPCs to mature neuron particularly in N3-4w. Moreover, the accumulation of mutant htt aggregates and NIs during *in vitro* differentiation significantly increased from NPCs to late neuron differentiation. On the other hand, the aggregation and NIs of mutant htt of undifferentiation TrES1 was not observed. Our finding, indicated that mutant htt have impact to neuron cell and minimum impact to peripheral tissue. Moreover, the aggregation and NIs of mutant htt were not observed in cardiomyocytes during spontaneous differentiation of TrES1 (data not show).

Stem cell carrying genetic disorder is a good model to study the progress and mechanism of the disease during *in vitro* study (Dimos et al., 2008; Mateizel et al., 2006; Metzler et al., 1999; Park et al., 2008). The pluripotentcy of ES cells is great advantage to allow differentiation into many lineages particularly to study neurodegenerative disease such as HD. The HD showed severe pathological specific to neuron cell type when compare with peripheral tissue (Bates et al., 1998a; Bates et al., 1998b; Li and Li, 2006; Li et al., 1993; Yang et al., 2008). *In vitro* differentiation of TrES1 to neuronal lineage show the accumulation and NIs as develop in early HD pathology. Moreover, the replicating of gradually progression of mutant htt pathogenesis is capable inTrES1 after *in vitro* differentiation which no other HD cell model either transient or stable expression of mutant htt in somatic cell (CHO and 293) or primary neuronal culture (PC12) have been achieve (Ciammola et al., 2006; Desai et al., 2006; Outeiro and Giorgini, 2006). HD-ES cells have been established from mouse (Heng et al., 2007; Lin et al., 2001; Menalled et al., 2003; Metzler et al., 1999; Wheeler et al., 2000) and human (Mateizel et al., 2006; Park et al.,

2008), however, the phenotype of HD hallmark along differentiation to neuron were not develop. hHD-ES cell lines did not report in detail about HD pathology. On the other hand, TrES1 could develop HD hallmark during differentiation to neuron cells which show the progression of HD cellular phenotype from N2 to N3-4w stage (Figure 5.7). Therefore, TrES1 is a useful pluripotent cell line for accurate interpretation of therapeutic efficacy of new drug test.

Teratoma was observed after undifferentiated TrES1 grafted into brain of SCID mice. Many cell types were observed in teratoma. On the other hand, teratoma formation was not found in the NPCs grafted hemisphere. It still continue differentiated to neuron. The results indicated that TrES1 have similar property to ES cells and can form teratoma which contained many cell types.

Number of HD cellular phenotype of TrES1 increase during differentiated to neuron could be because of the over expression of small htt fragments with expanded polyQ. Recent report showed that monkey carried similar mutant htt as TrES1 shows HD phenotype in early stage of their life (Yang et al., 2008). ES cell lines derived from iPS or human HD patients may not develop HD hallmark as express like TrES1 even expanded polyQ. The study in HD mouse found that full length htt is less toxic when compare to small htt fragments (Bates et al., 1998c; Schilling et al., 1999; Wang et al., 2008). So, HD-ES cells derived from HD patients may not be able to develop HD hallmark cellular feature without extended culture to allow accumulation of cellular defects.

5.6 Conclusion

These studies present the first NHPs hybrid cell line, TrES1, carried HD which is an inherited genetic disease. After *in vitro* differentiation, the progressions of HD cellular pathology along differentiation to neural cell were observed which other previous reported of HD-ES cell line can not achieve. Teratoma formation after TrES1 grafted to brain of

SCID mice show the pluripotency of TrES1 differentiated to ectoderm, endoderm and mesoderm cells. Thus, TrES1 is an alternative approach to produce pluripotent cell line that carry genetic disease which can be use to study the mechanism, pathology and the progression of disease during *in vitro* culture.

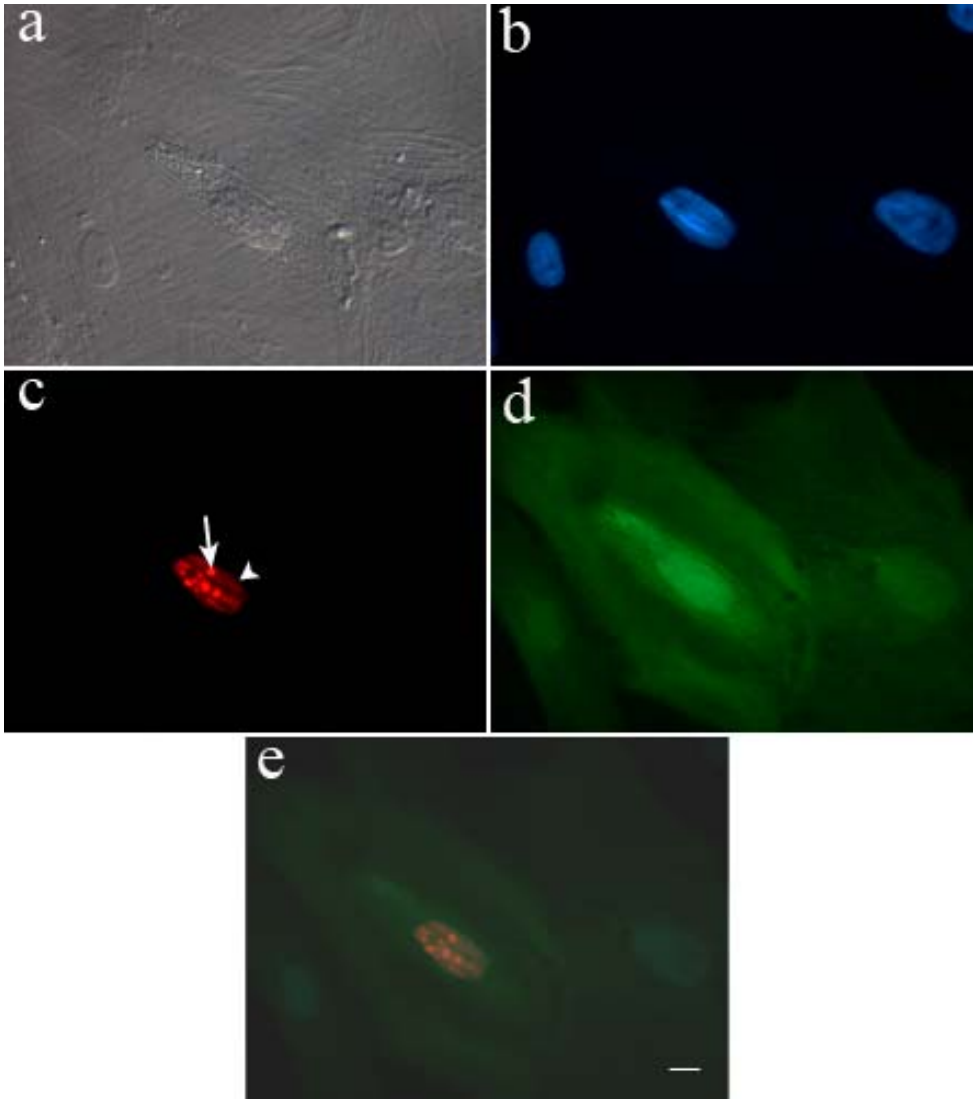

Figure 5.1 Immunohistochemistry staining of skin fibroblast cells that carry GFP gene and mutant Huntington. Morphology of monkey fibroblast cells (a). Cell nuclei of fibroblast cells stained with Hoechst (b). The expression of mutant Huntington results in aggregation (arrow head) and inclusion

(arrow) bodies detected by immunocytochemistry (c). GFP expression of fibroblast cell under fluorescence microscope (d). Merged view Hoechst staining, mutant htt (mEM48) and GFP (e). Scale bar: 5 μm.

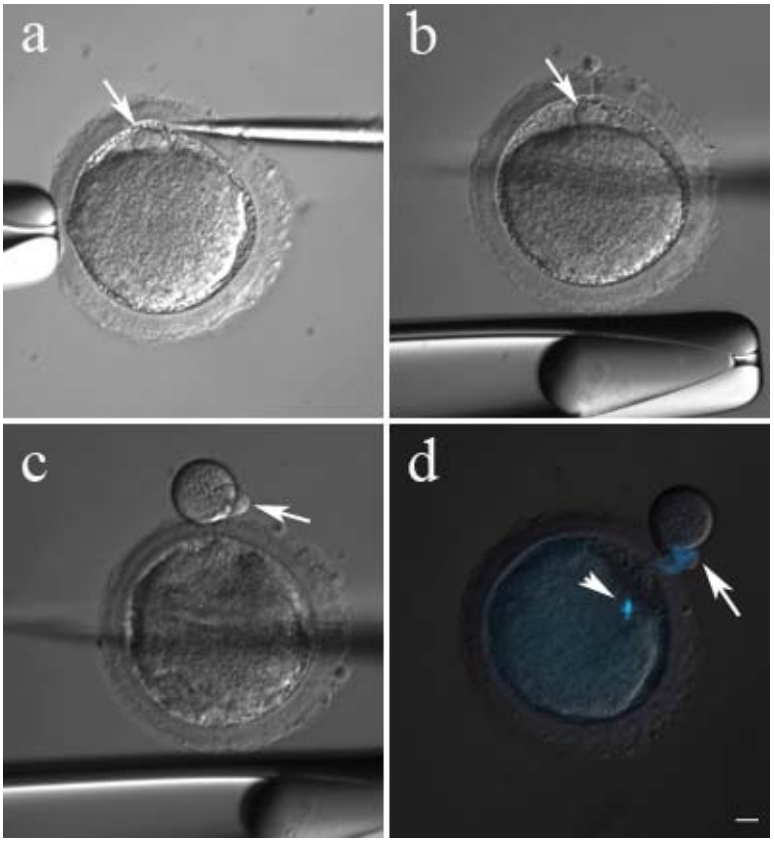

Figure 5.2 Removal of polar body from MII oocytes. Metaphase II oocytes, the 1st PB (arrow) were adjusted to 12 o'clock position. The zona pellucida above the first polar body was cut with glass needle (a). Gentle squeezing of polar body out though the slit of zona pellucida (b-c). Confirmation of the metaphase spindle (arrow head) by fluorescent microscopy (d). Scale bar: 10 μm.

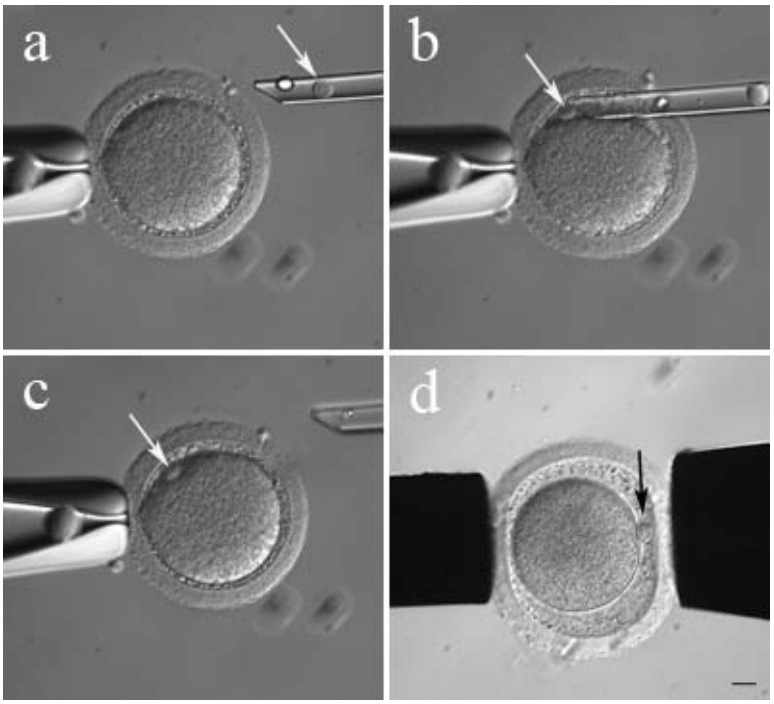

Figure 5.3 Donor cell (arrow) diameter 14-16 μm was selected and inserted into perivitelline space of oocytes (a-c). Somatic cell-cytoplast complexes (SCCCs) were fused by fusion electrode (d). Scale bar: 10 μm.

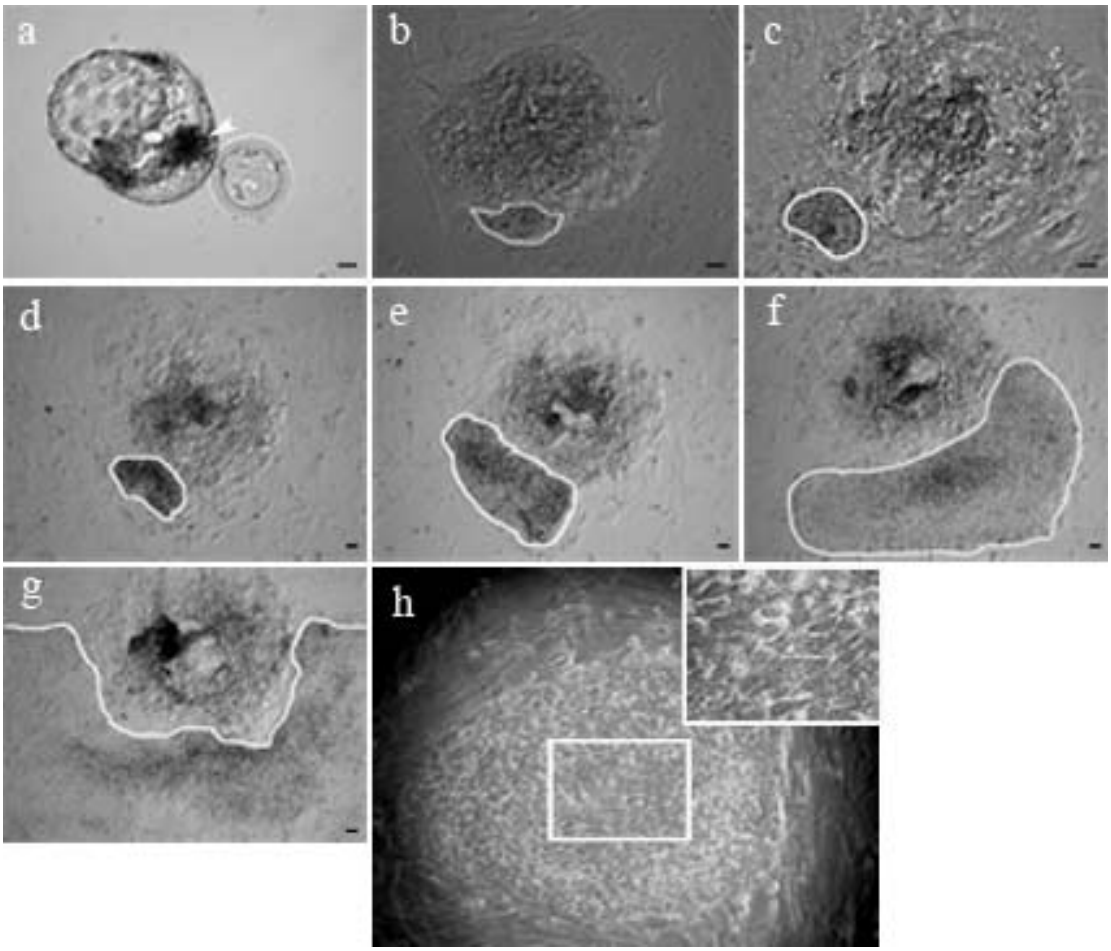

Figure 5.4 ES cell outgrowth derived from whole embryo culture (TrES1). Expanded blastocyst clearly shows ICM (arrow head) were selected to culture on feeder layer (a). White circle line indicate the edge of ICM outgrowth. On day 2 of culture, embryo flatten on feeder layer (b). ICM clump more packed and form circular-like shape on day 4 (c). On day 6 of culture, ICM outgrowth could be seen more clearly (d). Day 8 of culture, ICM outgrowth developed faster than the TE outgrowth (e). ICM outgrowth on day 9 (f) and day 12 (g) expanded faster. ES colony was form after mechanical dissection (h). high magnification of the selected region (inserted picture). Scale bar: 10 μm.

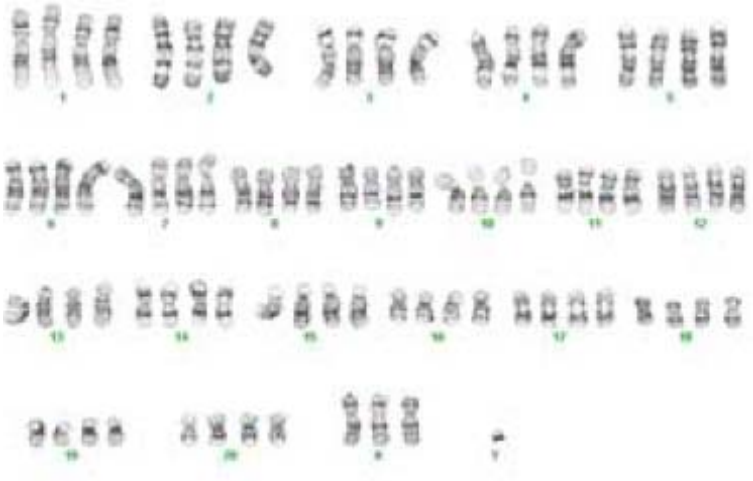

Figure 5.5 Cytogenetic analysis of TrES1 shows the tetraploid chromosome (84; XXXY).

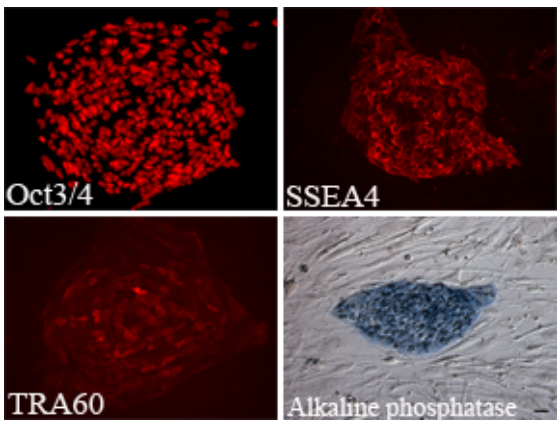

Figure 5.6 The expression of Oct3/4, SSEA4, TRA60 and Alkaline phosphatase of TrES1. Scale bar: 10 μm.

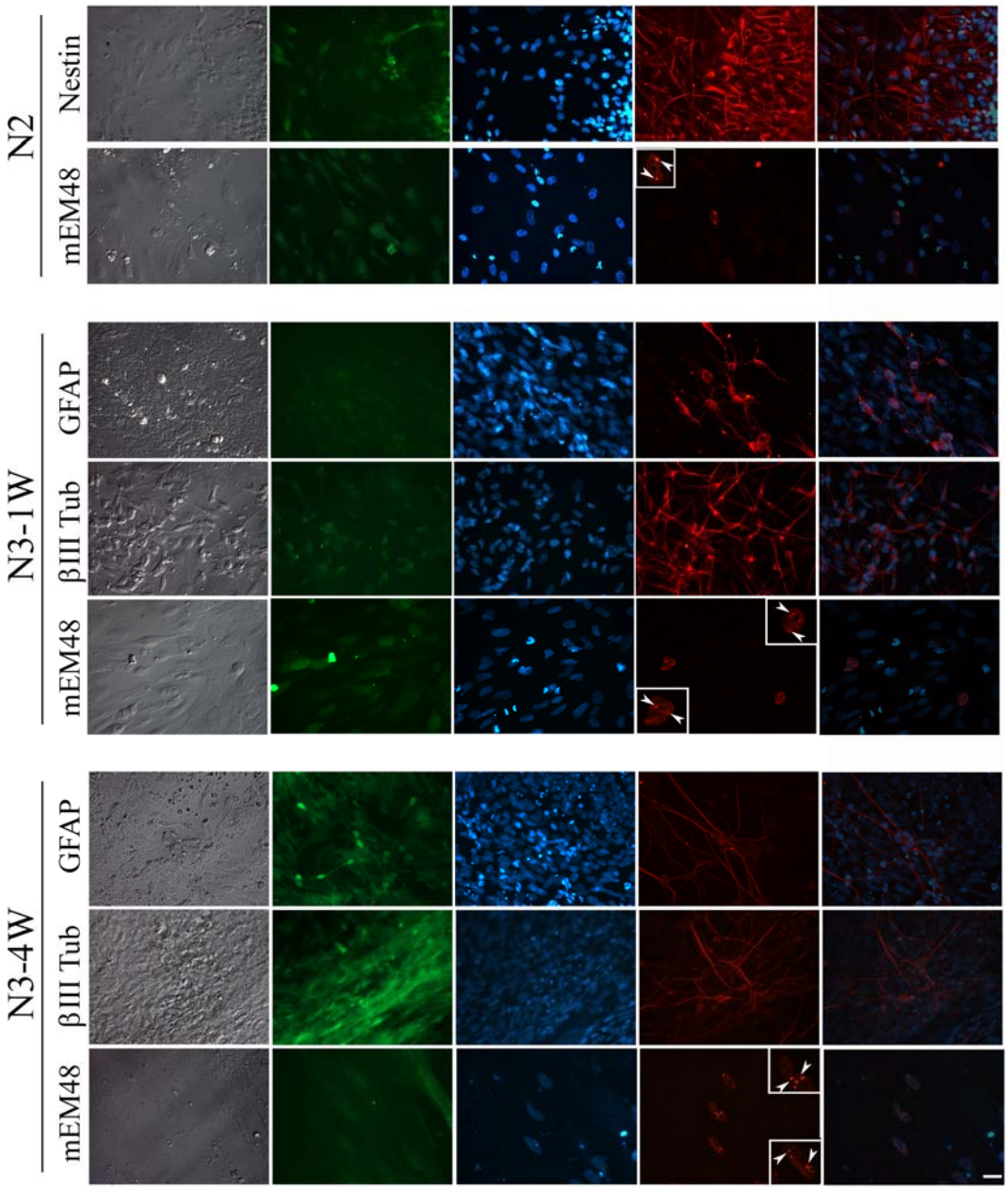

Figure 5.7 Immunostaining of *in vitro* differentiated TrES1 to neuron cell. *In vitro* differentiation to neuronal lineage of TrES1 with a step-wise differentiation protocol. Immunostaining of neuron at different differentiation stages: N2, N3-1 week (N3-1w), and N3-4 weeks (N3-4w) using specific antibodies to neural progenitor cells (nestin), glial fibrillary acidic protein (GFAP), and mutant htt (mEM48). At N2 stage, all cells were stained with nestin, however small number of cells were stained

positive with mEM48 (N2). GFAP, βIII-tubulin and mEM48 were stained at one week (N3-1w) and four week (N3-4w) after culture TrES1 at the N3 stage. First column-bright field images; second column-epifluorescent images of GFP; third column-DNA staining with Hoechst; fourth column-immunostaining with specific antibodies, and fifth column-overlay images of the third and fourth columns. Insets are images of selected nuclei with nuclear inclusions at higher magnification. Arrow head indicated nuclear inclusions (NIs). Scale bar: 10 μm.

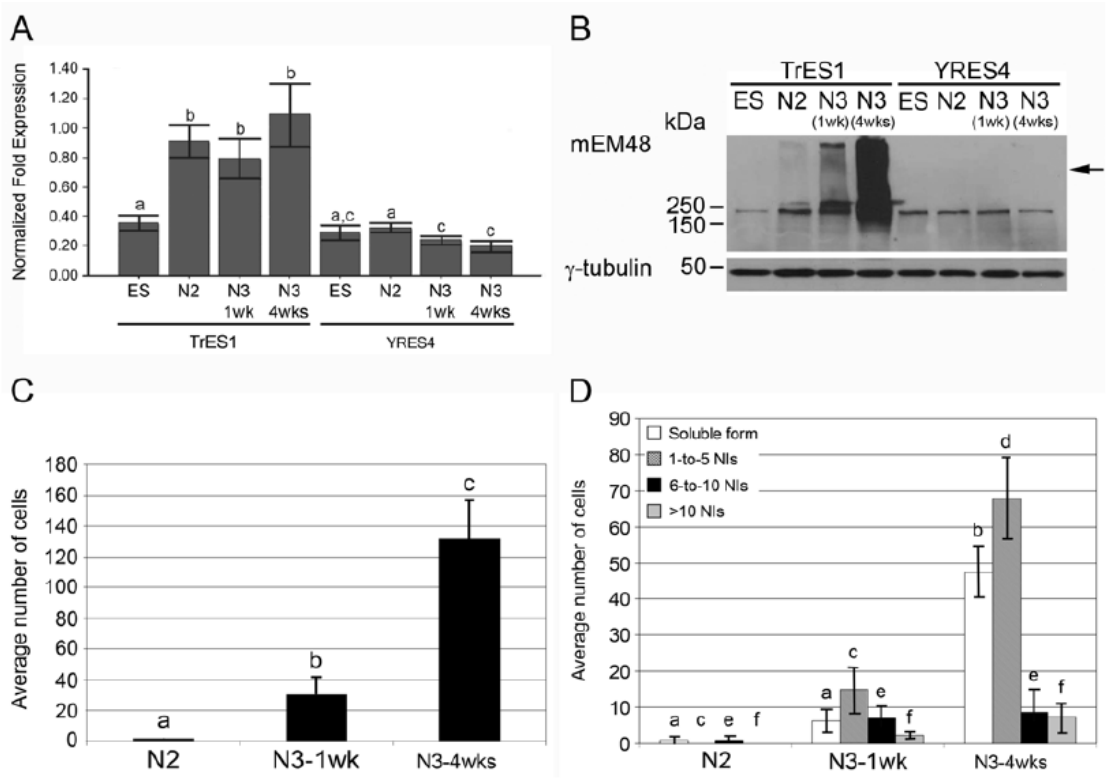

Figure 5.8 Expression pattern of htt in neural differentiated TrES1. Expression levels of htt at different neuron stages were determined by quantitative real time PCR (qRT-PCR). Non transgenic monkey ES cell line, YRES4, was used as a control (A). The expression levels of mutant htt in differentiated

TrES1 were significantly increased at N2, N3-1w and N3-4w when compared with undifferentiated TrES1 (ES) and YRES4 at all differentiation stages. The significant different indicate within the same columns at $P<0.05$. Western blot analysis was performed by using mEM48 antibody (B). Gradual increase of oligomeric transgenic mutant htt during neuronal differentiation of TrES1 were detected which TrES1 show the progresses of mutant htt during neural differentiation (N3-4w > N3-1w > N2). No high molecular weight of mutant htt aggregates were detected in undifferentiated ES cells and YRES4 at all stages. Number of cell expresses mutant htt was detected by mEM48 at differentiation step (C). The number of mEM48 positive cells was significantly higher in N3-4w (32.2%; 132±42.5; n=1484) > N3-1w (8.4%; 30.3±19.4; n=1078) > N2 (0.26%; 2±0; n=1271), respectively. The significant different indicate within the same columns at $P<0.05$. Degree of mutant htt expression was categorized into four groups: soluble form, 1-to-5 nuclear inclusions (NIs), 6-to-10 NIs, and more than 10 NIs (D). The significant different indicate within the same columns at $P<0.05$.

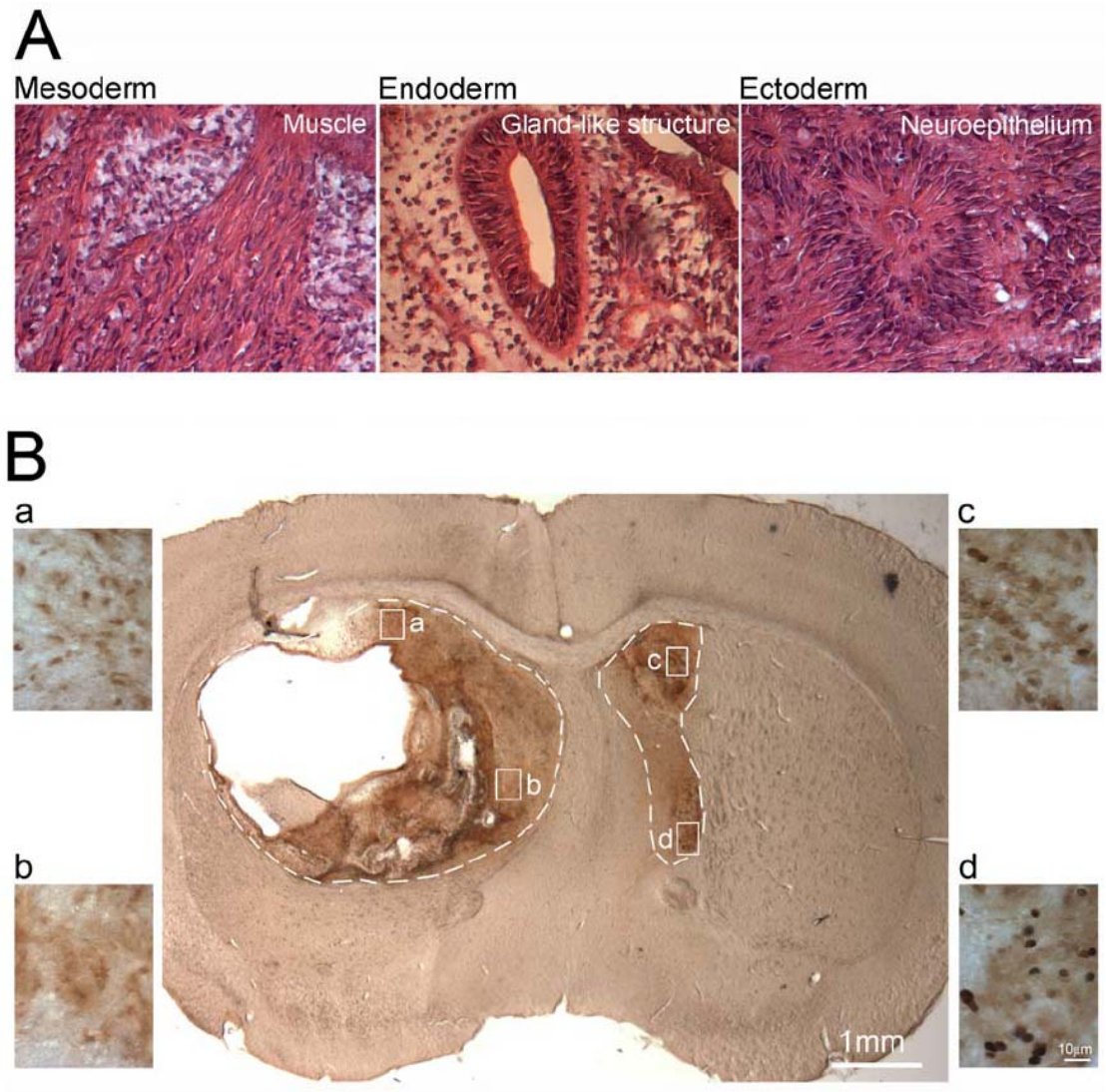

Figure 5.9 Teratoma formation and expression of mutant htt in striatal of SCID mice after graft of TrES1. Undifferentiated TrES1 cells were stereotaxically implanted into the striatum of SCID mice and recovered at 6 weeks for morphological and immunohistological analysis. (A) Teratoma was observed after grafted undifferentiated TrES1 into right hemisphere of SCID mice while neural progenitor cells (NPCs) which grafted in left hemisphere were smaller size of cell proliferation when compare to implanted undifferentiated TrES1. Teratoma was stained using hematoxylin and eosin. (B) Undifferentiated TrES1 was grafted in left

hemisphere and TrES1 derived NPCs was grafted in right hemisphere of SCID mice. The expression of mutant htt was detected by immunohistochemical staining using mEM48 antibody. The intense staining was observed at the right hemisphere which grafted by NPCs (B-c and B-d) when compare to left hemisphere which grafted by undifferentiated TrES1 (B-a and B-b).

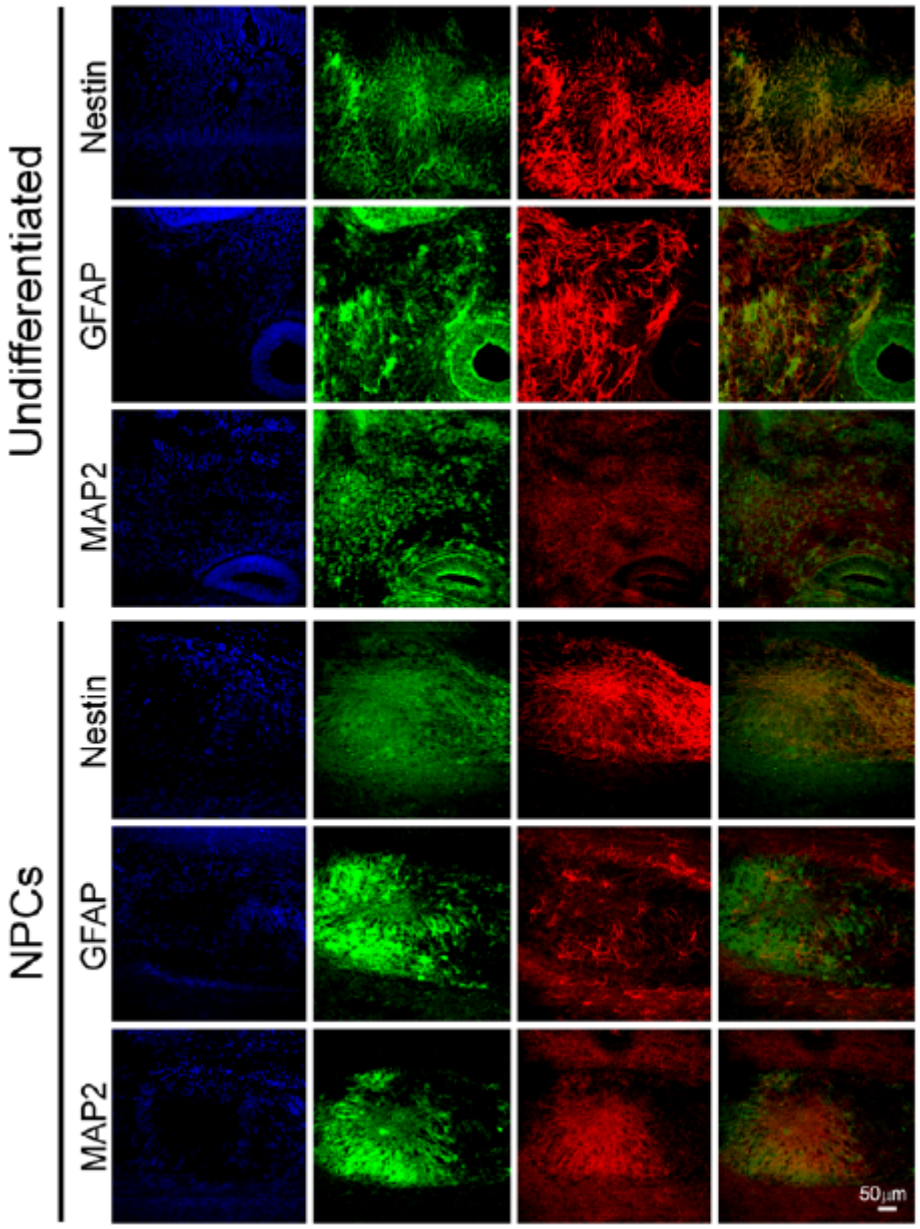

Figure 5.10 *In vivo* differentiation after implanted undifferentiated TrES1 and NPCs

derived TrES1. Undifferentiated and NPCs derived TrES1 were implanted into contralateral hemispheres of SCID mice for 6 weeks. The expression of nestin, GFA and MAP2 were observed in both hemispheres and co expression with GFP. However, the homogenous expressions were observed at the NPCs implanted hemisphere. Neuronal and non-neuronal tissues were observed in teratoma which implanted undifferentiated TrES1. However, co-expression of GFP were observed in both non-neuronal and neuron tissue. First column shows DNA staining; Second column shows epifluorescent images of GFP; Third column shows immunostaining using specific antibodies; Fourth column shows overlay images of GFP and immunostaining. Scale bar: 50 μm.

Table 5.1 Result of genotyping analysis of TrES1. Genotypes from 10 loci were assayed on genomic DNA of HD monkey skin fibroblast, lymphocyte of monkey oocyte donor and TrES1. All alleles present in the HD monkey skin fibroblasts and oocyte donor are presented in TrES1. The result indicating that TrES1 is a hybrid cell line that contained both genetic material from HD monkey skin fibroblast and monkey oocyte.

Locus	D9s261	D19s582	D16s403	D4s413	D5s108	D2s146	D3s1768	D6s493	D7s513	D13s1371
Donor	96/105	158/170	171/177	141/152	191/193	213/221	230/230	272/328	195/208	145/153
Recipient	103/105	167/175	167/169	141/150	179/189	208/210	210/226	266/269	193/193	169/174
TrES1	96/103/105	158/167/170/175	167/169/171/177	141/150/152	179/189/191/193	208/210/213/221	210/226/230	266/269/272/328	193/195/208	145/153/169/174

Table 5.2 The comparison of mitochondrial DNA sequence between TrES1 and oocyte donor**.** The sequence of mitochondrial DNA from HD monkey derived from skin fibroblast, lymphocyte of monkey oocyte donor and TrES1 were compared. The representative example of *Macaca mulatta* is position 371-731 from NCBI reference sequence NC_005943.

Donor	TTGGCA	CAAACA	CTACAA	CAAGAGG
Recipient	TTGACA	CAAGCA	CTATAA	CAACAGG
TrES1	TTGACA	CAAGCA	CTATAA	CAACAGG

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APPENDIX

mDPBS

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

PBS (-)

1) NaCl	10 g.
2) KCl	0.2500 g.
3) Na_2HPO_4	1.4400 g.
4) KH_2PO_4	0.2500 g.
5) Ultra pure water	1L.

Trypsin/EDTA

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

0.1% Gelatin

1) Gelatin	0.10 g.
2) PBS (-)	100 ml.

0.5% Pronase

1) Pronase	0.50 g.
2) mDPBS	50 ml.

Hoechst 33342 (stock)

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

α-MEM

1) α-MEM powder	1 g.
2) $NaHCO_3$	2.20 g.
3) Ultra pure water	1 L.

L-glutamine (stock)

1) L-glutamine	0.1462 g.
2) Ultra pure water	10 ml.

α MEM + 10% FBS

1.) α MEM (sterilized)	88 ml.
2). FBS (sterilized)	10 ml.
3). P-S (100x; sterilized)	1 ml.
4). L-Glutamine (200x; sterilized)	1 ml.

α MEM + 10%FBS + 20%DMSO

1.) α MEM + 10%FBS	8 ml.
2). DMSO	2 ml.

K buffer

1.) 10x PCR buffer	100 µl
2.) 50% Tween 20	10 µl
3.) Proteinase K	5 µl
4.) ddH_2O	890 µl

Monkey ES cell base solution

1.) Knockout DMEM (4˚C)	77.50 ml
2.) Knockout Serum (KSR) (20%)	20 ml
3.) NEAA (100x)	1 ml
4.) L-Glutamine	1 ml

Monkey ES cell outgrowth

1.) Base solution	8.9470 ml
2.) FBS	1 ml
3.) rhbFGF (1.5 µl/ml)	15 µl
4.) Activin A (1 µl/ml)	10 µl
5.) LIF (0.1 µl/ml)	1 µl

N1

1.) Knockout DMEM	9.750 ml
2.) NEAA (100x)	100 µl
3.) N2-supplement	100 µl
4.) L-Glutamine (200 mM)	50 µl

N2

1.) Knockout DMEM	9.750 ml
2.) NEAA (100x)	100 µl
3.) N2-supplement	100 µl
4.) L-Glutamine (200 mM)	50 µl
5.) rhbFGF (20 ng/ml)	5 µl

N3

1.) Knockout DMEM	9.750 ml
2.) FBS	100 µl
3.) B27 supplement	200 µl

Blocking solution

	Final conc.	100 ml
1). Triton X-100	0.2%	200 µl.
2). Sodium azide	3 mM	19.50 mg.
3). Saponin (mass/V)	0.1%	100 mg.
4). BSA (mass/V)	2%	2 g.
5). Horse/Donkey serum (V/V)	5%	5 ml.
6). PBS (-)		95 ml.

BIOGRAPHY

Chuti Laowtammathron was born in Khonkaen, Thailand on July 19th, 1977. He finished high school from Khonkanewitayayon School in Khonkaen. In 2000, he received Bachelors Degree (B.Sc.) in Animal Production Technology from Institute of Agricultural Technology, Suranaree University of Technology. In 2004, he received Master degree (M. Sc.) in the field of Animal Biotechnology at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. His research topic was "Cloning of bovine embryos by using ear fibroblasts as donor cell : Comparison of survival rate after freezing in several media". His master thesis was awarded as an outstanding master thesis from Thai Society of Biotechnology in 2007. During his Ph.D. study, he received a graduate student outstanding award from Suranaree University of Technology and also received a fellowship from National Center for Genetic Engineering and Biotechnology carried out research on "Production of bovine IVF-derived embryos from sexed-sorted semen" at Department of Animal Science, Faculty of Agriculture and Life Science, University of Wisconsin-Madison, USA during March 2005 - June 2005 and Feb 2006 – May 2006. His Ph.D. study was supported by the Royal Golden Jubilee (RGJ) Ph.D. Program of Thailand Research Fund. His Ph.D. thesis title was Establishment of embryonic stem cell lines from transgenic rhesus monkey blastocysts. Part of this work was presented as poster presentation in the RGJ-Ph.D. Congress X on April 3-5, 2009 at Jomtien Palm Beach Hotel and Resort, Pattaya, Chonburi, Thailand.