PRODUCTION OF TRANSGENIC HUNTINGTON'S BLASTOCYST FROM TRANSGENIC HUNTINGTON'S RHESUS MONKEY SPERM AND DERIVATION OF EMBRYONIC STEM CELL LINES

Kittiphong Putkhao

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2013

การผลิตเซลล์ตัวอ่อนระยะบลาสโตซิสที่เป็นโรคฮันทิงตันจากอสุจิของลิงวอก ดัดแปลงพันธุกรรมที่เป็นโรคฮันทิงตันเพื่อผลิตเซลล์ต้นกำเนิดตัวอ่อน

นายกิตติพงษ์ พุฒขาว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

PRODUCTION OF TRANSGENIC HUNTINGTON'S BLASTOCYST FROM TRANSGENIC HUNTINGTON'S RHESUS MONKEY SPERM AND DERIVATION OF EMBRYONIC STEM CELL LINES

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

Thesis Examining Committee

_____
(Assoc. Prof. Dr. Montarop Yamabhai)
Chairperson

_____
(Assoc. Prof. Dr. Rangsun Parnpai)
Member (Thesis Advisor)

_____
(Assoc. Prof. Dr. Mariena Ketudat-Cairns)
Member

_____
(Assoc. Prof. Dr. Anthony W.S. Chan)
Member

_____
(Asst. Prof. Panlada Tittabutr)
Member

_____
(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs

_____
(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

กิตติพงษ์ พุฒขาว : การผลิตตัวอ่อนระยะบลาสโตซิสท์ที่เป็นโรคฮันทิงตัน จากอสุจิของลิงวอกดัดแปลงพันธุกรรมที่เป็นโรคฮันทิงตันเพื่อผลิตเซลล์ต้นกำเนิดตัวอ่อน (PRODUCTION OF TRANSGENIC HUNTINGTON'S BLASTOCYST FROM TRANSGENIC HUNTINGTON'S RHESUS MONKEY SPERM AND DERIVATION OF EMBRYONIC STEM CELL LINES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 110 หน้า

มีรายงานแล้วว่า ลิงวอกดัดแปลงพันธุกรรมที่เป็นโรคโรคฮันทิงตัน (rHD-1) ซึ่งมีความผิดปกติของยีน huntingtin (*HTT*) ที่ทำให้เกิดสายโปรตีนกลูตามีนยาวกว่าปกติ (polyQ) ทำให้เกิดการกระตุกของกล้ามเนื้อ การเกร็งของกล้ามเนื้อ และการไม่สามารถควบคุมการเคลื่อนไหวของกล้ามเนื้อได้ ซึ่งคล้ายกันกับการเกิดโรคฮันทิงตันแบบปกติ ในการศึกษานี้ได้อธิบายการถ่ายทอดพันธุกรรมของลิงวอกดัดแปลงพันธุกรรมที่เป็นโรคโรคฮันทิงตัน โดยการผลิตตัวอ่อนด้วยวิธีฉีดอสุจิเข้าเซลล์ไข่ โดยใช้อสุจิจาก rHD-1 แล้วนำตัวอ่อนที่ได้ไปผลิตเป็นเซลล์ต้นกำเนิดตัวอ่อน (rHD-ESCs) จากผลการศึกษาพบว่า rHD-ESCs ได้รับการถ่ายทอดพันธุกรรมของยีน mutant *HTT* และ *GFP* จากอสุจิของลิงวอกดัดแปลงพันธุกรรม โดยคุณสมบัติของเซลล์ต้นกำเนิดตัวอ่อนถูกตรวจสอบด้วย specific makers การเหนี่ยวนำให้เซลล์เปลี่ยนแปลงไปเป็นเซลล์ประสาทและการเกิดเนื้องอกหลังจากการปลูกถ่ายเซลล์ต้นกำเนิด rHD-ESCs ลงในสมองหนู SCID การแสดงออกของ *GFP* ใน rHD-ESCs ตรวจสอบด้วยกล้องจุลทรรศน์ fluorescent และการแสดงออกของ mutant *HTT* ถูกตรวจสอบด้วยวิธี quantitative real time PCR (Q-PCR), DNA sequencing และ immunocytochemistry การแสดงออกของ mutant *HTT* ใน rHD-ESCs ยังพบว่ามีการแสดงออกของ intranuclear inclusions ซึ่งเป็นรอยโรคที่จำเพาะต่อ HD นอกจากนี้ยังพบ polyQ region ใน rHD-ESCs และในอสุจิของ rHD-1 อีกด้วย จากการศึกษาการถ่ายทอดพันธุกรรมของโรคในการศึกษานี้สามารถนำไปสู่การผลิตลิงจากลิงที่มี mutant *HTT* เพื่อนำไปใช้ศึกษาพัฒนาการของโรคในระยะยาว ซึ่งเป็นประโยชน์ต่อการพัฒนาตัวบ่งชี้ทางชีวภาพของโรคใหม่ๆ และนำไปสู่กระบวนการรักษาได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 25556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

KITTIPHONG PUTKHAO : PRODUCTION OF TRANSGENIC HUNTINGTON'S BLASTOCYST FROM TRANSGENIC HUNTINGTON'S RHESUS MONKEY SPERM AND DERIVATION OF EMBRYONIC STEM CELL LINES. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 110PP.

HUNTINGTON'S DISEASE/RHESUS MONKEY/EMBRYONIC STEM CELL

A transgenic Huntington's rhesus monkey (rHD1) has been reported that to carry the mutant *huntingtin (HTT)* gene with expanded polyglutamine (CAGs; polyQ) repeats, and it develop chorea, dystonia, and other involuntary motor deficiencies similar to HD. In this study, the germline transmission of rHD1 was demonstrated by using rHD1 sperm produced embryo by intracytoplsmic sperm injection (ICSI) technique. Subsequently, the embryos were used for derivation of rHD1 embryonic stem cells (rHD-ESCs). The rHD-ESCs inherited mutant *HTT* and green fluorescent protein (*GFP*) genes through the gametes of rHD1. Pluripotency of rHD-ESCs was determined by the expression of stem cell specific markers, *in vitro* neural differentiation, and the formation of teratoma in immune compromised mice. Expression of *GFP* in rHD-ESCs was confirmed by fluorescent microscopy, and the expression of mutant *HTT* was determined by quantitative real time PCR (Q-PCR), DNA sequencing, and immunocytochemistry. Mutant *HTT* and form intranuclear inclusion, a classic cellular feature of HD, were observed in rHD-ESCs. Additional expansion of the pathogenic polyQ region was also observed in rHD-ESCs and HD gametes. The confirmation of transgene inheritability in this study advances the

establishment of a cohort of monkeys with inherited mutant *HTT* for longitudinal disease analysis, and they could be useful for development of novel biomarkers and therapeutics.

School of Biotechnology

Academic Year 2012

Student's Signature _____

Advisor's Signature _____

Co-advisor's Signature _____

ACKNOWLEDGEMENTS

This work was supported by the Royal Golden Jubilee (RGJ) Ph.D. Program of Thailand Research Fund cooperated with Suranaree University of Technology and the part of the work that was done at the Yerkes National Primate Research Center, Emory University Medical School, USA.

I would like first to express my deepest gratitude and my sincerest appreciation to my Ph.D. supervisor, Assoc. Prof. Dr. Rangsun Parnpai, for his patience, warm encouragement and thoughtful guidance on my Ph.D. study and provided me a good scholarship that led me to the valuable experiences throughout the entire Ph.D. study. I would have been lost without him. I would also like to express my deeply-felt thank to my co-advisor, Assoc. Prof. Dr. Anthony W. S. Chan, for his enthusiasm, inspiration, and great effort s to provide me the clear and simple explanations on my thesis work. I would also like to thank my committee members, Assoc. Prof. Dr. Montarop Yamabhai, Assoc. Prof. Dr. Mariena Ketudat-Cairns, and Dr. Palada Tittabutr, for their valuable comments and partial corrections of this thesis. I wish to acknowledge to all the members in Chan's lab for discussions, technical support and recommendations: Dr. Dongming Zhao, Dr. Jannet Kocerha, Dr. Yan Xu, Jin-Jing Yang, In Ki Cho, Richard Carter, and Yuhong Liu at the Yerkes National Primate Research Center (YNPRC) as well as the veterinarian and animal resources staff at the YNPRC: Tim Chi and Adam Neumann for their thoughtful comments, assistance. My appreciation is also to Leslee Sinclair who had been kindly giving me many ideas and made me feel warm during my stay in Atlanta, GA, USA.

I would like to thank the member of Embryo Technology and Stem Cell Research Center, Suranaree University of Technology that support and encouragement me. I am indebted to my excellent colleagues, Dr. Chuti Laowtammathron, Dr. Chanchao Laotongpanich, Dr. Liang Yuan Yuan, Dr. Ye Danna, Ton Yoisungnern, Sumeth Imsoonthornruksa, Kanokwan Srirattana, Anawat Sangmalee, Nucharin Sripunya, Kwanrudee Keawmungkun, Wanwisa Phewsoi, in their beneficial advice to improve this work.

Most importantly, I would like to thank my parent, my brother and sister for having given me the opportunity to pursue my graduate studies and for their love and support.

Kittiphong Putkhao

CONTENTS

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ART	=	Assisted reproductive technique
^{o}C	=	Degree Celsius
RT	=	Room temperature
AI	=	Artificial insemination
ESC	=	Embryonic Stem cell
NHPs	=	Non human primates
NHP-ESCs	=	Non human primate embryonic stem cells
TE	=	Trophectoderm
ICM	=	Inner cell mass
MFFs	=	Mouse fetal fibroblasts
HD	=	Huntington disease
HTT	=	Huntingtin
AD	=	Alzheimer disease
GFP	=	Green fluorescent protein
WT	=	Wild type
ml	=	Milliliter
µg	=	Microgram
µl	=	Microlitre
mM	=	Millimolar
g	=	Gram
µsec	=	Microsecond
min	=	Minute

LIST OF ABBREVIATIONS (Continued)

rpm	=	Round per minute
UV	=	Ultraviolet
LN_2	=	Liquid nitrogen

CHAPTER I

INTRODUCTION

1.1 Introduction

Regarding nonhuman primate (NHP) exhibit greater similarity to human with physiology and neurobiology, therefore, they are widely used in many part of research such as drug development, treatment, and vaccines for the promotion of better health for human and NHP alike (Kaplan, 2004).

Cryopreservation is a process that preserves cells or whole tissues by cooling temperatures, such as -80°C or −196°C. At low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, when cryoprotectant solutions are not used, the cells being preserved are often damaged due to freezing during the approach to low temperatures or warming to room temperature. Cryopreservation plays a key role in long-term cell preservation, while keeping the cells or tissue “alive”. Vitrification is a process by which the cells are rapidly frozen without the formation of intracellular ice crystals and represents a milestone in cryopreservation techniques. In vitrification certain membrane permeable reagents are being used to aid the process without changing the integrity of the cells. Disruption of intracellular organelles, as well as formation of intracellular ice crystal, is a major hazard of the freezing process, which may result in loss of cell viability.

Developing an effective method to cryopreserve rhesus monkey sperm would contribute to maintaining the genetic diversity of the research population by improving breeding management through artificial insemination or other assisted reproductive technologies (ART) (Wolf, 2004). Moreover, there is currently a need for populations of NHP with specified disease susceptible genotypes. As above information, the cryopreservation of sperm can provide an opportunity to preserve valuable animal genetics. Although, many studies of NHP sperm cryopreservation has been done, however, results difficult to compare between laboratories because many factors used were different, such as protocol of freezing and thawing, freezing extenders, types of cryoprotectant, and skill of investigators.

Embryonic stem cells (ESCs) are self-renewing pluripotent cells that are derived from the inner cell mass (ICM) of mammalian blastocyst and are able to differentiate into all adult cell types (Brivanlou et al., 2003). Pluripotent ESCs have been isolated from mice, human and many species of animals. *In vitro*, these cells can be differentiated to a diverse range of functional progenitor and terminally differentiated cells. Characterization of this capability has led to recognition of the roles of growth factors and cell-cell and cell-extracellular matrix interactions in the determination of cell fate. The development of increasingly more sophisticated differentiation protocols for the formation of mature cell types will extend the utility of ESCs to allow the production of cell populations, such as those enriched in hematopoietic cells, cardiomyocytes, and neuronal cells, for use in cell replacement therapies for human disease. Moreover, if stem cell can cooperate with other technology such as gene transmission, molecular technology and embryo production

will become advantage to study in human disease such as Huntington (HD), Alzheimer (AD) and Parkinson diseases (PD).

1.2 References

Brivanlou, A.H., Gage, F.H., Jaenisch, T., Jessell, T., Melton, D., and Rossant, J. (2003). Stem cells. Setting standards for human embryonic stem cells. **Science** 300: 913-916.

Kaplan, J.R. (2004). Introduction: modeling women's health with nonhuman primates and other animals. **ILAR J** 45: 83-88.

Wolf, D.P. (2004). Assisted reproductive technologies in rhesus macaques. **Reprod Biol Endocrinol** 2: 37.

CHAPTER II

REVIEW OF LITERATURES

2.1 Taxonomy and general information of rhesus monkey (*Macaca mulatta*)

Rhesus monkey or rhesus macaque (*Macaca mulatta*) is a native species that occupying a great diversity of altitudes throughout Central, South, and Southeast Asia. This species diverged from ancestors of *Homo sapiens* and shares a last common ancestor with human about 25 million years ago (Rhesus macaque genome sequencing and analysis consortium, 2007). Rhesus monkeys were used for an extraordinary range of biomedical and basic research. Due to their close genetic, physiologic, and metabolic similarity to humans, this species serves as an essential research tool in neuroscience, behavioral biology, reproductive physiology, neuroendocrinology, endocrinology, cardiovascular studies, pharmacology and many other areas (Rogers et al., 2002). The scientific classification of rhesus macaques are show in below;

Kingdom:	Animalia
Phylum:	Chordata
Class:	Mammalia
Order:	Primates
Family:	Cercopithecidae
Genus:	*Macaca*
Species:	*M. mulatta*

2.2 The overview of non human primate sperm cryopreservation

The cryopreservation procedures have been developed to maintain spermatozoa, oocytes and embryos in various species of animals. The sperm cryopreservation technology is a one of ART that could preserve spermatozoa in many species of animals including some endangered species. The obvious development of cryopreservation just began after discovery of glycerol as cryoprotectant which could prevent fowl sperm cell from freezing method (Polge et al., 1949). Then the glycerol was employed to use as cryoprotectant in sperm freezing in many species such as boar (Curry et al., 2000; Holt et al 2005; Boe-Hansen et al., 2008), cattle (Chaveiro et al., 2006; Saragusty et al., 2008; Seidel Jr et al., 2008; Vera-Munoz et al., 2009) horse (Ball and VO, 2001) goat (Dorado et al., 2007) including NHPs sperm. NHPs especially, rhesus and cynomolgus monkey were most widely used for laboratory animals in medical, biological, and sperm cryopreservation research because their anatomical and physiological are similar to human. In NHP, cryopreservation of spermatozoa has been reported for several decades, such as lemurs (Clavert et al, 1986), marmosets (Holt et al, 1994; Morrell, 1997; Morrell et al, 1998), squirrel monkey (Denis et al, 1976), african green (Roussel and Austin, 1967), patas (Roussel and Austin, 1967), vervet (Seier et al, 1993; Conradie et al, 1994), cynomolgus (Cho and Honjo, 1973; Mahone and Dukelow, 1978; Tollner et al, 1990; Sankai et al, 1994; Feradis et al, 2001; Li et al, 2003; Li et al., 2005; Li et al., 2006), lion-tailed (Cranfield et al, 1988), rhesus (Roussel and Austin, 1967; Leverage et al, 1972; Sanchez-Partida et al, 2000; Si et al, 2000, Si et al., 2004; Si et al., 2006; Leibo et al., 2007; Dong et al., 2008; Dong et al., 2009(a,b)), stumptail (Roussel and Austin, 1967), and tibetan (Chen et al, 1994), baboons (Kraemer and Vera Cruz, 1969); chimpanzees (Roussel

and Austin, 1967; Gould and Styperek, 1989; Younis et al, 1998; Kusunoki et al, 2001); and gorillas (Lambert et al, 1991; Lanzendorf et al, 1992; Pope et al, 1997). However, artificial insemination (AI) has not been successful by using frozen-thawed sperm in NHP and live births were only reported through either intrauterine insemination (Tollner et al., 1990 and Sanchez et al., 2000) or intracytoplasmic sperm injection (ICSI) (Yeoman et al., 2005). The failure of vaginal insemination with frozen sperm methods may indicate that frozen-thawed sperm was incapable of swimming up through the cervical canal to meet the egg which may be due to barriers created by the interaction of sperm with cervical mucus or length and complexity of the cervical canal of NHP that sperm must traverse (Taranta et al., 1990) or sperm frozen-thawed may cause subtle damage with no obvious effects on sperm motility. Thus, freezing and thawing method still need more improvement for sperm cryopreservation in NHP.

Although many studied have been done in NHP sperm cryopreservation but difficult to compare results between laboratories because factors used were different such as, freezing-thawing protocols, freezing extenders, types of cryoprotectant, and skill of investigators. The major impact that affect to NHP sperm cryopreservation could divided into two parts. (1) Indirect, by influencing of the sperm viability in original ejaculated such as season and method of sperm collection. (2) Direct, by affecting the ability of sperm to withstand mechanical injury during cooling and thawing such as type and proportion of cryoprotectant added

2.2.1 Season

The reproductive season of rhesus monkey occur between September to May (Leibo et al., 2007; National Primate Research Centre, California, CNPRC). Although, seasonality of semen production and ejaculation quality may occur,

however there are few studies on primate sperm cryopreservation include detail of season. During breeding season, free-ranging rhesus monkey produce semen which normal in volume, quality and physical characteristics, and whose cellular fraction consists of normal structural (Zamboni et al., 1974). In contrast, out of breeding season, the animals are difficult to ejaculate following electro-stimulation or they produce only minimal volumes of seminal fluid. These seasonal variations in semen production are expression of equally drastic changes in the seminiferous tubules lead to meiotic spermatocytes, spermatids and differentiating spermatozoa are exceptional and are mostly undergoing degeneration. However, normal semen parameters in ejaculates collected could achieved from animal outside of breeding season if an animal kept long term under laboratories conditions that reduced seasonal effect (Okamoto, 1994, Dong et al., 2008, 2009(b)). Except the seasonal effect, the time of semen collection during the day also affect semen quality. The early morning produces the best sample of semen collection because later morning the male may have already masturbated to the point of depleting sperm numbers in the ejaculate (Vandevoort, 2004)

2.2.2 Semen collection methods

Methods for semen collection in NHP are well established and have been in use for more than three decade in the National Primate Center in the United State. The most commonly used, method is penile electro-stimulation that results in high quality semen with good sperm count and sperm motility (Vandevoort, 2004).

The Rectal probe method, sperm has been used successfully in primate as well as many other species. The ejaculation occurred by stimulation of intermittent charges of 10-15 volts at a frequency of between 3-5 impulses per second by using a

monophasic alternating current of 60 cycles (Massubayashi et al., 1982). However, no births have resulted from AI technique with chimpanzee or marmoset monkey sperm obtained by rectal probe method (Gould and Young, 1996). Many factors may affect sperm quality such as anesthesia, accurate probe placement, and operator skill (Massubayashi et al., 1982).

Artificial vagina method is a natural ejaculation method by using dummies for collection. This method has been successful in chimpanzee (Hardin et al., 1974) and orangutans (VandeVoort et al., 1993) but difficult to perform in other species, which may be due to their greater intelligence. However, artificial vagina method is difficult to collect without contamination because the male did not controlled by chair restraint or anesthesia.

The Electro-stimulation method, the direct penile electro-stimulation method was pioneered by Mastroianne and Manson (1963) using metal foil electrodes and square wave pulses. This method was improved with the use of EKG gel electrodes, which greatly reduced the risk of burn injury (Sarason et al., 1991). Animals are not anesthetized for this procedure but are trained for chair restraint. Most reports indicate that larger semen volumes and higher numbers of sperm per ejaculate compared to the rectal probe method (Vandevoort, 2004). The direct penile method might achieves better stimulation of the entire reproductive tract and fewer problems with contamination.

2.2.3 Cryopreservation extenders

The addition of a cryoprotectant into the semen sample is needed in order to protect spermatozoa from cold shock. Numerous extenders in combination with different components have been used for minimizing a physical and chemical stresses

derived from cooling, freezing and thawing of sperm cells (Purdy, 2006). In general, the extenders for sperm cryopreservation can be divided into two types: (1) permeable and (2) non-permeable cryoprotectants.

2.2.3.1 Permeable cryoprotectant, is a compounds that use against cryoinjury of the cells during freezing and thawing processes. Intracellular ice crystals, osmotic and chilling injury are induced during temperature reduction that lead to sperm damages, namely cytoplasmic fracture, effects on the cytoskeleton and genome related structures. The major changes that occurs during freezing are ultrastructual, biochemical and functional, which impair sperm transport and survival in the female reproductive tract and reduces fertility (Salamon and Maxwell, 2000). Therefore, permeable cryoprotectants are useful because membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperature, reduced intracellular ice formation, and increased survival to cryopreservation (Holt, 2000). The most permeable cryoprotectants that are commonly used in sperm cryopreservation are glycerol, ethylene glycol, dimethyl sulfoxide (DMSO) and propylene glycol. Although, penetrating cryoprotectants are beneficial for sperm cryopreservation but the mechanism in freezing and thawing remain unknown. In NHP sperm cryopreservation; that used glycerol, ethylene glycol, DMSO and propylene glycol have been reported in gorillas (Gould and Styperek, 1989), baboon (Kraemer et al., 1969), squirrel monkey (Denis et al., 1976), cynomolgus monkeys (Feradis et al., 2001, Li et al., 2005, Li et al., 2006), chimpanzee (Younis et al., 1998), marmoset (Morrell et al., 1997), and rhesus monkeys (Si et al., 2004, Dong et al., 2009(a,b)). Some report found that glycerol and ethylene glycol are the best in post-thawed sperm result. In contrast, DMSO and acetamide were the worse than

glycerol and ethylene glycol but better than propylene glycol in cynomolgus monkey sperm cryopreservation (Li et al., 2005). In rhesus monkey sperm cryopreservation, the glycerol and ethylene glycol offer the best protection than DMSO and propylene glycol (Si et al., 2004). Previous studies indicate that the glycerol and ethylene glycol present less toxicity to the spermatozoa than other cryoprotectants.

2.2.3.2 Non-Permeable cryoprotectant, is a compounds that protect cells from cryoinjury like a permeable cryoprotectants but the chemicals unable pass through the cell membrane but attached onto cell membranes (Aisen et al., 2002) to reduce cells damage from swelling or osmotic shock during freezing and thawing process (Dong et al., 2009(a)). The non-penetrating cryoprotectants include; egg yolk, non-fat skimmed milk and different types of sugar. It is difficult to determine which non-penetrating cryoprotectants are the best for sperm cryopreservation because of variation in very adding to different types of sugar among species.

*Egg yolk***:** has been used in sperm cropreservation in many species, however, the mechanism against cryoinjury during freezing and thawing remain unclear. Recently, the interest on protective role of egg yolk has been resumed and several mechanisms involving in its function of sperm protection have been proposed (Bergeron et al., 2006). However, these hypotheses have not been tested and verified, and its protection mechanism remains elusive. Dong and colleague (2009(a)) reported that 20% and 30% egg yolk concentration in extender resulted in higher post-thawed sperm motility than 40% and 50% of egg yolk, especially at 1 hour after thawed in rhesus monkey sperm cryopreservation. Pace and Graham reported that egg yolk was the main protection component because bull sperm could survive after frozen in 20% egg yolk without permeable cryoprotectants (Pace and Graham, 1974).

Additionally, Dong and colleague (2009(a)) showed that rhesus monkey sperm could survive after frozen in 20%-40% concentration of egg yolk without glycerol.

*Sugar types***:** the supplement of sugars in extenders as a non-penetrating cryoprotectant has demonstrated beneficial effects on the cryosurvival of spermatozoa. Sugars provide cryoprotection outside sperm membrane by stabilizing cell membranes via the loose, electrostatic binding of saccharide hydroxyl groups to phosphate groups on the membrane lipid head and cell dehydration which reduces intracellular ice crystal formation (Chen et al., 1993). Sugar also prevent of liposome fusion and leakage during dehydration and rehydration (Crowe et al., 1986). In different cryoprotective effects have been suggested for different sugar (Yildiz et al., 2000). In NHP sperm cryopreservation, most commonly used of sugars are glucose, lactose, and raffinose (Morrell and Hodges, 1998). Monosaccharide (glucose) and disaccharide (lactose) showed more effective than trisaccharide (raffinose) in post-thawed sperm motility, plasma membrane function, and acrosome integrity (Si et al., 2004). On the other hand, some types of sugars damage the spermatozoa acrosome during freezing and thawing. In freezing of dog spermatozoa, glucose, lactose, and raffinose increase the percentage of damaged acrosome after freezing and thawing but fructose, galactose, xylose, treharose, maltose, and sucrose reduced percentage of damaged acrosome (Yildiz et al., 2000). Furthermore, the combination of sugars for sperm cryopreservation has been tested. Yildiz and colleague (2000) reported that the combination between monosaccharide, disaccharide, and trisaccharide of sugars could provide better protection when compared with trisaccharide alone and the combination of monosaccharide and disaccharide provide better protection than monosaccharide or disaccharide alone. These results indicate that the cryoprotective effects of sugars may species dependent.

2.2.4 Cryopreservation methods

In general, there are three freezing methods that commonly used in NHP sperm cryopreservation; these methods are (1) programmed freezing (slow freezing), (2) straw freezing, and (3) pellet freezing method (Morrell and Hodges, 1998). The programmed method used low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock (Barbas and Mascarenhas, 2009). The cost is higher than other methods because its need freezing machine. Freezing in pellet and straw are rapid with reduced cold shock during freezing. The straw freezing has been widely used in sperm cryopreservation (Tollner et al., 1990, and Sankai et al., 1994). The comparison of the three freezing methods in NHP spermatozoa cryopreservation has been reported by Morrell, (1997). They found that there was no difference among the three freezing method in post-thawed sperm motility in marmoset sperm. However, in cynomolgus and rhesus monkey post-thawed sperm motility are varies. In cynomolgus and rhesus monkey performed by straw freezing technique were 50.03% and 44.69% sperm motility, respectively (Li et al., 2006) but in pellet freezing technique showed higher (85.0%) sperm motility in rhesus monkey sperm cryopreservation (Sanchez-Partida et al., 2000). The reason for differences in sperm motility reported by Morrell (1997), Sanchez-Partida et al., (2000), and Li et al., (2006) need to be investigated further.

2.2.4.1 Cooling rates, are most critical variable influence to sperm cryosurvival and prerequisite for an optimal sperm cryopreservation protocol. Difference species of spermatozoa respond differently to cooling and thawing (Grobfeld et al., 2008). The cooling rate is depending on the freezing procedure. *Programmable freezer;* is convenient and appropriate for freezing in large quantities

of sperm and involves the use of computerized automatic cell freezer where various cooling rates can be preset such as freezing straws in one step by freezing at −80 ^{o}C for 7–15 min and then plunging the straws into liquid nitrogen or customized by operator such as 4 to −5^{o}C at 4 ^{o}C/min, −5 to −110 ^{o}C at 25 ^{o}C/min and −110 to −140 ^{o}C at 35 ^{o}C/min and then plunged straw into liquid nitrogen (Baebas et al., 2006). *Pellet freezing*; is rapid and inexpensive but inventory management is problematic because the actual sperm samples cannot be labeled (Purdy, 2006). Once the sperm sample is cooled, aliquots 0.2 ml are disperse into indentation on the well of dry ice (-80◦C) for 60-90 second, then transferred and store in liquid nitrogen (Sanchez et al., 1992). The pellet freezing method could result 85% of post-thaw sperm motility in rhesus monkey (Sanchez et al., 2000). *Straw freezing;* refer to loading extended semen into plastic straws and seal the tip which are then place in liquid nitrogen vapor for specified time before plunging into liquid nitrogen. The cooling temperature of this method are varies by space between straw and liquid nitrogen surface. The straw freezing is a solidification of solution at low temperature; the solution will cool and changes into a glassy and not created ice crystal by used extreme elevation in viscosity during cooling. The straw freezing is commonly used in NHP sperm cryopreservation. Dong and colleague reported that high post thaw sperm motility in rhesus monkey was observed by using freezing straw procedure (Dong et al., 2009(a,b)). While also achieved in cynomolgus monkey sperm cryopreservation (Li et al., 2005, Li et al., 2006).

2.2.4.2 Thawing rates, is determined by the method that used to freeze sperm. The cooling rate has been sufficiently high to induce intracellular freezing or low enough to produce cell dehydration. The fast thawing is required to prevent re-

crystallization of any intracellular ice present in the spermatozoa. (Barbas and Mascarenhas, 2009). Sperm pellets should thaw in a dry test tube at 37^{o}C while the thawing straws may be performed using various methods (Evans and Maxwell, 1987). Traditionally, a straw is thawed by placing it in a 37^{o}C water bath for 30 second to 1 min. In NHP sperm cryopreservation, various thawing temperatures have been used. The rapid (37^{o}C water bath for 30s or 75^{o}C water bath for 5s) and slow thawing method (straw were left in goblets full of liquid nitrogen and the let liquid nitrogen bubbled off from goblets at room temperature (RT) for 10 min) in rhesus sperm cryopreservation has been tested. The rapid thawing showed higher post-thawed sperm motility than slow thawing method (Dong et al., 2009(a)). These results indicate that rapid thawing would aid recovery of motile sperm.

The attention of temperature and timing becomes much more critical in fast thawing rate because it exposes during less time to the concentrated solute and cryoprootectant, and restoration of the intra and extracellular equilibrium is more rapid than with slower thawing (Fisher et al., 1987).

2.3 Non human primate embryonic stem cells

Embryonic stem (ES) cells are cells derived from an early embryonic stage during which the cellular machinery is geared toward rapid expansion and diversification, simultaneously possess both the ability for unlimited self-renewal and the potential to differentiate into derivatives of all three germ layers. Even after months and years of growth in the laboratory, ES cells retain the ability to differentiate to different cell types, including muscle cells, heart cells, neurons, and hematopoietic cells (Thomson et al., 1998; Amit et al., 2000).

The fist murine ES cells was prepared over 30 years ago (Evans and Kaufman, 1981; Martin, 1981) that paved the future success in the isolation of primate ES cells (Thomson et al., 1995) and human ES cells in 1998 (Thomson et al., 1998). Much of the anticipated surrounding human ES cells were extrapolated from pioneer experiments in the mouse system. However, recent results in human ES cells have supported the notion that human ES cells will have an important impact on medical science. These advances include the differentiation of human ES cells into various types, comprising neurons, cardiac cells, vascular cells, hematopoietic cells, pancreatic cells, hepatic cells, and placental cells (Keller, 2005). The derivation of new ES cells lines under various conditions (Cowan et al., 2004; Ludwig et al., 2006); and the establishment of protocols that allow genetic modification of these cells (Eiges et al., 2001; Zwaka and Thomson 2003). Although, human ES cells offer tremendous potential in regenerative medicine; traditional method may animal derive product that impact clinical application of ES cell.

In NHP-ES cells establishment have been reported in the common marmoset (*Callithrix jacchus*) (Thomson et al., 1996), cynomolgus monkey (*Macaca fascicularis*) (Suemori et al., 2001), and baboon (*Papio ursinus*) (Simerly et al., 2009), and rhesus monkey (Thomson et al., 1995, Kuo et al., 2003, Mitalipov et al., 2006, Byrne et al., 2007, Navara et al., 2007, Rajesh et al., 2007, Dighe et al., 2008, Wianny et al., 2008, and Laowtammathron et al., 2010).

ESC lines from different species were claimed to exhibit different properties 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 ESC colonies

vary in different animal species such as mouse ESCs 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 ESCs are differ from the murine ESCs in the expression pattern of stem cell markers and cytokine factors that are important for maintaining at undifferentiated stage (Table 2.1).

2.3.1 Extrinsic factors maintenance of undifferentiated state of ES cells

2.3.1.1 Leukemia Inhibitory Factor (LIF)

When mouse ES cell lines derived in the early 1980s, the cells were propagated in co-culture with feeder layer cells (Evans and Kaufman, 1981; Martin, 1981). This methodology was adopted from the protocols for embryonal carcinoma (EC) cells. Feeder cells support ES cells through the secretion of a signal that prevents differentiation. While, ES cells can be maintained in feeder cells condition medium. Fractionation of conditioned media has led to the identification of LIF in culture media can substitute the need of co-culture with feeder layer cells (Smith et al. 1988; Williams et al., 1988).

LIF belongs to the family of interleukin 6 (IL-6)-type cytokines (Rose-John, 2002). This family of cytokines stimulates cells through the gp130 receptor, which works as a heterodimer together with a ligand specific receptor (LIF-R). Activation of gp130 leads to the activation of the Janus associated tyrosine kinase (JAK) and to activation of signal transducer and activator of transcription (STAT) proteins, their translocation to the nucleus, binding to DNA and subsequent activation of transcription.

The ability of LIF to prevent differentiation of mouse ES cells is dependent upon activation of STAT3. In addition to the activation of STAT3, at undifferentiated state, LIF also activates ERKs (extracellular receptor kinases) (Burdon et al., 1999). The activation of ERKs promotes differentiation. Thus, the balance between the activation of STAT3 and ERK signals determines the fate of the ES cell.

In human and monkey ESCs, co-culture with feeders is requires while the addition of LIF is not necessary (Table 2.2). LIF does not act on human cells (Layton et al., 1994) and other factors secreted from the mouse embryonic feeder (MEF) cells must be presence in order to maintain pluripotency. LIF inability responds to LIF signaling pathway in human and monkey ESCs but rather than activation of STAT3 pathway which in mouse ES cells is sufficient for self-renewal (Daheron et al., 2004).

2.3.1.2 Bone morphogenetic protein 4 (BMP4)

BMP4 is a member of the TGF-beta super family of polypeptide signaling molecules. BMP4 was suggested to be involved in the prevention of mouse ESC differentiation (Ying et al. 2003). Although LIF is sufficient to prevent mouse ESC differentiation in the presence of serum, when serum is removed, even in the presence of LIF, neural differentiation occurs. Since serum contains unknown factors and some of them prevent ESC differentiation. Addition of BMP4 to the media support serum-free culture but only in the presence of LIF, other, spontaneous non-neural lineage differentiation occurs. Withdrawal of both BMP4 and LIF results in neural differentiation. Therefore LIF is needed to maintain the undifferentiating capacity of BMP4, which in the absence of LIF actually drives the cells to differentiate.

Table 2.1 Comparison of murine, monkey and human ESCs properties.

Pluripotent marker	**Murine ESC**	**Monkey ESC**	**Human ESC**
AP	+	+	+
Oct-4	+	+	+
SSEA-1	+	-	-
SSEA-3	-	+	+
SSEA-4	-	+	+
TRA1-60	-	+	+
TRA1-81	-	+	+
Feeder cell dependent	Yes	Yes	Yes
Factor which aid in stem cell self-renewal	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	N/A

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

Moreover, BMP4 supports self-renewal of mouse ESC through the activation of Smad proteins. Another report showed that BMP4 also supports self-renewal by inhibition of ERK and p38 mitogen-activated protein kinase (MAPK) (Qi et al. 2004). However, the addition of BMP4 to the media does not enhance self-renewal of mouse ESCs. In fact, BMP4 drives the cells to differentiate to trophoblast cells (Xu et al. 2002). The difference in the abilities to create trophoblast has raised the notion that human and mouse ESCs may be derived from different stages in development, and thus their self-renewal capacity may be affected by different factors

2.3.1.3 Basic fibroblast growth factor (bFGF)

bFGF has been shown to be required for the routine culture of human and monkey ESCs in the presence of serum replacement (Table 2.2) (Amit et al. 2000). In serum-free media without with bFGF, spontaneous differentiation occurs. In addition, bFGF enhances the cloning efficiency of the cells (Amit et al. 2000). Although, the addition of bFGF is not required for the propagation of mouse ESCs, it was shown to be important for different pluripotent cells between human or monkey with mouse. bFGF is needed to convert the transient proliferating population of primordial germ cells to an indefinitely proliferating population of EG cells (Matsui et al. 1992; Resnick et al. 1992). However, in the case of mouse EG cells, once the EG cells culture is established, bFGF is no longer (Matsui et al. 1992). Investigations that examined the expression of different elements of signaling pathways in human ESCs have shown the presence of elements of FGF signaling, including all four FGF receptors and certain components of their downstream cascade, which are enriched in undifferentiated human and monkey ESCs in comparison to their differentiated derivatives (Bhattacharya et al. 2004; Dvash et al. 2004; Ginis et al. 2004; Rao and

Stice 2004). bFGF is expressed by human ESCs, but apparently its level is not enough to prevent differentiation.

Table 2.2 Cytokines required for ESCs self-renewal.

Protein	Mouse ESCs	Human ESCs	Monkey ESCs
LIF	Replaces the need for feeder cells	No apparent role in self-renewal	No apparent role in self-renewal
BMP-4	Replaces the need for serum presence	Drives to trophectoderm differentiation	Drives to trophectoderm differentiation
bFGF	Not required for their propagation	Required for propagation in serum replacement media	Required for propagation in serum replacement media

(Adapted from Darr and Benvenisty, 2004).

2.4 Huntington's disease (HD)

2.4.1 General information of Huntington's disease

Huntingtons's disease also known as Huntington's chorea was discovered by George Huntington (1872). The global incidence of HD varies from 3-7 per 100,000 people of Western European and 1 per 1,000,000 people of Asia and Africa. HD is an autosomal dominant inherited neurodegenerative disorder featuring progressive development of chorea, psychiatric disturbances and cognitive impairment due to neuronal cells loss in the basal ganglia and the cerebral cortex. HD patient has uncontrollable of body movement, which include continues and irregular twisting and jerking movement. HD is more common as adult onset while juvenile (Hannan, 2004).

Abnormal polyglutamine (CAG) extensions in the IT-15 (interesting transcript number 15) gene located at chromosome encode the *huntingtin* (*HTT)* protein is a major cause of HD (Nance, 1996). The extend of CAG repeat determine early juvenile or late onset (adult) of HD. Normal population has 37 CAG repeats, while in HD patients has more than 37 CAG repeats (Li et al., 1999). Huntingtin is expressed ubiquitously, with the highest levels found in the brain, particularly in cortical layers II and V and in the Cerebellar Purkinje cells (Difiglia et al., 1995). HD characterization cellular pathology of HD includes the aggregation of mutant *HTT* that lead to the formation of intranuclear aggregation or called the inclusion bodies (IBs). One possible cases is the involvement of the caspase partway that cleaved mutant *HTT* to fragment and form inclusion. Another HD characterization is the death of striatum and cortical neuron

2.4.2 Huntington's disease pathways

The wild-type (WT) *HTT* is cleaved, remains in cytosol associated with vesicles and microtubules that function as cytoskeleton anchor or the transport of mitochondria (Hoffner et al., 2001). The mutation of *HTT* causes a conformational change and abnormal folding of the protein, which affect the nucleus and mitochondria of neural cell. The *HTT* protein interacts with other proteins, HIP1 (Huntingtin's I interactor Protein-1) and HAP1. The number of CAG repeats in the *HTT* gene determines how the *HTT* protein interacts with HIP1 and HAP1. As repeat numbers increase, *HTT* protein binds less to HIP1 and more to HAP1. Free HIP1 binds to a hitherto unknown polypeptide, HIPPI (HIP1 Protein I interactor), which has partial sequence homology to HIP1 and similar tissue and subcellular distribution. The availability of free HIP1 is modulated by polyglutamine length within *HTT*, with disease-associated polyglutamine expansion favouring the formation of pro-apoptotic

HIPPI-HIP1 heterodimers. This heterodimer can recruit procaspase 8 into a complex of HIPPI, HIP1 and procaspase 8, and launch apoptosis through components of the extrinsic cell-death pathway (Steffan et al., 2000). Therefore, neuron communication by neurotransmitter would not occur smoothly (Figure 2.1).

The expanded polyglutaminein *HTT* alters protein conformation, resulting in aberrant protein interactions, including interactions of the expanded polyglutamine with cellular proteins containing short polyglutamine stretches. CBP (CREB Binding Protein) is a co-activator for CREB (cAMP Response Element-Binding Protein)-mediated transcription and CREB-mediated gene transcription promotes cell survival, and CBP is a major mediator of survival signals in mature neurons by interacting with different transcription factors including histone acetylase. The expanded CAG interact with the short glutamine repeat in CBP, interfering with CBP function, causing transcriptional abnormalities (Figure 2.1) and leading to cellular toxicity. Mutant Huntingtin also interacts directly with the acetyltransferase, blocking this activity lead to transcriptional repression that in turn leads to neurodegeneration (Steffan et al., 2000).

2.4.3 The effect of gender on Huntington's disease

Gender is known to influence the transmission of trinucleotide repeats in human disease. However, the molecular basis for the parent of origin effect associated with trinucleotide repeat expansion is not known. Meritt et al (1969) first observed that disproportionate number of HD cases inherited from fathers. Although, meiotic instability may occur in both maternal and paternal transmission, in paternal transmission there is propensity toward larger repeat expansion. For maternal transmission, nearly equal numbers of expansion and contractions are seen, and the

shift are small ranging from 1 to 3 repeats but in the paternal transmission are prone to large increase in size (Duyao et al., 1993). Which corresponding with Chong et al, (1997) reported that alterations in the length of trinucleotide repeat after transmission are distinctly dependent on the gender of the transmitting parent.

Kovtun and colleagues (2000) had been studied the effect of gender on CAG repeats instability by using mouse model, the results showed that the CAG tend to expand in male and contract in female offspring even though the disease allele is inherited from the same father. Although, the male and female offspring have difference CAG repeat expansion, but gender dependent repeat change cannot be originated from events in the germ cell. Repeat length might be influenced by post-zygotically in the embryos. Because of if the germ cell of the father provides the information for the repeat length in the progeny, then the repeat size in the progeny should reflect the frequency and distribution of repeat size found in the sperm of the father but distribution of repeat length in the progeny did not faithfully reflect the distribution of repeat present in the sperm of the founding fathers. These data supported that the expansion and contraction of CAG repeats in the progeny is influenced by the gender of the embryo. Although HD shows a paternal bias for transmission of larges expansion, however, the mechanism of parent of origin effect for this disease has, until recently, remained elusive.

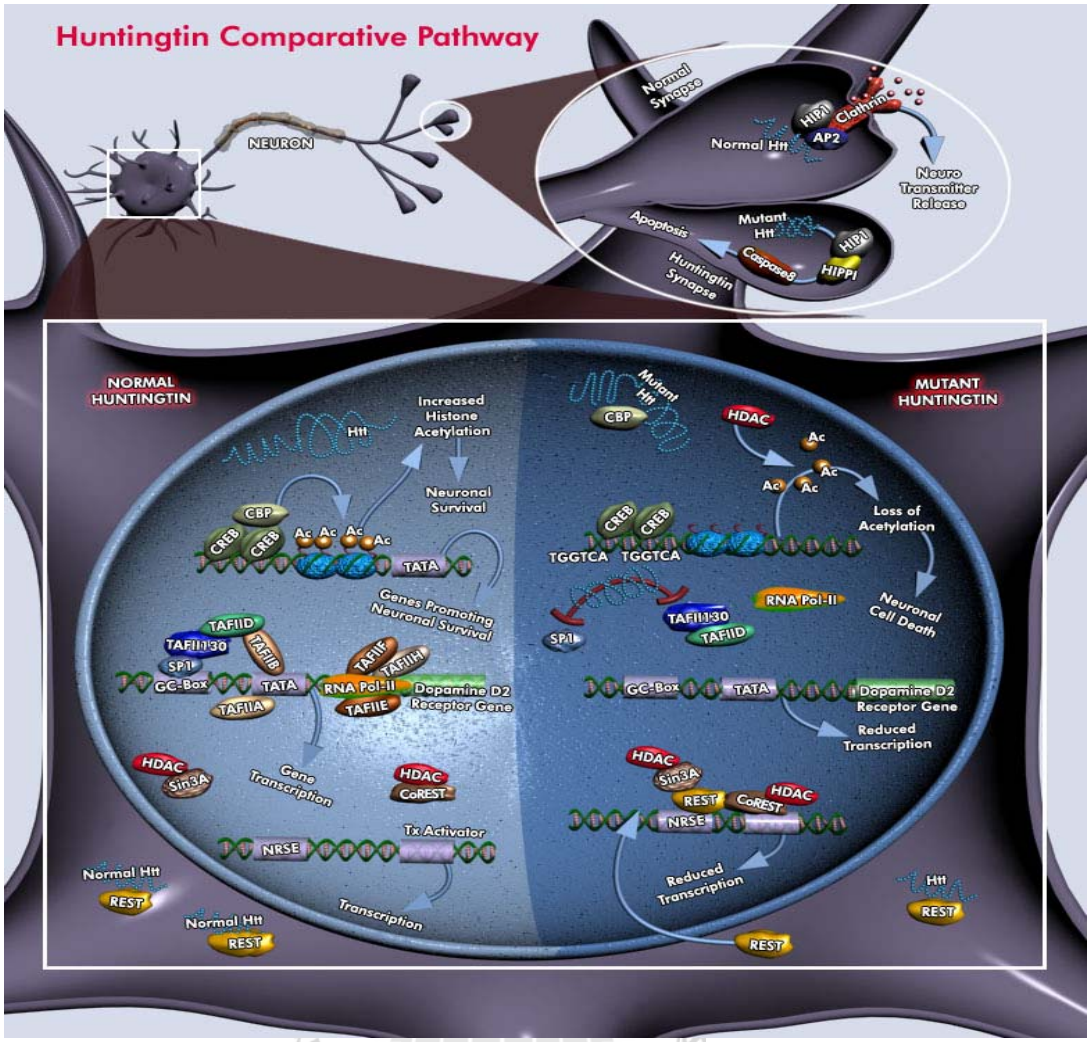


Figure 2.1 The functions of the normal and mutant huntingtin protein. In normal huntingtin, it can form a complex with the proteins Hip1, clathrin and AP2, which crucial in allowing neurons to communicate by secreting neurotransmitters. In HD patient, the expended CAG weakens *HTT*'s interaction with Hip1, which is then free to bind the protein Hippi, and so to activate apoptosis through caspase-8. The expanded CAG also interfering CBP function, causing transcriptional abnormalities and it interacts directly with the acetyltransferase that lead neurodegeneration. (https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=232).

2.4.4 CAG expansion in germ cells of HD

The analysis of testicular cells of human HD patients revealed that high frequencies of repeat expansion of considerable magnitude were present in mitotic diploid germ cells before meiosis (Yoon et al., 2003). This instability occurred in at least two phases; pre-meiotic and post-meiotic cells. However, the proportion of large expansions in post-meiotic spermatids or spermatozoa was greater supporting that expansion events occurred before and possibly after meiosis (Pearson, 2003). The CAG expansion event must occur at some point between segregation of the primordial germ cells, development to puberty, or during the life long post puberty spermatogonial stem cell division (Figure 2.2). If CAG expansion events occur only during post-pubertal cycling of spermatogonia, sperm produced at advanced ages would have larger expansions than sperm produced at the onset of puberty. A limitation of CAG repeat to cycling spermatogonia would be expected to show paternal age effect on the progress of CAG expansion in HD progeny, as has been observed in transgenic mice (Savouret et al., 2003). However, some report showed that, the inability to detect a paternal age effect in HD affected fathers with CAG instability is likely paucity effect of transmission by older HD parents (Leeflang et al., 1999). Thus, difficult to prove the paternal age effect is affect to HD CAG instability or its association with mutations occurring during spermatogonial cycling.

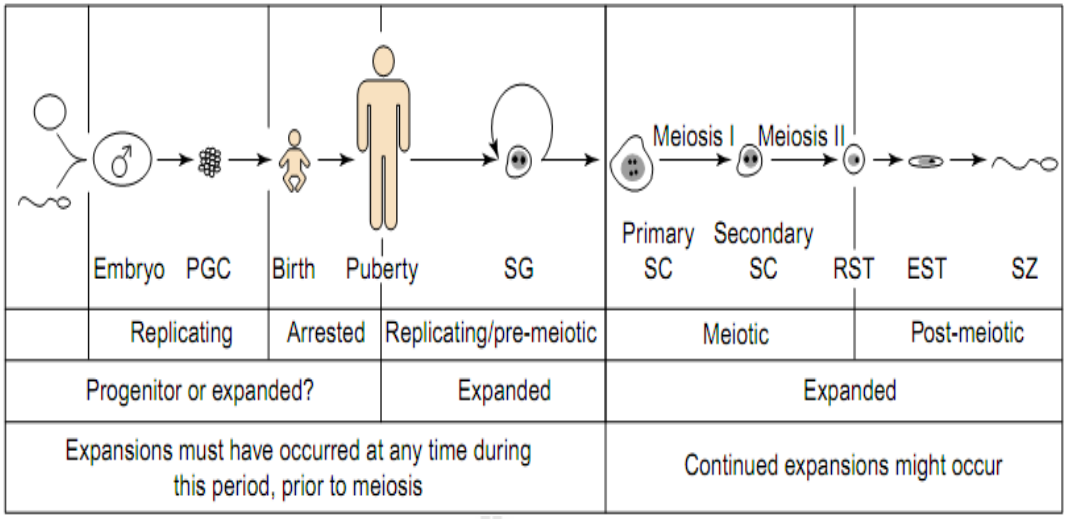


Figure 2.2 Expansion of Huntington CAG repeats during spermatogenesis. Instability of CAG repeat can occur in primordial germ cells through to fertilization and post-natal growth. As expansion products are evident in pre-meiotic diploid cells. Further expansions can involve meiotic recombination or post meiotic genome maintenance. The high number of life-long spermatogonial cell divisions might drive the paternal mutation bias of HD. Abbreviations: PGC, primordial germ cell; SG, spermatogonia; SC, spermatocyte; RST, round spermatid; EST, elongating spermatid; SZ, spermatozoa (Pearson, 2003).

2.5 References

Aisen, E.G., Medina, V.H., and Venturino, A. (2002). Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. **Theriogenology** 57: 1801-1808.

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

Ball, B.A., and VO, A. (2001). Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. **J Androl** 22: 6.

Barbas, J. P., Marques, C.C., Baptista, M.C., Vasques, M.I., Pereira, R., and Cavaco, G.A. (2006). Reproduction in the Serrana goat breed: seasonal and individual factors affecting fresh and frozen semen performance, in vivo and in vitro fertility. In: Animal products from the Mediter-ranean Area. Edited by JMC Ramalho Ribeiro, AEM Horta, C Mosconi and A Rosati. **EAAP Publication** 119: 337-342.

Barbas, J.P., and Mascarenhas, R.D. (2009). Cryopreservation of domestic animal sperm cells. **Cell Tissue Bank** 10: 49–62.

Bergeron, A., and Manjunath, P. (2006). New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. **Mol Reprod Dev** 73: 1338-1344.

Bhattacharya, B., Miura, T., Brandenberger, R., Mejido, J., Luo, Y., Yang, A.X., Joshi, B.H., Ginis, I., Thies, R.S., Amit, M., Lyons, I., Condie, B.G., Itskovitz-

Eldor, J., Rao, M.S., and Puri, R.K. (2004). Gene expression in human embryonic stem cell lines: unique molecular signature. **Blood** 103: 2956-2964.

Boe-Hansen, G.B., Christensen, P., Vibjerg, D., Nielsen, M.B.F., and Hedeboe, A.M. (2008). Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. **Theriogenology** 69: 728-736.

Burdon, T., Stracey, C., Chambers, I., Nichols, J., and Smith, A. (1999). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. **Dev Biol** 210: 30-43.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., Nelson, M., Sanger, W.G., Gokhale, S., Wolf, D.P., and Mitalipov, S.M. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. **Nature** 450: 497-502.

Chaveiro, A., Liu, J., Engel, B., Critser, J.K., and Woelders, H. (2006). Significant variability among bulls in the sperm membrane permeability for water and glycerol: Possible implications for semen freezing protocols for individual males. **Cryobiology** 53: 349-359.

Chen, J.C., Ji, W.Z., Yang, S.C., and Shi, L.M. (1994). Semen cryopreservation in the Tibetan macaque (*Macaca thibetana*) comparison of different cooling programs and freezing media. **Acta Zoologica Sinica** 40: 174-181.

Chen, Y., Foote, R.H., and Brockett, C.C. (1993). Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. **Cryobiology** 30: 423-431.

Cho, F., and Honjo, S. (1973). A simplified method for collecting and preserving cynomolgus macaque semen. **Jpn J Med Sci Biol** 26: 261-268.

Chong, S.S. (1997). Contribution of DNA sequence and CAG size to mutation frequencies of intermediate alleles for Huntington disease: evidence from single sperm analysis. **Hum Mol Genet** 6: 301-309.

Clavert, A., Cranz, C., Brun, B., and Rumpler, Y.A (1986). Method for sperm cryopreservation of the lemur fulvus. **Primate Rep** 14: 101.

Conradie, E., Oettle, E.E., and Seier, J.V. (1994). Assessment of acrosomal integrity of vervet monkey spermatozoa after cryopreservation. **J Med Primatol** 23: 315-316.

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

Cranfield, M.R., Kempske, S.E., and Schaffer, N. (1988). The use of in vitro fertilization and embryo transfer techniques for the enhancement of genetic diversity in the captive population of the lion-tailed macaque. **Int Zoo Yearbook** 27: 149-159.

Crowe, L.M., Crowe, J.H., Womersley, C., Reid, D., Appel, L., and Rudolph, A. (1986). Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. **Biochim Biophys Acta** 861: 131-140.

Curry, M.R., Kleinhans, F.W., and Watson, P. F. (2000). Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. **Cryobiology** 41: 167-173.

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

Denis, L.T., Poindexter, A.N., Ritter, M.B., Seager, S.W.J., and Deter, R.L. (1976). Freeze preservation of squirrel monkey sperm for use in timed fertilization studies. **Fertil Steril** 27: 723-729.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J.P., Carraway, R., Reeves, S.A., Boyce, F.M., and Aronin, N. (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. **Neuron** 14: 1075-1081.

Dighe, V., Clepper, L., Pedersen, D., Byrne, J., Ferguson, B., Gokhale, S., Penedo, M.C.T., Wolf, D.P., and Mitalipov, S.M. (2008). Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes. **Stem Cells** 26: 756-766.

Dorado, J., Rodriguez, I., and Hidalgo, M. (2007). Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. **Theriogenology** 68: 168-177.

Dong, Q., Rodenburg, S.E., Huang, C., and VandeVoort, C.A. (2008). Cryopreservation of rhesus monkey (*Macaca mulatta*) epididymal spermatozoa before and after refrigerated storage. **J Androl** 29: 283-292.

Dong, Q., Hill, D., and VandeVoort, C.A. (2009(b)). Interactions among pre-cooling, cryoprotectant, cooling, and thawing for sperm cryopreservation in rhesus monkeys. **Cryobiology** 59: 268-274.

Dong, Q., Correa, L.M., and VandeVoort, C.A. (2009(a)). Rhesus monkey sperm cryopreservation with TEST-yolk extender in the absence of permeable cryoprotectant. **Cryobiology** 58: 20-27.

Duyao, M., Ambrose, C., Mters, R., Noveletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., and Abbott, M. (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. **Nat Genet** 4: 387-392.

Dvash, T., Mayshar, Y., Darr, H., McElhaney, M., Barker, D., Yanuka, O., Kotkow, K.J., Rubin, L.L., Benvenisty, N., and Eiges, R. (2004). Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. **Hum Reprod** 19: 2875-2883.

Eiges, R., Schuldiner, M., Drukker, M., Yanuka, O., Itskovitz-Eldor, J., and Benvenisty, N. (2001). Establishment of human embryonic stem cell-transfected clones carrying a maker for undifferentiated cells. **Curr Biol** 11: 514-518.

Evans, G., and Maxwell, W. (1987). Salamon's artificial insemination of sheep and goats. **Butterworth, Sydney** 194.

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

Feradis, A.H., Pawitri, D., Suatha, I.K., Amin, M.R., Yusuf, T.L., and Sajuthi, D. et al. (2001). Cryopreservation of epididymal spermatozoa collected by needle biopsy from cynomolgus monkeys (*Macaca fascicularis*). **J Med Primatol** 30: 100-106.

Fisher, P.S., Ainsworth, L., and Fairfull, R.W. (1987). Evaluation of a new diluent and different processing procedures for cryopreservation of ram semen. **Theriogenology** 28: 599-607.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Car-penter, M.K., Itskovitz-Eldor, J., and Rao, M.S. (2004). Differences between human and mouse embryonic stem cells. **Dev Biol** 269: 360-380.

Gould, K.G., and Styperek, R.P. (1989). Improved methods for freeze preservation of chimpanzee sperm. **Am J Primatol** 18: 275-284.

Gould, K.G., and Young, L.G. (1996). Functional parameters of chimpanzee Pan troglodytes sperm from ejaculates collected by rectal probe electrostimulation and by artificial vagina. **Am J Primatol** 39: 115-122.

Großfeld, R., Sieg, B., Struckmann, C., Frenzel, A., Maxwell, W.M.C., and Rath, D. (2008). New aspects of boar semen freezing strategies. **Theriogenology** 70: 1225-1233.

Hannan, A.J. (2004). Molecular mediators, environmental modulators and experience-dependent synaptic dysfunction in Huntington's disease. **Acta Biochimica Polonica** 51: 415-430.

Hardin, C., Leigherr, G., and Fairchild, O. (1974). Artificial Insemination in Chimpanzees. **International Zoo Yearbook** 15: 132-134.

Hoffner, G., Kahlem, P., and Djian, P. (2001). Perinuclear localization of huntingtin as a consequence of its binding to microtubules through an interaction with β-tubulin: relevance to Huntington's disease. **J Cell Sci** 115: 941-948.

Holt, W.V., Medrano, A., Thurston, L.M., and Watson, P.F. (2005). The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. **Theriogenology** 63: 370-382.

Holt, W.V., Wilton, L.J., and Marshall, V.S. (1994). Cryopreservation of spermatozoa from the marmoset monkey (*Callihrix jacchus*). In Bradley, M., Cummins, J. Eds. , Proc. 7th Symp. **Spermatology** 9: 17.

Holt, W.V. (2000) Basic aspects of frozen storage semen. Anim Reprod Sci, 62, 3-22.

Isachenko, E. (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. **Reprod Biomed Online** 6(2): 191-200.

Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. **Genes Dev** 19: 1129-1155.

Kovtun, I.V., Therneau, T.M., and McMurray, C.T. (2000). Gender of embryo contributes to CAG instabikity in transgenic mice containing a Huntington's disease gene. **Hum Mol Genet** 9: 2767-2775.

Kraemer, D.C., and Vera Cruz, N.C. (1969). Collection, gross characteristics and freezing of baboon semen. **J Reprod Fertil** 20: 345-348.

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

Kusunoki, H., Daimaru, H., Minam, S., Nishimoto, S., Yamane, K., and Fukumoto, Y. (2001). Birth of a chimpanzee (*pan troglodytes*) after artificial insemination with cryopreserved epididymal spermatozoa collected postmortem. **Zoo Biol** 20: 135-143.

Lambert, H., Citino, S., Collazo, I., and Jeyendran, R.S. (1991). Penetration of zona-free hamster oocytes by ejaculated cryopreserved gorilla spermatozoa. **Fertil Steril** 56: 1201-1203.

Lanzendorf, S.E., Holmgren, W.J., Schaffer, N., Hatasaka, H., Wentz, A.C., and Jeyendran, R.S. (1992). In vitro fertilization and gamete micromanipulation in the lowland gorilla. **J Assist Reprod Genet** 9: 358-364.

Laowtammathron, C., Cheng, E.C.H, Cheng, P.H., Snyder, B.R., Yang, S.H., Johnson, Z., Lorthongpanich, C., Kuo, H.C, Parnpai, R., and Chan, A.W.S. (2010). Monkey hybrid stem cells develop cellular features of Huntington's disease. **BMC Cell Biol** 11.

Layton, M.J., Owczarek, C.M., Metcalf, D., Clark, R.L., Smith, D.K., Treutlein, H.R., and Nicola, N.A. (1994). Conversion of the biological specificity of murine to human leukemia inhibitory factor by replacing 6 amino acid residues. **J Biol Chem** 269: 29891-29896.

Leeflang, E.P. (1999). Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. **Hum Mol Genet** 8: 173-183.

Leibo, S.P., Kubisch, H.M., Schramm, R.D., Harrison, R.M., and VandeVoort, C.A. (2007). Male-to-male differences in post-thaw motility of rhesus spermatozoa after cryopreservation of replicate ejaculates. **J Med Primatol** 36: 151-163.

Leverage, W.E., Valerio, D.A., Schultz, A.P., Kingsbury, E., and Dorey, C. (1972). Comparative study on the freeze preservation of spermatozoa: primate, bovine and human. **Lab Anim Sci** 22: 882-889.

Li, H., Li, S.H., Cheng, A.L., Mangiarini, L., Bates, G.P., and Li, X.J. (1999). Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. **Hum Mol Genet** 8: 1227-1236.

Li, Y., Si, W., Zhang, X., Dinnyes, A., and Ji, W. (2003). Effect of amino acids on cryopre-servation of cynomolgus monkey (*Macaca fascicularis*) sperm. **Am J Primatol** 59: 159-165.

Li, Y., Cai, K., Kovacs, A., and Ji, W. (2005). Effects of various extenders and permeating cryo-protectants on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. **J Androl** 26: 387-395.

Li, Y., Cai, K., Li, J., Dinnyes, A., and Ji, W. (2006). Comparative Studies With Six Extenders for Sperm Cryopreservation in the Cynomolgus Monkey (*Macaca fascicularis*) and Rhesus Monkey (*Macaca mulatta*). **Am J Primatol** 68: 39-49.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Marian, S.P., Llanas, R.A., and Thonson, J.A. (2006). Derivation of human embryonic stem cells in defined conditions. **Nat Biotechnol** 24: 185-187.

Mahone, J.P., and Dukelow, W.R. (1978). Semen preservation in Macaca fascicularis. **Lab Anim Sci** 28: 556-561.

Mastroianni, L., Jr., and Manson, W. A. (1963). Collection of monkey semen by electroejaculation. **Jr Proc Soc Exp Biol Med** 112: 1025-1027.

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

Matsubayashi, K. (1982). Comparison of the two methods of electroe-jaculation in the Japanese monkey (*Macaca fuscata*). **Jikken Dobutsu** 31: 1-6.

Matsui, Y., Zsebo, K., and Hogan, B.L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. **Cell** 70: 841-847.

Merritt, A.D., Conneally, P.M., Rahman N.F., and Drew, A.L. (1969). Juvenile Huntington's chorea. **Excerpta Medica Int Congr Ser** 175: 645-673.

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

Morrell, J.M., Nubbemeyer, R., Heistermann, M., Rosenbusch, J., Kuderling, I., Holt, W.V., and Hodges, J.K. (1998). Artificial insemination in *Callithrix jacchus* using fresh or cryopreserved sperm**. Anim Reprod Sci** 52: 282-390.

Morrell, J.M. (1997). Cryopreservation of marmoset sperm (*Callihrix jacchus*). **Cryoletters** 18: 45-54.

Morrell, J.M., and Hodges, J.K. (1998). Cryopreservation of non-human primate sperm: priorities for future research. **Anim Reprod Sci** 53: 43-63.

Nance, M.A. (1996). Huntington's disease; Another chapter rewritten. **Am J Hum Genet** 59: 1-6.

Navara, C.S., Mich-Basso, J.D., Redinger, C.J., Ben-Yehudah, A., Jacoby, E., Kovkarova-Naumovski, E., Sukhwani, M., Orwig, K., Kaminski, N., Castro, C.A., Simerky, C.R., and Schatten, G. (2007). Pedigreed primate embryonic stem cells express homogeneous familial gene profiles. **Stem Cells** 25: 2695-2704.

Okamoto, M. (1994). Annual sperm concentration variation in semen collected by electroejaculation in the cynomolgus monkey (*Macaca fascicularis*). **Exp Anim** 43: 25-31.

Pace, M.M., and Graham, E.F. (1974). Components in egg yolk which protect bovine sperm during freezing. **J Anim Sci** 39: 1144-1149.

Pearson, C.E. (2003). Slipping while sleeping? Trinucleotide repeat expansions in germ cells. **TRENDS in Mol Med** 9: 11.

Polge, C., Smith, A.U., and Parkes, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature** 164: 666.

Pope, C.E., Dresser, B.L., Chin, N.W., Liu, J.H., Loskutoff, N.M., Behnke, J., Brown, C., MCrae, M.A., Sinoway, C.E., Campbell, M.K., Cameron, K.N., Owens, O'D.M., Johnson, C.A., Evans, R.R., and Cedars, M.I. (1997). Birth of a lowland gorilla (*Gorilla gorilla gorilla*) following in vitro fertilization and embryo transfer. **Am J Primatol** 41: 247-260.

Purdy, P.H. (2006). A review on goat sperm cryopreservation. **Small Ruminant Research** 63: 215-225.

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

Rajesh, D., Chinnasamy, N., Mitalipov, S.M., Wolf, D.P., Slukvin, I., Thomson, J.A., and Shaaban, A.F. (2007). Differential requirements for hematopoietic commitment between human and rhesus embryonic stem cells. **Stem Cells** 25: 490-499.

Rao, R.R., and Stice, S.L. (2004). Gene expression profiling of embryonic stem cells leads to greater understanding of pluripotency and early evelopmental events. **Biol Reprod** 71: 1772-1778.

Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse pri-mordial germ cells in culture. **Nature** 359: 550-551.

Rogers, J., Katze, M., Bumgarner, R., Gibbs, R.A., and Weinstock, G.M. (2002). White paper for complete sequencing of the rhesus macaque (*Macaca mulatta*) genome. **Rhesus Macaque White Paper**.

Rose-John, S. (2002). GP130 stimulation and the maintenance of stem cells. **Trends Biotechnol** 20: 417-419.

Roussel, J.D., and Austin, C.R. (1967). Preservation of primate spermatozoa by freezing. **J Reprod Fertil** 13: 333-335.

Salamon, S., and Maxwell, W.M. (2000). Storage of ram semen. **Anim Reprod Sci**: 62: 77-111.

Sanchez-Partida, L.G., Maxwell, W.M., Paleg, L.G., and Setchell, B.P. (1992). Proline and glycine betaine in cryoprotective diluents for ram spermatozoa. **Reprod Fertil Dev** 4: 113-118.

Sanchez-Partida, L.G., Maginnis, G., Dominko, T., Martinovich, C., McVay, B., Fanton, J., and Schatten, G. (2000). Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. **Biol Reprod** 63: 1092-1097.

Sankai, T., Terao, K., Yanagimachi, R., Cho, F., and Yoshikawa, Y. (1994). Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). **J Reprod Fertil** 101: 273-278.

Saragusty, J., Gacitua, H., Zeron, Y., Rozenboim, I., and Arav, A. (2008). Double freezing of bovine semen. **Anim Reprod Sci** 115: 10-17.

Sarason, R.L., VandeVoort, C.A., Mader, D.R., and Overstreet, J.W. (1991). Electro-ejaculation by direct penile stimulation of restrained but unanesthetized macaques. **J Med Primatol** 20: 122-125.

Savouret, C. (2003). CTG repeat instability and size variation timing in DNA repair-deficient mice. **EMBO J** 22: 2264-2273.

Seidel Jr, G.E., and Schenk, J.L. (2008). Pregnancy rates in cattle with cryopreserved sexed sperm: Effects of sperm numbers per inseminate and site of sperm deposition. **Anim Reprod Sci** 105: 129-138.

Seier, J.V., Conradie, E., Oettle, E.E., and Fincham, J.E. (1993). Cryopreservation of vervet monkey semen and recovery of progressively motile spermatozoa. **J Med Primatol** 355-359.

Si, W., Zheng, P., Tang, X., He, X., Wang, H., Bavister, B.D., Ji, W. (2000). Cryopreservation of rhesus macaque (*Macaca mulatta*) spermatozoa and their functional assess-ment by in vitro fertilization. **Cryobiology** 41: 232-240.

Si, W., Zheng, P., Li, Y., Dinnyes, A., Ji, W. (2004). Effect of glycerol and dimethyl sulfoxide on cryopreservation of rhesus monkey (*Macaca mulatta*) sperm. **Am J Primatol** 62: 301-306.

Si, W., Wang, H., Reid, C., Hildebrandt, T.B., Ji, W. (2006). Effect of Sugar Type on the Survival of Frozen-Thawed Rhesus Monkey (*Macaca mulatta*) Sperm. **Am J Primatol** 68: 103-108.

Simerly, C.R., Navara, C.S., Castro, C.A., Turpin, J.C., Redinger, C.J., Mich-Basso, J.D., Jacoby, E.S., Grund, K.J., McFarland, D.A., Oliver, S.L., Ben-Yehudah,

A., Carlisle, D.L., Frost, P., Penedo, C., Hewitson, L., and Schatten, G. (2009). Establishment and characterization of baboon embryonic stem cell lines: An Old World Primate model for regeneration and transplantation research. **Stem Cell Res** (Epub ahead of print).

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

Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E., and Thompson, L.M. (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. **Proc Natl Acad Sci USA** 12: 6763-6768.

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

Taranta, A.F., VandeVoort, C.A., and Overstreet, J.W. (1990). Intrauterine insemination with ultrasound guidance in the Cynomolgusmonkey (*Macaca fascicularis*). **J Med Primato**.

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

Thomson, J.A., Kalishman, J., and Golos, T.G. (1996). Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. **Biol Reprod** 55: 254 -259.

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

Tollner, T.L., VandeVoort, C.A., Overstreet, J.W., and Drobnis, E.Z. (1990). Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). **J Reprod Fertil** 90: 347-352.

VandeVoort, C. (2004). High quality sperm for nonhuman primate ART: production and assessment. **Reprod Biol Endocrinol** 16: 33.

VandeVoort, C.A., Neville, T.L., Tollner, T.L., and Farichild, O. (1993). Non-invasive semen collection from an adult Orangutan. **Zoo Biology** 12: 257-266.

Vera-Munoz, O., Amirat-Briand, L., Diaz, T., Vasquez, L., Schmidt, E., Desherces, S., Anton, M., Bencharif, D., and Tainturier, D. (2009). Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl and Bioxcell. **Theriogenology** 71: 895-900.

Wianny, F., Bernat, A., Huissoud, C., Marcy, G., Markossian, S., Cortay, V., Giroud, P., Leviel, V., Kennedy, H., Savatier, P., and Dehay, C. (2008). Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein. **Stem Cells** 26: 1444-1453.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Met-calf, D., Nicola, N.A., and Gough, N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. **Nature** 336: 684-687.

Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C., Zwaka, T.P., and Thomson, J.A. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. **Nat Biotechnol** 20: 1261-1264.

Yeoman, R.R., Mitalipov, S., Gerami-Naini, B., Nusser, K.D., and Wolf, D.P (2005). Low temperature storage of rhesus monkey spermatozoa and fertility evaluation by intracytoplasmic injection. **Theriogenology** 63: 2356-2371.

Yildiz, C., Kaya, A., Aksoy, M., and Tekeli, T. (2000). Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. **Theriogenology** 54: 579-585.

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

Yoon, S.R., Dubeau, L., Young, M.D., Wexler, N.S., and Arnheim, N. (2003). Huntington disease expansion mutations in humans can occur before meiosis is completed. **PNAS** 100: 8834–8838.

Younis, A.I., Rooks, B., Khan, S., and Gould, K.G. (1998). The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. **J Androl** 19: 207-214.

Zamboni, L., Conaway, C.H., and Van Pelt, L. (1974). Seasonal changes in production of semen in free-ranging rhesus monkeys. **Biol Reprod** 11: 251-267.

Zwaka, T.P., and Thomson, J.A. (2003). Homologous recombination in human embryonic stem cells. **Nat Biotechnol** 21: 319-321.

CHAPTER III

THE CRYOPRESERVATION OF TRANSGENIC HUNTINGTON'S RHESUS MONKEY SPERM

3.1 Abstract

The cryoprotective effects of three different extenders, TRIS, TEST, and TTE, on WT rhesus monkey (*Macaca mulatta*) sperm cryopreservation with glycerol as cryoprotectant have been compared. Sperm motility and viability were examined to evaluate integrity of frozen-thawed sperm and the best extender was selected for transgenic HD rhesus monkey (rHD1) sperm cryopreservation. The results showed that all freezing extender were not different in post-thaw sperm motility ($P>0.05$), however, sperm viability in TEST and TTE exhibited significant higher than TRIS extender ($P<0.05$). The TEST was chosen for rHD1 sperm cryopreservation. The result showed that post-thawed rHD1 sperm motility and viability was not difference compared with WT control group ($P>0.05$). The present study demonstrate that TEST and TTE were excellent extenders and suitable for rhesus monkey sperm cryopreservation and no detectible differences of post-thawed sperm motility and viability between rHD1 and WT in TEST extender.

3.2 Introduction

The cryopreservation technology is one of the ART that contribute preserved spermatozoa of many animal species. Since, the discovery of glycerol as cryoprotectant agent (CPA) for sperm cryopreservation (Polge et al., 1949), glycerol have been the major CPA for sperm cryopreservation in many species including; NHP such as lemurs (Clavert et al, 1986), marmosets (Holt et al, 1994; Morrell, 1997; Morrell et al, 1998), squirrel monkey (Denis et al, 1976), African green (Roussel and Austin, 1967), patas (Roussel and Austin, 1967), vervet monkey (Seier et al, 1993; Conradie et al, 1994), cynomolgus monkey (Cho and Honjo, 1973; Mahone and Dukelow, 1978; Tollner et al, 1990; Sankai et al, 1994; Feradis et al, 2001; Ng et al., 2002; Li et al, 2003; Li et al., 2005; Li et al., 2006), Japanese monkey (Sankai et al, 1997), lion-tailed monkey (Cranfield et al, 1988), rhesus monkey (Roussel and Austin, 1967; Leverage et al, 1972; Sanchez-Partida et al, 2000; Si et al, 2000, 2004; Si et al., 2006; Dong et al., 2008; Dong et al., 2009), stumptail monkey (Roussel and Austin, 1967), Tibetan (Chen et al, 1994), baboons (Kraemer and Vera Cruz, 1969); chimpanzees (Roussel and Austin, 1967; Gould and Styperek, 1989; Younis et al, 1998; Kusunoki et al, 2001); and gorillas (Lambert et al, 1991; Lanzendorf et al, 1992; Pope et al, 1997). Among NHP, rhesus monkey is an essential research model in the development of sperm cryopreservation due to their high close genetic, physiologic, and metabolic with humans. Although, extensive studies on rhesus monkey sperm cryopreservation has been reported; however, sperm cryopreservation efficacy varies tremendously among laboratories because different freezing method, extenders type, and species used which difficult to compare results between laboratories and still need more optimal protocol for sperm cryopreservation.

Huntington's disease (HD) is an inherited neurodegenerative disorder resulting from an expanded CAG (cytosine-adenine-guanine) encodes the polyglutamine (HD Collaborative Research Group., 1993) that lead to motor dysfunction, psychiatric disturbances and cognitive impairment. The transmission of trinucleotide repeats in human disease including HD has influenced from gender (Kovtun et al., 2000) by the transmission tends to be higher effect from paternal than maternal (Chong et al, 1997). However, the mechanism of parent of origin effect in HD has not been clearly and HD genetics transmissions still need more data to make understanding. Therefore, the cryopreservations of HD genetic especially in paternal sperm become important because they increase opportunity to learn more about HD including; germ line transmission, CAG expansion and HD pathology. Thus, the goal of present study was to cryopreserved HD rhesus monkey sperm by using optimal cryopreservation protocol.

3.3 Materials and Methods

A total 5 ejaculated were collected from single male of WT rhesus monkey and used for studied efficiency of 3 freezing extenders (TRIS, TEST, and TTE). The extender that offers satisfactory results was selected for rHD1 sperm cryopreservation. A total 4 ejaculated were collected compared with WT control.

3.3.1 Animals model

The rhesus monkeys were housed and maintained at Yerkes National Primate Research Center (YERKES). All animal procedures were conducted according to a guideline provided by institutional animal care and committee protocols

at Emory University. All documented research in this study was conducted in accordance to the Guide for the Care and Use of Laboratory Animals.

3.3.2 Generation of transgenic HD rhesus monkey (rHD1).

rHD1 were provided by Yang and colleagues (2008). Briefly, the high titer lenti viruses carrying exon1of human *HTT* with 84 CAG repeats and green fluorescent protein (*GFP*) gene under the regulation of human poly ubiquitin C promoter were injected into the perivitelline space of metaphase II arrested monkey oocytes followed by ICSI. The resultant embryos were transferred into surrogate females for generation of transgenic monkeys. Transgenic status was confirmed by PCR.

3.3.3 Sperm cryopreservation extender

Each cryopreservation extenders (TRIS, TEST, and TTE) were divided into two parts (with and without glycerol). The composition of each extender was listed in Table 1. The primary semen diluting extender (without glycerol) was centrifuged at 7,000 × g under 4^{o}C for 1 h to remove yolk granules (Sakai et al., 1994). Supernatant were filtered with 0.2 μm filter and stored at -80°C. The extender was thawed at 37°C in water bath and equilibrated at room RT before used. The secondary extender is a mixture of the primary extender and glycerol.

3.3.4 Semen collection

A single male of WT and rHD1 rhesus monkey were used for semen collection. Ejaculated semen was collected once a week in the morning. The males were trained to chair restraint and semen was collected by direct penile stimulation with stimulator equipped with EKG pad electrodes (30–50V, 20 ms duration 18 pulse/sec). The ejaculated semen was kept at RT for 20 min to liquefy. Liquid portion

of semen was transferred into 15 mL conical tube and washed with Tyrode's albumin lactate-pyruvate-HEPES (TALP-HEPES) medium supplemented with 4 mg/mL bovine serum albumin (BSA) (Bavister et al., 1983), after that they was centrifuged at 112 × g at RT for 7 min. The sperm concentration, motility and viability were determined and recorded.

3.3.5 Sperm cryopreservation

Washed WT rhesus monkey sperm was divided into three groups for three different extenders. The sperm was diluted with primary extender in 15 mL conical tube and kept in 500 mL beaker containing RT water and incubated in 4°C refrigerator for 2 h. Equal volume of pre-cooled secondary extender (with glycerol) was added and incubated for 30 min at 4°C (Sankai et al., 1994). The final concentration of glycerol was 8%, 3% and 5% in TRIS, TEST and TTE, respectively. Final sperm concentration was 30×10^6 cells/mL. Sperm was then loaded into 0.25 mL straws by using 1 mL syringe and sealed with plug powder. Straws were plunged directly into 4°C water. The straws were laid horizontal on an aluminum rack and then transferred to styrofoam box containing liquid nitrogen (LN_2) and incubated for 8 min at positioned 4 cm above the surface of LN_2. LN_2 was added up 1 cm every 2 min until final distant between surface of LN_2 and straw is 1 cm. Straws were then plunged directly into LN_2 and storage.

For rHD1 monkey sperm cryopreservation, the extender that has best post-thawed sperm motility and viability in WT was selected for freezing. The cryopreservation process was preformed following the protocol described above. Sperm samples were stored in LN_2 at a minimum of 7 days before evaluation.

Table 3.1 The composition of three extenders.

Chemicals	Vender/catalog no.	TRIS	TTS	TES
TES	Sigma/ T0772	-	172 mM	47.8 mM
Tris	Fisher/ BP152	250 mM	84.7 mM	-
Tris-HCl	Fisher/ BPE153	-	-	12.7 mM
Glucose	Sigma/ G8270	-	55.5 mM	111 mM
Citric acid	Sigma/ C2404	87.2 mM	-	-
D-fructose	Sigma/ F0127	69.4 mM	-	-
Lactose	Sigma/ L3625	-	-	55.4 mM
Raffinose	Sigma/ R0250	-	-	3.36 mM
Distill water	-	50 ml	50 ml	50 ml
pH	-	7.2-7.4	7.2-7.4	7.2-7.4
Egg yolk	-	20%	20%	20%
Glycerol	Sigma/ G7793	8%	3%	5%

3.3.6 Sperm thawing

Frozen sperm were thawed after storage in LN_2 at least 7 days. Straws of frozen sperms were thawed in a 37°C water bath for 1-2 min. Thawed sperms washed in 15 ml conical tube with 4 mL of TALP-HEPES medium supplemented with 4 mg/mL BSA (Bavister et al., 1983) by centrifugation at 112 × g under RT for 7 min. Deposit was re-suspended by gently pipetting, after that sperm motility and viability were determined and recorded.

3.3.7 Sperm motility evaluation

Ten µL thawed semen was dropped onto a 37°C pre-warmed slide and covered with a 22 mm square cover-glass. The slide was visualized with 10x positive phase objective on a microscope. Then sperm motility was determined by three operators. The sperm motility recovery was calculated by the following formula: (post-thaw motility % × 100)/ pre-freeze sperm motility %.

3.3.8 Sperm viability evaluation

The sperm viability test was assessed by using LIVE/DEAD® Sperm Viability Kit (Invitrogen). The pre-freeze or post-thawed sperms concentration was adjusted to 2.5×10^6 mL^{-1}. Sperm samples were incubated with 5 µL of 20 µM SYBR 14 dye and final concentration was 100 nM. Samples were incubated at 37°C water bath for 5-10 min in the dark room and then they were incubated with 5 µL of 2.4 mM Propidium Iodide (PI) at 12 µM final concentration then incubated for 5 min at 37°C water bath in the dark room. After that, two µL of stained sperm were dropped on slide coated with 0.1% poly-D-lysine (Sigma) and covered with a 22 mm square cover-glass. The sperm stained with a bright green were evaluated to be alive and red were regarded as dead (Figure 3.5). Sperm viability was calculated by the following formula: (number of live sperm x 100)/ number of total sperm count. The sperm viability recovery was calculated by the following formula: (viability of post-thawed sperm % × 100)/ pre-freeze sperm motility %.

3.3.9 Statistical analysis

Sperm motility and viability were analyzed between each treatment group using general linear model. This test was used to analyze significant differences between freezing extenders in WT frozen sperm and between WT and rHD1 frozen sperm. 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.

3.3 Results

3.4.1 Effect of extender on WT rhesus monkey sperm cryopreservation

The post-thawed motility and motility recovery of WT rhesus monkey sperm were showed in Table 3.2. The sperm motility and motility recovery after thawed in TEST group has slightly higher than other groups. However, there was no different among of three extenders both in motility (54.78 ± 3.88%, 50.16 ± 7.52%, and 46.99 ± 4.82%, in TEST, TTE, and TRIS respectively) and motility recovery (60.69 ± 5.58%, 53.99 ± 6.70%, and 52.51 ± 7.4%, in TEST, TTE, and TRIS respectively).

The viability and viability recovery of post-thawed WT rhesus monkey sperm were showed in the Table 3.3. The sperm viability after thawed in TEST group (60.75 ± 2.85%) significant higher than TRIS group (48.79 ± 3.46%) but no different from TTE group (54.85 ± 3.34%) while TRIS group no different with TTE group.

3.4.2 rHD1 sperm cryopreservation

The comparisons of three extenders by using WT rhesus monkey sperm for cryopreservation revealed that TEST extender has slightly better than other extenders. Therefore, TEST was selected for cryopreservation of rHD1 sperm. The motility and motility recovery of post-thawed rHD1 sperm were showed in the Table 3.4. The rHD1 sperm motility (53.67 ± 8.96%) and motility recovery (56.53 ± 9.89%) were showed no significant different with control group (58.06 ± 2.68%, in motility and 65.19% ± 4.29%**,** in motility recovery).

The sperm viability and viability recovery of rHD1 sperm was showed in the Table 3.5. The post-thawed rHD1 sperm viability was not different from control

group (58.18 ± 1.74% and 61.74 ± 3.46%, respectively). However, viability recovery of rHD1 sperm showed significant lower than control group (62.97. 1.57% and 78.48 ± 3.40%, respectively).

Table 3.2 The motility analysis of post-thawed spermatozoa in TRIS, TTS, and TES extenders**.**

Fresh sperm motility, %	Extender	Post-thaw sperm motility, % [a]	Motility recovery rate, % [a]
87.12 ± 4.56	TRIS	46.99 ± 4.82	52.51 ± 7.43
	TTS	54.78 ± 3.88	60.69 ± 5.58
	TES	50.16 ± 7.52	53.99 ± 6.70

[a] Values with in column were not significantly different (P>0.05).

Table 3.3 A viability analysis of post-thawed spermatozoa in TRIS, TTS, and TES extenders**.**

Extender	Post-thaw sperm viability, %	Viability recovery rate, %
TRIS	48.79 ± 3.46[b]	60.19 ± 3.62[b]
TTS	60.75 ± 2.85[a]	75.23 ± 4.17[a]
TES	54.85 ± 3.34[ab]	67.81 4.01[ab]

[a,b] Values with in column were significantly different (P<0.05).

Table 3.4 Comparison of frozen-thawed rhesus sperm motility in rHD1 and WT group**.**

Sperm types	Fresh sperm motility, % [a]	Post-thaw sperm motility, % [a]	Motility recovery rate, % [a]
rHD1	95.37 ± 1.01	53.67 ± 8.96	56.53 ± 9.89
WT	86.60 ± 2.48	58.06 ± 2.68	65.19 ± 4.29

[a] Values with in column were not significantly different (P>0.05).

Table 3.5 Comparison of frozen-thawed rhesus sperm viability in rHD1 and WT group**.**

Sperm types	Post-thaw sperm viability, %	Viability recovery rate, %
rHD1	58.18 ± 1.74	62.97. 1.57[b]
WT	61.74 ± 3.46	78.48 ± 3.40[a]

[a,b] Values with in column were significantly different (P<0.05).

3.5 Discussion

This study investigated the effect of freezing extenders on rhesus monkey sperm cryopreservation by compared three extenders. The composition of the extender plays a key role in sperm cryopreservation. The extenders studied in our experiments have been employed in research on NHP. Previous studies obtained satisfactory results for TTE in cynomolgus (Sankai et al., 1994) and rhesus (Si et al., 2000) monkey, TEST in rhesus monkey (Dong et al., 2008, 2009), and TRIS in rhesus monkey (our laboratory, unpublished results). Post-thawed sperm motility was around ≥ 50% for the TEST and TTE in previous studies; however, there were difference among both of species and freezing methods used. In this study, the results show that the three extenders (TRIS, TEST, and TTE) offered good cryopretection during sperm freezing in rhesus monkey. Although, TEST showed slightly better than other extenders; however, no significant difference in sperm motility were found for each extenders.

Many factors affect to success of sperm cryopreservation which includes; procedure of freezing and thawing techniques, extenders, types of cryoprotectant, and skill of investigators. The cryoprotectant is a major factor that influences sperm

survival during sperm cryopreservation. The glycerol is a commonly used for permeating cryoprotectant in NHP sperm freezing; however, its toxicity can cause loss in sperm motility and fertility if their concentration not available (Li et al., 2005). There are many reports suggested that 3-5% glycerol offered suitable concentration for cynomolgus monkey (Sankai et al., 1994) and rhesus monkey (Si et al., 2004, Li et al., 2005, 2006, and Dong et al., 2008, 2009) sperm cryopreservation. In the present study, the results show that 3% (TEST) and 5% (TTE) of glycerol concentration provided good cryoprotection than 8% (TRIS), which proved that high concentrations of permeating cryoprotectant resulting in higher toxicity to the sperm cells. Although, glycerol as penetrate cryoprotectants are beneficial for sperm cryopreservation in suitable concentration; however, the mechanism of them behind the sperm cryoprotection remains unknown.

In this study also conducted on cryopreservation of rHD1 sperm by using TEST extender. The results show that sperm motility and viability of post-thawed has no significant difference between rHD1 and WT group. Which, HD might be not impact to spermatozoa during freezing and thawing process.

Huntington' is a neurodegenetive disorder cause from CAG repeat expansion and could inherit to next generation. Gender and age of parent is known to influence the transmission of CAG repeats to next generation in HD. However, the molecular basis for the parent of origin effect associated with CAG repeat expansion is not known. The paternal transmission there is propensity toward larger CAG repeat expansion but maternal transmission nearly equal numbers of CAG expansion (Meritt et al., 1969, Duyao et al., 1993, Chong et al, 1997) in progeny. Although, cause of CAG expansion tend to effect from paternal than maternal; however, distribution of

repeat length in the progeny did not faithfully reflect the distribution of repeat present in the sperm of the founding fathers (Kovtun et al., 2000). The repeat length progeny might not only affected from sperm but may be also including the step of post-zygotically in the embryos. From above information implies that the mechanism of parent of origin effect in HD has not been clearly and need more data to understanding. Therefore, HD sperm cryopreservations in this study are useful for using in the future study of HD such as germ line transmission, CAG expansion in different age of paternal sperm and HD pathology and which might brings us closer to effective treatments in HD.

In conclusion, the present study showed that freezing extender TEST and TTE were excellent extenders suitable for rhesus monkey sperm cryopreservation and no detectible differences of post-thawed sperm motility and viability between rHD1 and WT in TEST extender.

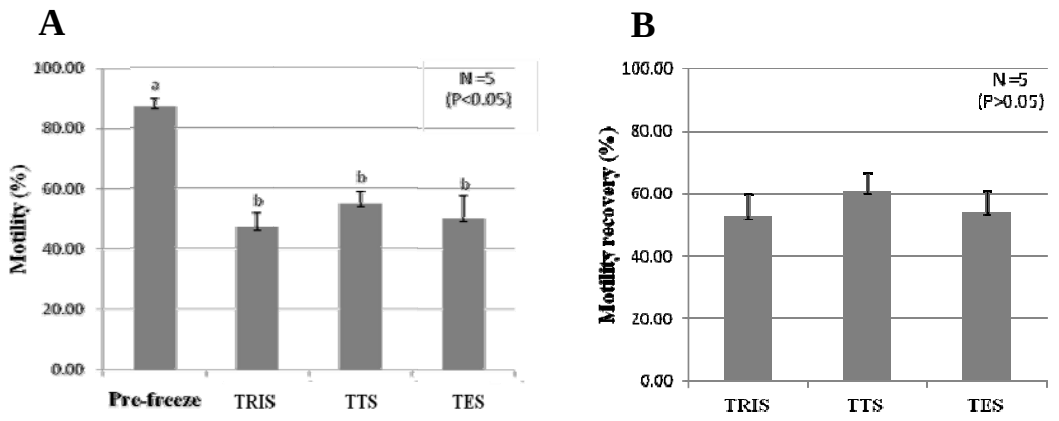


Figure 3.1 The motility (A) and motility recovery (B) of rhesus monkey spermatozoa after frozen in various extenders (TRIS, TTS, and TES). a, b, indicates significant difference (P<0.05) compare to other values.

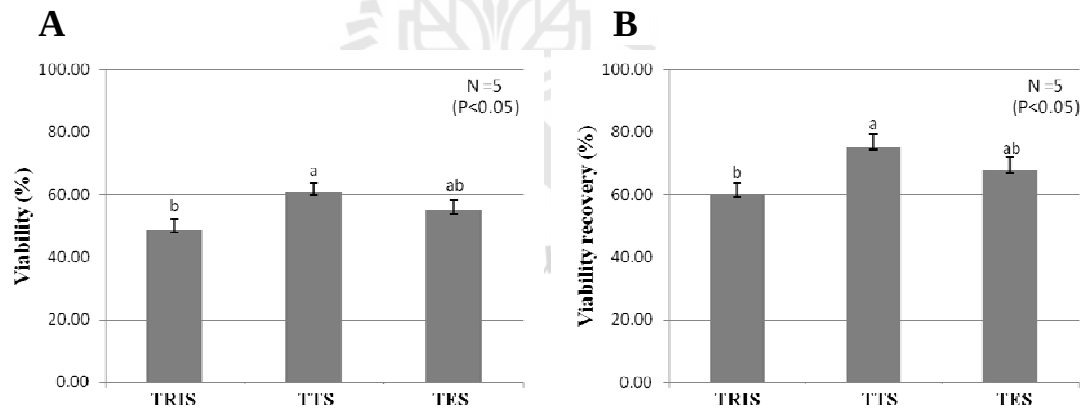


Figure 3.2 The viability (A) and viability recovery (B) of rhesus monkey spermatozoa after frozen in various extenders (TRIS, TTS, and TES). a, b, indicates significant difference (P<0.05) compare to other values.

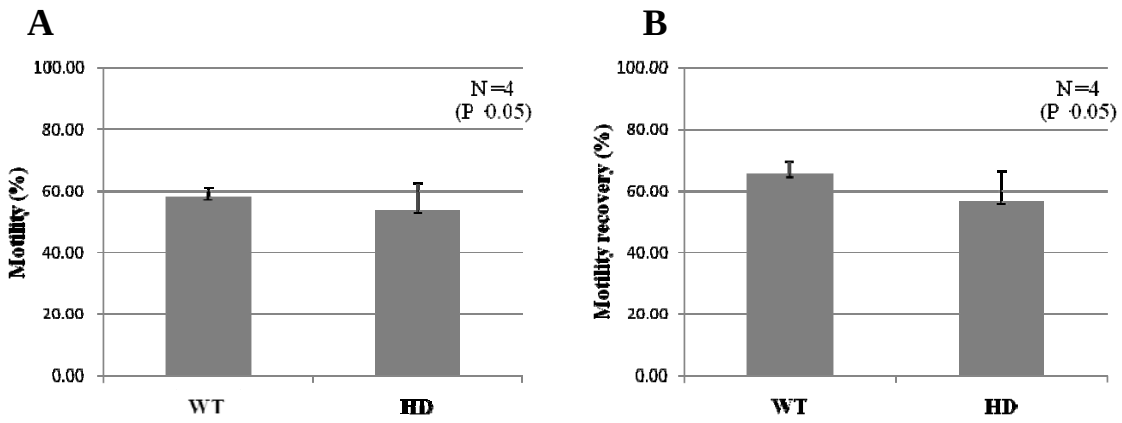


Figure 3.3 The motility (A) and motility recovery (B) of WT and rHD1 sperm after frozen (P>0.05).

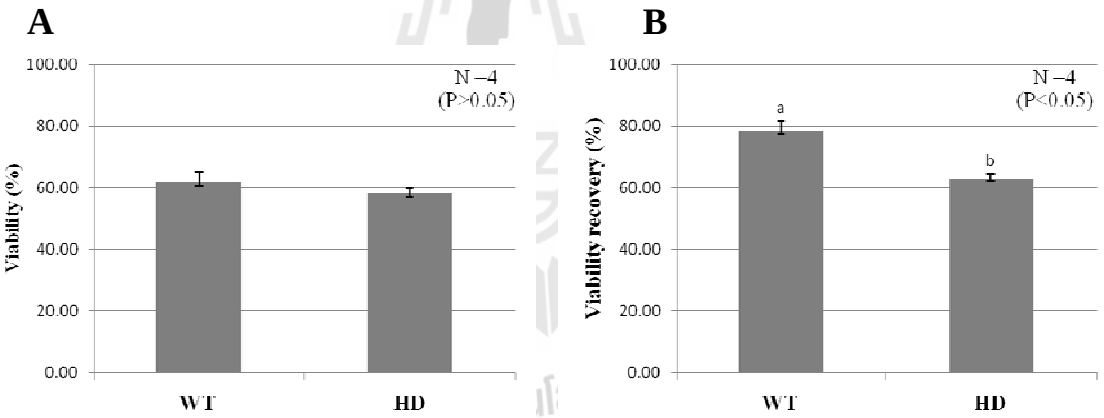


Figure 3.4 The viability (A) and viability recovery (B) of WT and rHD1 sperm after frozen. a, b indicates significant difference (P<0.05) compare to other values.

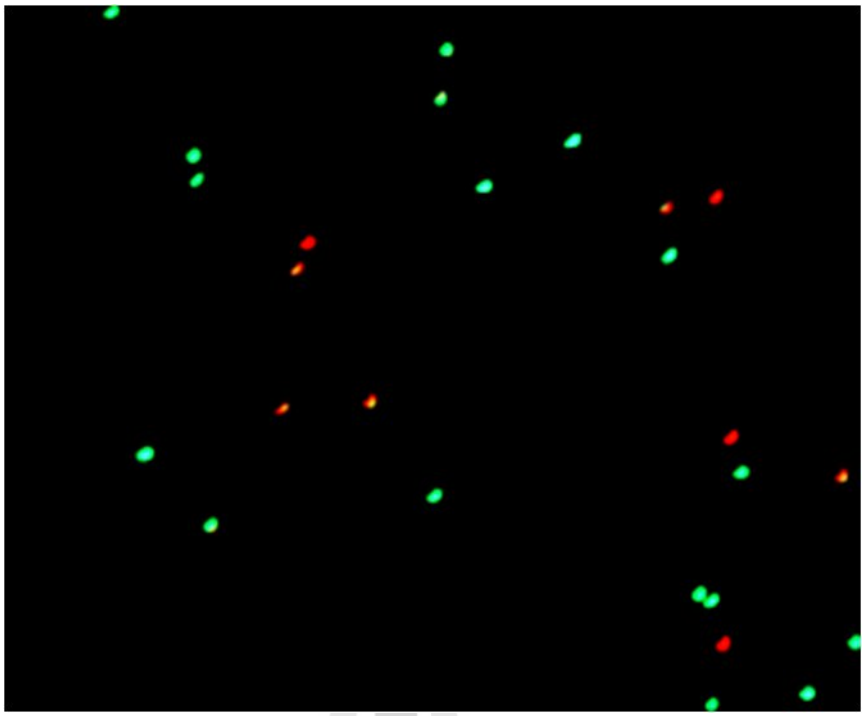


Figure 3.5 Sperm viability assessment. Alive sperm stained with SYBR 14 dye (green color) and dead sperm stained with PI (red color).

3.7 References

Bavister, B.D., Leibfried, M.L., and Lieberman, G. (1983). Development of preimplantation embryos of the golden hamster in a defined culture medium. **Biol Reprod** 28: 235-247.

Chen, J.C., Ji, W.Z., Yang, S.C., and Shi, L.M. (1994). Semen cryopreservation in the Tibetan macaque (*Macaca thibetana*) comparison of different cooling programs and freezing media. **Acta Zoologica Sinica** 40: 174-181.

Cho, F., and Honjo, S. (1973). A simplified method for collecting and preserving cynomolgus macaque semen. **Jpn J Med Sci Biol** 26: 261-268.

Chong, S.S. (1997). Contribution of DNA sequence and CAG size to mutation frequencies of intermediate alleles for Huntington disease: evidence from single sperm analysis. **Hum Mol Genet** 6: 301-309.

Clavert, A., Cranz, C., Brun, B., and Rumpler, Y.A. (1986). Method for sperm cryopreservation of the lemur fulvous. **Primate Rep** 14: 101.

Conradie, E., Oettle, E.E., and Seier, J.V. (1994). Assessment of acrosomal integrity of vervet monkey spermatozoa after cryopreservation. **J Med Primatol** 23: 315-316.

Cranfield, M.R., Kempske, S.E., and Schaffer, N. (1988). The use of in vitro fertilization and embryo transfer techniques for the enhancement of genetic diversity in the captive population of the lion-tailed macaque. **Int Zoo Yearbook** 27: 149-159.

Denis, L.T., Poindexter, A.N., Ritter, M.B., Seager, S.W.J., and Deter, R.L. (1976). Freeze preservation of squirrel monkey sperm for use in timed fertilization studies. **Fertil Steril** 27: 723-729.

Dong, Q., Rodenburg, S.E., Huang, C., and VandeVoort, C.A. (2008). Cryopreservation of rhesus monkey (*Macaca mulatta*) epididymal spermatozoa before and after refrigerated storage. **J Androl** 29: 283-292.

Dong, Q., Correa, L.M., and VandeVoort, C.A. (2009). Rhesus monkey sperm cryopreservation with TEST-yolk extender in the absence of permeable cryoprotectant. **Cryobiology** 58: 20-27.

Duyao, M., Ambrose, C., Mters, R., Noveletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., and Abbott, M. (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. **Nat Genet** 4: 387-392.

Feradis, A.H., Pawitri, D., Suatha, I.K., Amin, M.R., Yusuf, T.L., and Sajuthi, D. (2001). Cryopreservation of epididymal spermatozoa collected by needle biopsy from cynomolgus monkeys (*Macaca fascicularis*). **J Med Primatol** 30: 100-106.

Gould, K.G., and Styperek, R.P. (1989). Improved methods for freeze preservation of chimpanzee sperm. **Am J Primatol** 18: 275-284.

HD Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. **Cell** 72: 971-983.

Holt, W.V., Wilton, L.J., and Marshall, V.S. (1994). Cryopreservation of spermatozoa from the marmoset monkey (*Callihrix jacchus*). In Bradley, M., Cummins, J. Eds. Proc. 7th Symp. **Spermatology** 9: 17.

Kovtun, I.V., Therneau, T.M., and McMurray, C.T. (2000). Gender of embryo contributes to CAG instabikity in transgenic mice containing a Huntington's disease gene. **Hum Mol Genet** 9: 2767-2775.

Kraemer, D.C., and Vera Cruz, N.C. (1969). Collection, gross characteristics and freezing of baboon semen. **J Reprod Fertil** 20: 345-348.

Kusunoki, H., Daimaru, H., Minam, S., Nishimoto, S., Yamane, K., and Fukumoto, Y. (2001). Birth of a chimpanzee (*Pan troglodytes*) after artificial insemination with cryopreserved epididymal spermatozoa collected postmortem. **Zoo Biol** 20: 135-143.

Lambert, H., Citino, S., Collazo, I., and Jeyendran, R.S. (1991). Penetration of zona-free hamster oocytes by ejaculated cryopreserved gorilla spermatozoa. **Fertil Steril** 56: 1201-1203.

Lanzendorf, S.E., Holmgren, W.J., Schaffer, N., Hatasaka, H., Wentz, A.C., and Jeyendran, R.S. (1992). In vitro fertilization and gamete micromanipulation in the lowland gorilla. **J Assist Reprod Genet** 9: 358-364.

Leverage, W.E., Valerio, D.A., Schultz, A.P., Kingsbury, E., and Dorey, C. (1972). Comparative study on the freeze preservation of spermatozoa: primate, bovine and human. **Lab Anim Sci** 22: 882-889.

Li, Y., Si, W., Zhang, X., Dinnyes, A., and Ji, W. (2003). Effect of amino acids on cryopre-servation of cynomolgus monkey (*Macaca fascicularis*) sperm. **Am J Primatol** 59: 159-165.

Li, Y., Cai, K., Kovacs, A., and Ji, W. (2005). Effects of various extenders and permeating cryoprotectants on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. **J Androl** 26: 387-395.

Li, Y., Cai, K., Li, J., Dinnyes, A., and Ji, W. (2006). Comparative Studies With Six Extenders for Sperm Cryopreservation in the Cynomolgus Monkey (*Macaca fascicularis*) and Rhesus Monkey (*Macaca mulatta*). **Am J Primatol** 68: 39-49.

Mahone, J.P., and Dukelow, W.R. (1978). Semen preservation in Macaca fascicularis. Lab. **Anim Sci** 28: 556-561.

Merritt, A.D., Conneally, P.M., Rahman, N.F., and Drew, A.L. (1969). Juvenile Huntington's chorea. **Excerpta Medica Int Congr Ser** 175: 645-673.

Morrell, J.M. (1997). Cryopreservation of marmoset sperm (*Callihrix jacchus*). **Cryoletters** 18: 45-54.

Morrell, J.M., and Hodges, J.K. (1998). Cryopreservation of non-human primate sperm: priorities for future research. **Anim Reprod Sci** 53: 43-63.

Ng, S.C., Martelli, P., Liow, S.L., Herbert, S., and Oh, S.H. (2002). Intracytoplasmic injection of frozen-thawed epididymal spermatozoa in a nonhuman primate model, the cyno-molgus monkey (*Macaca fascicularis*). **Theriogenology** 58: 1385-1397.

Polge, C., Smith, A.U., and Parkes A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature** 164: 666.

Pope, C.E., Dresser, B.L., Chin, N.W., Liu, J.H., Loskutoff, N.M., Behnke, J., Brown, C., MCrae, M.A., Sinoway, C.E., Campbell, M.K., Cameron, K.N., Owens, O'D.M., Johnson, C.A., Evans, R.R., and Cedars, M.I. (1997). Birth of a lowland gorilla (*Gorilla gorilla*) following in vitro fertilization and embryo transfer. **Am J Primatol** 41: 247-260.

Roussel, J.D., and Austin, C.R. (1967). Preservation of primate spermatozoa by freezing. **J Reprod Fertil** 13: 333-335.

Sanchez-Partida, L.G., Maginnis, G., Dominko, T., Martinovich, C., McVay, B., Fanton, J., and Schatten G (2000). Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. **Biol Reprod** 63: 1092-1097.

Sankai, T., Terao, K., Yanagimachi, R., Cho, F., and Yoshikawa, Y. (1994). Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). **J Reprod Fertil** 101: 273-278.

Sankai, T., Shimizu, K., Cho, F., and Yoshikawa, Y. (1997). In vitro fertilization of follicular oocytes by frozen-thawed spermatozoa in Japanese monkeys (*Macaca fuscata*). **Lab Anim Sci** 47: 58-62.

Seier, J.V., Conradie, E., Oettle, E.E., and Fincham, J.E. (1993). Cryopreservation of vervet monkey semen and recovery of progressively motile spermatozoa. **J. Med Primatol** 355-359.

Si, W., Zheng, P., Tang, X., He, X., Wang, H., Bavister, B.D., and Ji, W. (2000). Cryopreservation of rhesus macaque (*Macaca mulatta*) spermatozoa and their functional assessment by in vitro fertilization. **Cryobiology** 41: 232-240.

Si, W., Zheng, P., Li, Y., Dinnyes, A., and Ji, W. (2004). Effect of glycerol and dimethyl sulfoxide on cryopreservation of rhesus monkey (*Macaca mulatta*) sperm. **Am J Primatol** 62: 301-306.

Si, W., Wang, H., Reid, C., Hildebrandt, T.B., and Ji, W. (2006). Effect of Sugar Type on the Survival of Frozen-Thawed Rhesus Monkey (*Macaca mulatta*) Sperm. **Am J Primatol** 68: 103-108.

Tollner, T.L., VandeVoort, C.A., Overstreet, J.W., and Drobnis, E.Z. (1990). Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). **J Reprod Fertil** 90: 347-352.

Yang, S.H., Cheng, P.H., Banta, H., Piotrows ka-Nitsche, K., Yang, J.J., Cheng, E.C., Snyder, B., Larkin, K., Liu, J., Orkin, J., Fa ng, Z.H., Smith, Y., Bachevalier, J., Zola, S.M., Li, S.H., Li, X.J., and Chan, A.W. (2008). Towards a transgenic model of Huntington's disease in a non-human primate. **Nature** 453: 921-924.

Younis, A.I., Rooks, B., Khan, S., and Gould, K.G. (1998). The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. **J Androl** 19: 207-214.

CHAPTER IV

HD EMBRYO PRODUCTION AND EMBRYONIC STEM CELL ESTABLISHMENT

4.1 Abstract

Transgenic Huntington's rhesus monkey (rHD1) carry a mutant *HTT* gene with expanded polyglutamine repeats (CAGs; polyQ), develop chorea, dystonia and other involuntary motor deficiencies similar to HD. Germline transmission of rHD1 by production of embryos using rHD1 sperm and subsequently derivation of rHD1 embryonic stem cells (rHD-ESCs) were demonstrated. rHD-ESCs inherited mutant *HTT* and *GFP* genes through the gametes of rHD1. Pluripotency was determined by the expression of stem cell specific markers, *in vitro* neural differentiation and the formation of teratoma in immune compromised mice. Expression of *GFP* was confirmed by fluorescent microscopy and the expression of mutant *HTT* was determined by quantitative real time PCR (Q-PCR), sequencing, and immunocytochemistry. rHD-ESCs express mutant *HTT* and form intranuclear inclusion, a classical cellular feature of HD. Additional expansion of the pathogenic polyQ region was also observed in rHD-ESCs and HD gametes. The confirmation of transgene inheritability in this study advances the establishment of a cohort of monkeys with inherited mutant *HTT* for longitudinal disease analysis and development of novel biomarkers and therapeutics.

4.2 Introduction

Huntinton's disease is an inherited neurodegenerative disorder resulting from an expanded CAG encodes the polyglutamine, polyQ, stretches at the N- terminus of the *HTT* protein (HD Collaborative Research Group., 1993). HD is a devastating disease resulted in motor dysfunction, psychiatric disturbances and cognitive impairment. The expression of HD in patient presented after onset of symptom at mid-life about 15 to 20 years later onset depending on length of the poly Q, which CAG repeats below 37 are considered unaffected (Li et al., 1999). The HD pathological could be found in spiny striatal neurons and could be characterized by accumulation of oligomeric mutant *HTT*, the formation of inclusion body (IB), and progressive neuronal death. Although, the genetic alteration that lead to HD has been identified but, however, the function of *HTT* remain largely unknown.

HTT protein is plays key roles in early embryo development (Nasir et al., 1995, Zeitlin et al., 1995), and expressed widely in the body with highest expression in the brain and the testis. However, the brain is the primary target of damage (Gutekunst et al., 1999, Davies et al., 2001, Li et al., 2002, Rubinsztein et al., 2002). To elucidate the mechanism of HD pathogenesis, stem cells could be a suitable model because they have the ability to differentiate into multiple cell lineages, which is appropriate to study the tissue specific pathogenesis. Although, HD-ESCs have been established from mouse (Wheeler et al., 2000, Lin et al., 2001, Menalled et al., 2003, Heng et al., 2007) and human (Mateizel et al., 2006, Park et al., 2008), however, the HD-ESC have not been generate from germline transmission. And phenotypes of HD in neuron differentiation also have not been developed.

Nonhuman primate share close genetic, physiologic, and metabolic similarity to humans, therefore, their serves as an essential research tool particularly in human diseases (Rogers et al., 2002). A transgenic NHP which carries a genetic defect leading to human disease such as HD will not only recapitulate human conditions but also the pathogenic events that are critical for investigating disease pathogenesis and can used to develop novel biomarkers and treatments (Chan et al., 2004, Yang et al., 2008). Thus, the objectives of this study were established HD-ESC lines from transgenic HD rhesus monkey (rHD1) sperm by using ICSI technique to produced HD rhesus monkey embryos and examined the expression of HD pathological features after differentiated to neural cells. These cell lines could be useful for stem cell models for studying HD. The confirmation of germline transmission through the gametes, which is the critical step in the development of transgenic primate models of human inherited genetic diseases were also studied.

4.3 Materials and Methods

4.3.1 Animals models

The rhesus monkeys were housed and maintained at Yerkes National Primate Research Center (YERKES). All animal procedures were conducted according to a guideline provided by institutional animal care and committee protocols at Emory University. All documented research in this study was conducted in accordance to the Guide for the Care and Use of Laboratory Animals.

4.3.2 Rhesus monkey ovarian stimulation and oocytes collection

The ovarian stimulations were performed as described by Laowtammathron et al. (2010). Briefly, the cycling female monkey was superovulated by subcutaneous

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

Cumulus-oocyte complexes (COCs) were collected around 37 hours 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 COCs was diluted immediately with TALP-HEPES medium (Bavister et al., 1983) supplemented with 10 iu/ml heparin sodium salt (MP Biochemicals heparin)

Cumulus cells were stripped off from the COCs 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), and MII (extrusion of the first polar body). The 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 17β estradiol and 3 μg/ml progesterone, at 37°C under humidified atmosephere of 5% CO_2, 5% O_2 and 90% N_2 for 3-4 hours. Numbers and stages of oocytes after collection from monkey aging from 5 to 15 years old were recorded.

4.3.3 Generation of transgenic HD rhesus monkey (rHD1).

rHD1 were provided by Yang and colleagues (2008). Briefly, the high titer lenti viruses carrying exon1of human *HTT* with 84 CAG repeats and *GFP* gene under the regulation of human poly ubiquitin C promoter were injected into the perivitelline space of MII arrested monkey oocytes followed by ICSI. The resultant embryos were transferred into surrogate females for generation of transgenic monkeys. Transgenic status was confirmed by PCR.

4.3.4 Rhesus monkey semen collection

A WT and rHD1 rhesus monkey was used for semen collection. Ejaculated semen was collected once a week in the morning. The males were trained to chair restraint and semen was collected by direct penile stimulation with stimulator equipped with EKG pad electrodes (30–50V, 20 ms duration 18 pulse/sec). The ejaculated semen was kept at RT for 20 min to liquefy. Liquid portion of semen was transferred into 15 mL conical tube and washed with TALP-HEPES medium supplemented with 4 mg/mL BSA (Bavister et al., 1983), after that they was centrifuged at 112 × g at RT for 7 min. The sperm concentration, motility and viability were determined and recorded.

4.3.5 Intracytoplasmic sperm injection (ICSI) and embryos culture

The embryos were produced by ICSI method. The experiment was divided into two groups by compared between rHD1 and WT sperms fertilized with WT rhesus monkey oocytes. The ICSI was carried out using micromanipulator under inverted microscope (Olympus). The sperm was individually immobilized by scoring the mid piece and single sperm was aspirated from sperm droplet and moved to a droplet containing MII oocytes (with first polar body). The oocyte was captured with

the holding pipette and immobilized with its polar body at either the 6 or 12 O'clock position, then, spermatozoa was injected into the cytoplasm. Injected oocytes were transferred to HECM-9 medium (Zheng et al., 2001) and incubated at 37°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 7-8 days. The 5% FBS was added to HECM-9 medium on second day of culture. Fresh media were replaced every two days.

4.3.6 Feeder cells preparation

Pregnant mice at day 13 post coitum were sacrificed by cervical dislocation. The uterine horns were dissected out from the placenta and each fetus was separated. Head and internal organs were removed and washed several times in PBS to remove blood. The fetus tissues were minced to small pieces and suspended in Trypsin/EDTA at 37°C with gentle shaking for 15 min. The Trypsin/EDTA was neutralized with culture medium and transferred 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 was carefully collected and subjected to low speed centrifugation for 5 min. The cell pellet was then resuspend and culture in DMEM (Invitrogen) supplemented with 10% FBS (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 dead cells. At sub-conference, MFF were trypsinized and cultured. MFFs were frozen at passage 1 or 2 of culture. The MFFs were inactivated with 5 μg/ml mitomycin C (Sigma) for three hours followed by a thorough wash before plating.

4.3.7 Establishment of HD-ESC

Rhesus monkey embryos at stage expanded and hatching blastocysts were selected for ESC establishment. The embryos for ESC establishment were derived from 2 sources; ICSI-WT and ICSI-HD. The embryos were washed several times in TALP-HEPES (Bavister et al., 1983) and placed in TALP-HEPES drop. The inner cell mass (ICM) isolation was manipulated under inverted microscope by hold the embryo with both sides on holding pipette. The ICMs was positioned at 3 o'clock position (Figure 3.3.1a). The ICM were separated from trophectoderm cell (TE) by XYClone laser (Hamilton Throne, Inc.) in condition; 100% power, 150 pulses/sec, 200 pulse width (μs) and 10 sec duration. The isolated ICMs were washed in ES medium several times before plated on feeder cells. Four days after plating, the attachments of the ICM were recorded. Half of the ES medium was replaced every other day. The outgrowth morphology was observed daily for 10 to 14 days before mechanically sub-cultured.

3.3.8 Establishment and maintenance of monkey ESCs

ICMs outgrowths that showed ES like morphology were mechanically sub-cultured. Only the distinctive ESCs morphology were mechanically dissociated into small pieces and plated on fresh MFFs which were pre-incubated with ES medium for 3 hr. Two days after sub-culture, ESCs morphology was observed and only the colonies that exhibited distinct monkey ES-like cells morphology were selected for sub-culture.

3.3.9 ESCs characterization

4.3.9.1 Immunocytochemistry for ESCs markers

ES medium was removed and the ESCs were washed several times in PBS without Ca^{2+} and Mg^{2+}. The ES cells were fixed with 4%

paraformaldehyde (PFA) for 15 min at RT and washed several times with PBS. The alkaline phosphatase staining was done by using an alkaline phosphatase staining kit (Vector® Blue Alkaline Phosphatase Substrate Kit III; SK-5300).

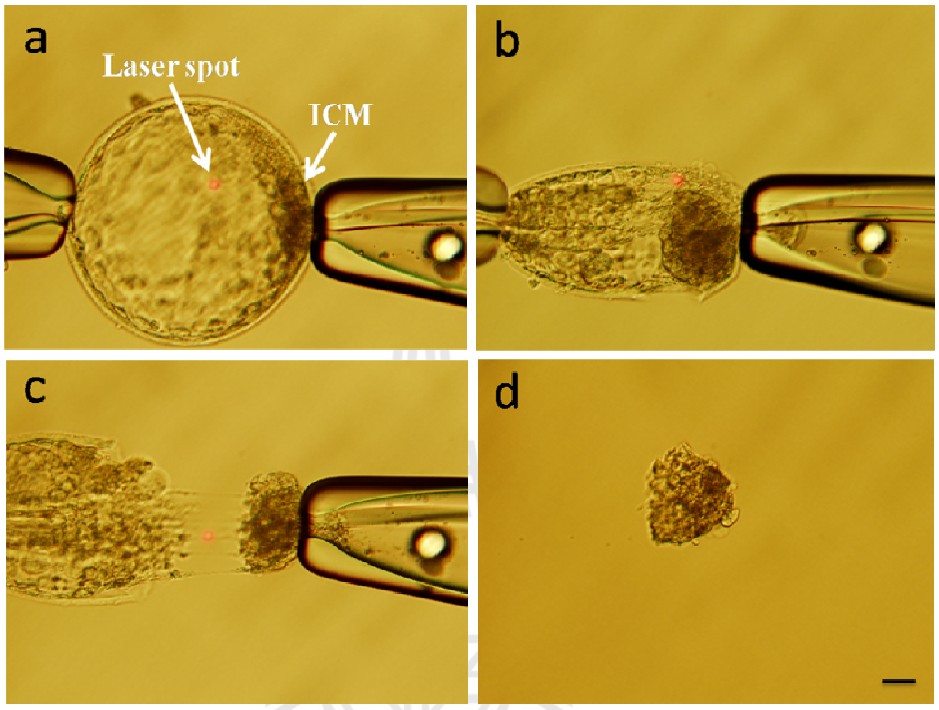


Figure 4.3.1 The ICMs isolation by XYClone laser. ICM region was positioned at 3 o'clock (a). ICM and TE cells were separated by move the embryo through the red spot of laser (b, c). ICMs clump surrounded with small amount of TE cells (d). Scale bar: 20 µm.

The cell surface markers including stage specific embryonic antigen (SSEA)-3, SSEA-4, tumor related antigen (TRA)-1-60, and TRA-1-81 were examined following standard protocols. The ES cells were blocked in PBS containing 4% goat serum for 1 h at RT. Then, cells samples were incubated with the primary antibodies including rat monoclonal antibody anti-SSEA-3 (1:200 dilutions, Chemicon, MAB 4303), mouse monoclonal antibody anti-SSEA-4 (1:200 dilution, Chemical, MAB 4304), mouse monoclonal antibody anti-TRA1-60 (1:200 dilution,

Chemicon, MAB 4360), mouse monoclonal antibody anti-TRA1-81 (1:200 dilution, Chemicon, MAB 4381). For nuclear markers, ESCs were permeabilized by 0.2% Triton-X and 0.1% Tween 20 for 1 h. Then incubated with primary nuclear antibodies including mouse monoclonal antibody anti-Oct3/4 (1:200 dilution, Santa cruz biotechnology, inc., SC-5279), goat monoclonal antibody anti Nanog (1:200 dilution, Santa cruz biotechnology, inc., SC-30329), rabbit monoclonal antibody anti SOX-2 (1:200 dilution). The ESCs were incubated with primary antibody overnight at 4˚C. After primary antibody were washed, ESCs were incubated with appropriate secondary antibody conjugated with either Alexa Flour 488 (1:1000 dilution, Invitrogen) or Alexa Flour 594 (1:1000 dilution, Invitrogen) or Rhodamine (1:1000 dilution, Invitrogen) or Texas red (1:1000 dilution, Invitrogen) for 1 h. ESCs were co-stained with Hoechst 33342 (5μg/ml) for 5 min and then examined under fluorescence microscopy (Olympus).

4.3.9.2 Immunostaining of mutant *HTT*

The aggregation mutant *HTT* was detected by staining with EM48 antibody. Differentiated rHD-ESCs were fixed using 4% PFA for 15 min, permeabilized, and blocked. The sample was then incubated with primary antibody mEM48 (1:50) at 4°C overnight. After washing with PBS, the samples were processed with avidin-biotin using the Vectastain Elite ABC kit (Vector Laboratories), and immediately stained with DAB (Vector Laboratories) for 30–40 sec. Cell samples were examined and captured by MetaMorph software (Universal Imaging). For fluorescent imaging, a secondary antibody conjugated with Alexa Red (Molecular Probe) was used for detection of the primary antibody. DNA was counterstained with Hoechst 33342 (5μg/ml).

4.3.9.3 Cytogenetic analysis

ES cells colonies were passaged into a T-25 flask and treated with colcemid (KaryoMax®) for 20 minutes to block cells at the metaphase. Cells were dislodged with 0.05% Trypsin-EDTA, centrifuged and gently resuspended in hypotonic 0.075 M KCL solution for 20 min. 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 20-30 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).

4.3.9.4 Embryoid body (EB) formation

The ESCs colonies were mechanically detached from feeder cells and carefully transferred into 35 mm non-attachable dish containing ES culture medium without FGF-2. Half of the medium was replaced every other day. The detached ES colonies were cultured for 5-7 days to form EBs.

4.3.9.5 *In vitro* differentiation to neuronal lineage

Embryoid bodies were transferred into 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. After that the cells were cultured in N3 medium for another 7 days to enhance maturation of neurons. The N1 medium

composed of KO-DMEM supplemented with minimum essential amino acid, 200 mM of L-glutamine and N2 supplement (Invitrogen). The N2 medium composed of N1 medium supplemented with 20 ng/mL bFGF. The N3 medium composed of KO-DMEM supplemented with 1% FBS and B27 supplement (Invitrogen). The neural progenitor cells were stained with Nestin. Successful differentiation of neuronal cell types was confirmed by the expression of neuron specific βIII tubulin, GFAP and MAP2.

4.3.9.6 *In vivo* differentiation of ES cells

Undifferentiated of ESCs colonies were collected by mechanical dissociation and resuspensed in 50 µl of DPBS. Then ES cells suspension were injected into kidney of severe compromised immune deficient (SCID) mice. The resulting tumors at 8-10 weeks of development were fixed with 4% PFA and histological examined after paraffin embedding. All animal procedures were approved by the IACUC at the Emory University.

4.3.9.7 Genomic DNA isolation and genotyping PCR

Genomic DNA (gDNA) was extracted from a cell pellet generated from all HD-ESCs using the Promega Wizard Kit (Qiagen). Additionally, gDNA was extracted from sperm with phenol-chloroform extraction and precipitation with isopropanol. For genotyping of polyQ, 50 ng of gDNA was amplified by PCR at an annealing temperature of 68° for 40 cycles with the following primers; HD32-Forward 5'-CTACGAGTCCCTCAAGTCCTTCCAGC-3' and MD177-Reverse 5'-GACGCAGC AGCGGCTGTGCCTG -3'. All products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide.

4.3.9.8 Sequencing of polyQ

Five hundred ng of total RNA was extracted from the all HD-ESC and rHD1 sperm and reverse-transcribed to cDNA using High Capacity Reverse Transcription Kit (Applied Biosystems). The *HTT* transcript was amplified from the cDNA by PCR at an annealing temperature of 67° for 40 cycles with the following primers; HD32-Forward 5'-CTACGAGTCCCTCAAGTCCTTCCAGC-3' and MD177-Reverse 5'-GACGCAGCAGCGGCTGTGCCTG-3'. All PCR products were electrophoresed on a 1.5% agarose gel and target bands were gel purified, cloned into the pGEM-T easy vector (Promega), and subsequently sequenced at Genewiz Corporation with T7 and SP6 primers. Both the expanded polyQ mutant *HTT* transgene and the endogenous *HTT* gene were cloned into the pGEM-T easy vector and sequenced.

4.3.9.9 *HTT* mRNA expression analysis by quantitative real-time PCR (qPCR)

Five hundred ng of total RNA from all cells was reverse transcribed to cDNA. Quantitation of *HTT* mRNA expression was performed using custom-designed gene-specific Taqman assays (forward sequence - 5'GCCGCTGCTG CCTCA4'3; reverse sequence– 5'TGCAGCGGCTCCTCAG'3; probe sequence - 5'CCGCCGCCCCCGC C'3) in a 1X final reaction of Taqman Gene Expression Master Mix (Applied Biosystems.) All qPCR results were first normalized with the geometric mean of two endogenous controls, beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase and *(GAPDH)* (custom Taqman assay for *ACTB* forward sequence - 5'GCCGTCTTCCCCTCCAT3'*;* reverse sequence – 5'CTGACCCATGCC CACCA T'3; probe sequence –

5’CACGCCCTGGTGCCTG’3; *GAPDH* measured with commercially available rhesus Taqman assay from ABI). The *ACTB-GAPDH* normalized qPCR results were then further normalized to the express of the endogenous *HTT* transcript via a Taqman assay designed to exon 10-11 of *HTT* (forward sequence 5’AGCCCTGTCCTTTCAAGAAAACAA’3; reverse sequence- 5’CA TCCTCCAAGGCTTCTTCTTCT’3; probe sequence -5’ CCTAAGAGCACT TTGCCTTT’3).

4.4 Results

4.4.1 Production of HD embryos

A rHD1 monkey that carrying exon 1 of the human *HTT* gene with 29 CAG repeats and a *GFP* gene under the regulation of human polyubiquitin C promoter was generated by lentiviral transfection of matured oocyte followed by *in vitro* fertilization, culture and embryo transfer into surrogate female as described previously (Yang et al., 2008). To determine successful germline transmission of the mutant *HTT* and *GFP* transgenes, *In vitro* culture, ICSI, rHD1 sperm followed by *in vitro* embryo culture and derivation of ESCs from the ICMs of the resulted blastocyst stage embryos were performed. A total of 106 oocytes were retrieved by laparoscopy from two stimulated females, 35-37 hours after hCG injection. The MII oocytes were randomly divided into two groups; 70 MII oocytes were ICSI using rHD1 sperm and 36 MII oocytes were ICSI using WT sperm (control group). The cleavage rates of HD and WT rhesus monkey embryos were not significant different (Table 4.1). Embryos development from cleavage to molula stage (Figure 4.1) of HD embryos (93.4%;

fertilized, 90.8%; cleavage; 90.8% 8 cell stage, and 76.3%; molula stage) was slightly higher than WT embryos (86.7%; fertilized, 86.7%; cleavage; 83.3% 8 cell stage, and 76.7%; molula stage) but not statistically significant different. The gene transfer was confirmed by expression of GFP in HD rhesus monkey embryos (Figure 4.2).

4.4.2 Outgrowths of ES like cells from HD embryos

The ICMs of expanded blastocysts were separated from TE cells by using laser. After that the ICMs were cultured on MFFs feeder layer in ES medium. The ICMs and small amount of TE cells were attached onto feeder layer 2 to 3 days after cultured. In early stage, the TE cells started to grow on the feeder layer faster than the ICMs outgrowth and the overgrowth of TE cell tends to inhibit ICMs outgrowth. However, ICM grow aggressively after 7. The ICMs isolated using the laser technique could reduce the overgrowth of TE cells because most of the TE cells were discarded.

A total of 45 HD blastocyst embryos were dissected and cultured (Table 4.2). Twenty-one embryos (21/45; 46.67%) attached onto the feeder layer. Seven out of 21 embryos (7/21; 15.56%) could further proliferate (Figure 4.3). After 10 to 14 days of culture, ICM outgrowths were passaged by mechanically. The ICM outgrowths were cut into small clumps of cells using fined glass pipette and transferred onto freshly prepared MFFs feeder layer which were preincubated with ES medium. After several sub-cultured, all 7 outgrowths (rHD-ES-1, 2, 3, 4, 5, 6, and 7) showed ES like cells morphology.

A total of seven rHD-ESC lines were established from blastocysts derived from rHD1 sperm. Three of rHD-ESC lines were male and four were female with normal karyotype and diploid set of 42 chromosomes (Figure 4.7 and Table 4.3).

Table 4.1 HD and WT embryo development.

Group	No. embryo	Cleavage (%)	No. (%) embryo developed to			
			8 cell	Mor	ComMor	BL
HD	76	71 (93.4)	69 (90.8)	58 (76.3)	55 (72.4)	45 (59.2)
WT	30	26 (86.7)	25 (83.3)	23 (76.7)	20 (66.7)	18 (60.0)

Values with in column are not significantly different (P>0.05).

Abbreviation 8-C: eight cell stage embryo,

Mor: morula stage embryo,

ComMor: compacted morula stage embryo,

BL: blastocyst stage embryo.

Table 4.2 Establishment of rHD-ES cells from HD embryos.

No. ICM cultured	No. (%) attached on feeder layer	No. (%) outgrowth	Passage number	
			1-5 (%)	> 5 (%)
45	21 (46.67%)	7 (15.56%)	7 (15.56%)	7 (15.56%)

4.4.3 Monkey embryonic stem cells characteristic and pluriporency

After mechanical sub-cultured more than 10 passages, all outgrowths (rHD-1, 2, 3, 4, 5, 6, and 7) showed ES cells morphology. All HD-ESC lines were confirmed for their ES cells properties by ES cell markers expression analysis. The results showed that all HD-ES cells lines expressed common ES cell markers including Oct-4, Nanog, SOX-2, SSEA-4, TRA1-60 and alkaline phosphatase, however, expression of SSEA-3 could not observe (Figure 4.4). The pluripotency of ES cells were determined by *in vitro* differentiation to neuronal cells. The neuron

differentiation by step-wise protocol was used in this study. Immunostaining was performed at different stages of neural differentiation using specific markers. At the end of N2 stage, expression of nestin was observed in neural progenitor cells (Figure 4.5). The matured neurons were found after induction in the end of N3 stage by immunostaining with βIII tubulin, MAP2 and GFAP (Figure 4.5).

Table 4.3 Karyotype of rHD-ESCs

Line	Passage number	Sex	Karyotype
rHD-1	62	M	42XY
rHD-2	60	F	42XX
rHD-3	53	F	42XX
rHD-4	49	F	42XX
rHD-5	52	M	42XY
rHD-6	47	F	42XX
rHD-7	45	M	42XY

The undifferentiated ES cell was implanted into the kidney capsule of SICD mice for teratoma formation. Animals were euthanized at 8 to 10 weeks post-implantation and teratomas were recovered for histological study. Teratoma had differentiated into tissue of different germ layers such as gut tube epithelial (endoderm), cartilage (mesoderm) and hair follicle (ectoderm) (Figure 4.6).

4.4.4 Expansion of mutant *HTT* in rHD-ESCs

All rHD-ESC lines established from rHD1 carrying exon 1 of the human *HTT* gene with 29 CAG repeats and a GFP gene, which was confirmed by PCR

(Figure 4.8). However, the express of mutant *HTT* in each rHD-ESC vary among cell lines. Sperm from rHD1 carry 28 to 48 CAG repeats and rHD-ESC lines carry 28-130 CAG repeats suggesting possible germ line transmission of polyQ (Table 4.4). To further confirm if rHD-ESCs carry the human mutant *HTT* transgene, sequence analysis of the PCR amplicon flanking the polyQ region was aligned and compared with the following genes; *HTT* sequences of four control rhesus monkey, rhesus *HTT* sequence from the University of California at Santa Cruz (UCSC) genome data base, and rhesus monkey *HTT* from the 7 rHD-ES cell lines (Figure 4.9). All rHD-ES cell lines carry the human specific *HTT* sequence with variations in the number of CAG repeats (Table 4.4).

4.4.5 Expression of mutant *HTT* in rHD-ESCs derived neuronal differentiation

Although, mutant *HTT* express ubiquitously in the body; the expression level of mutant *HTT* is higher in neuronal cells with mutant *HTT* aggregate nuclear inclusion (Yang et al., 2008). The expression level of mutant *HTT* in undifferentiated rHD-ES cells were determined by q-PCR analysis (Figure 4.10a) and immunostaining of *in vitro* derived neural cells by using antibody specifically recognizing mutant *HTT* (Figure 4.10b). The expression of mutant *HTT* was detected in undifferentiated rHD-ES cells with increased expression as neural differentiation (N3 stage) progresses in rHD-ES1, 2, 3 and 7 when compared with endogenous *HTT* and WT control (Figure 4.10a). The expression level of *HTT* in rHD-ES5 was not up-regulated (Figure 4.10a). It is not clear whether the expression of mutant *HTT* in rHD-ES5 is impacted by the expanded polyQ or if aberrant gene rearrangement occurred. While protein level of aggregated mutant *HTT* was too low to be detected by western blot analysis,

aggregated mutant *HTT* and intranuclear inclusions in neural differentiated rHD-ESCs was detected by immunostaining with mEM48 antibody (Figure 4.10b).

Table 4.4 Summary of rHD-ESCs. All rHD-ESCs carry the human specific *HTT* sequence with variations in the number of CAG repeats.

rHD-ES cell Lines	**Mutant *HTT***	**Endogenous rhesus *HTT* CAG#**	**Mutant *HTT* CAG#**
rHD-ES1	+	10	30-48
rHD-ES2	+	10	28
rHD-ES3	+	10	30
rHD-ES4	+	10	29-30
rHD-ES5	+	10	130
rHD-ES6	+	10	29-30
rHD-ES7	+	10	28-29
rHD1-sperm	+	10	28-48

#: repeat number.

4.5 Discussion

A male transgenic HD rhesus monkey, rHD1, that carrying exon 1 of the human *HTT* gene with 29 CAG repeats and a *GFP* gene under the regulation of human polyubiquitin C promoter was used for HD embryo production. Development of HD embryos was no different from the control group (WT).

The *HTT* protein is important for embryogenesis, craniofacial formation, and forebrain development (Reiner et al., 2003). The deletion of the *HTT* gene in mice

resulted in early embryonic lethality (Duyao et al., 1995, Nasir et al., 1995, Zeitlin et al., 1995), which may due to the disorganization of embryo and gastrulation (Nasir et al., 1995). The *HTT* gene may play an important role as regulatory molecules underlying gastrulation in vertebrates (Copp, 1995) or vesicular transport of nutrients (Reiner et al., 2003). In this study, demonstrated that a rhesus monkey embryo with mutant *HTT* and expanded CAG repeats has no impact on early embryo development until blastocyst stage. HD dose not critically disrupt embryos function because homogenetic HD mutations do not exhibit embryonic lethality (Wexler et al., 1987, Myers et al., 1989, Gusella and MacDonald et al., 1996).

The establishment of ESC lines from rHD1 embryos was produced by ICSI. Seven rHD-ESC lines (rHD-ES1-7) were established (Table 4.2) and pluripotent based on differentiation to neural cell and the formation of teratoma in the kidney capsule of SCID mice has been done. A rHD-ESC lines carry mutant *HTT* and *GFP* transgene (Figure 4.8). The development of HD caution pathology was observed (rHD-ES1, 2, 3, and 7) in neural cells *in vitro* differentiation from rHD-ESC. Increased expression of mutant *HTT* was observed as neural differentiate progress aggregates and NIs was also observed in neural differentiation. Mutant *HTT* (rHD-ES5) carry 130 CAG repeats but the expression level of mutant *HTT* was not upregulated compared to control ES level which not clear whether expression of mutant *HTT* is impacted by expanded polyQ or if aberrant gene rearrangement occurred because some reported found that, the long full length mutant *HTT* is less toxic when compare with small *HTT* fragments in HD mouse (Bates et al., 1998; Schilling et al., 1999; Wang et al., 2008).

A transgenic animal is a good model for studying disease progression and pathologies. Transgene can be inheriting through the germ cells similar to the inheritable pattern of human genetic disorders (Schilling et al., 1999, Chan et al., 2001, Yang et al., 2008). NHP is an important in modeling diseases such as HD because various systems in the body are impacted during the course of the disease, which include motor functions, psychiatric and metabolic disturbance (Tabrizi et al., 2011). NHP also share similar motor repertoire that allows the evaluation of fine movement control that no other model system can offer (Courtine et al., 2007). However, rhesus monkey reaches pubertal age at around four years old. It is a relatively long physiological event compared to most of the laboratory species. Therefore, pluripotent stem cell is an alternative model (Park et al., 2008) because not need long study event. rHD-ESCs in present study that derived from rHD1monkey inheritance and express mutant *HTT* and GFP transgenes (Figure 4.2) including develop HD cellular feature (Figure 4.10), which could serve as a unique tool for further evaluation of HD pathogenic mechanism, and could be a useful stem cell model for studying HD, the confirmation of germline transmission through the gametes is an important and critical step in the development of transgenic primate models of human inherited .

4.6 Conclusion

rHD1, the first transgenic HD rhesus monkeys, has reached pubertal age and germline transmission was confirmed by using his sperm to fertilize mature monkey oocytes followed by the production of transgenic embryos (Figure 4.1 and 4.2) and subsequent derivation of rHD-ESCs. rHD-ESCs inherit and express both the mutant *HTT* and GFP transgenes and develop unique HD cellular features, including

intranuclear inclusions, as they differentiated toward neuronal lineage. While rHD-ESCs could be a useful stem cell model for HD study, the confirmation of germline transmission through the gametes is an important and critical step in the development of transgenic primate models of human inherited genetic diseases. Overall, the successful inheritability of the mutant *HTT* transgene in our nonhuman primate model can now lead to the establishment of second generation HD rhesus monkeys for studies of HD pathogenic trajectories and much needed development of novel treatments.

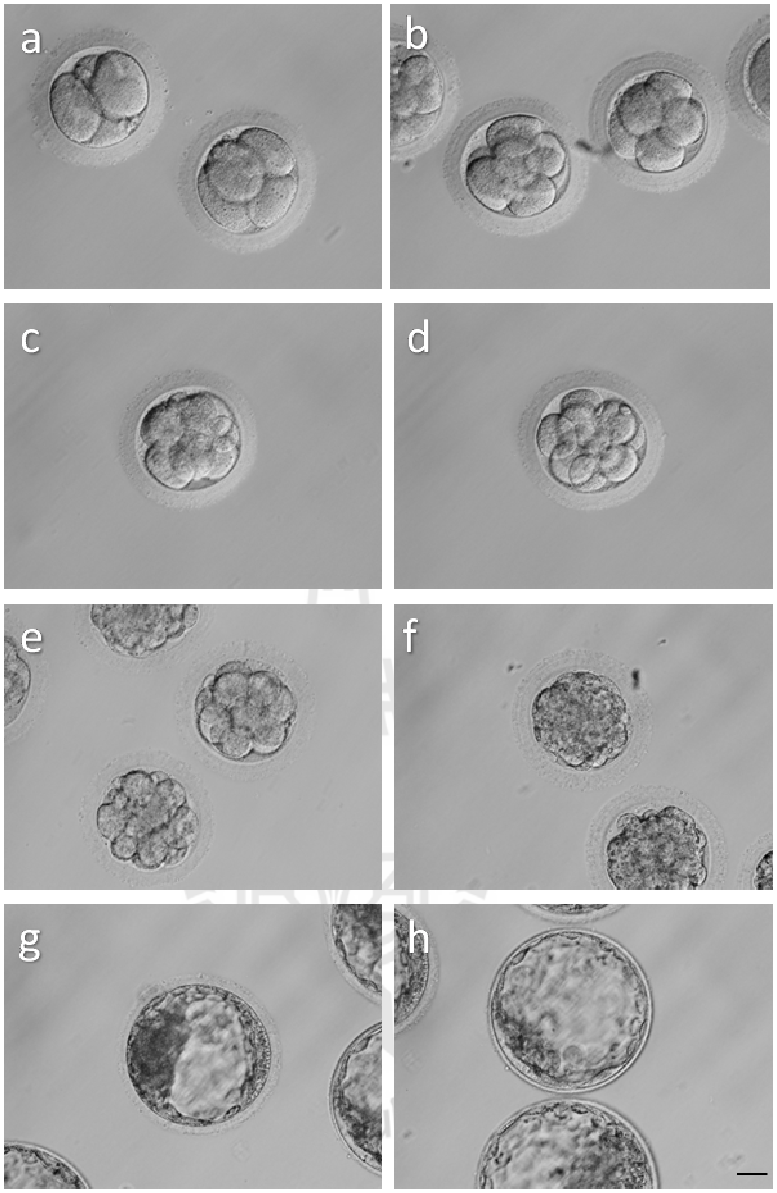


Figure 4.1 The HD rhesus monkey embryos at 4 cell (a), 6 cell (b, c), 8 cell (d), morula (e), compacted morula (f), blastocyst (g) and expanded blastocyst stages (f). Scale bar: 20 μm.

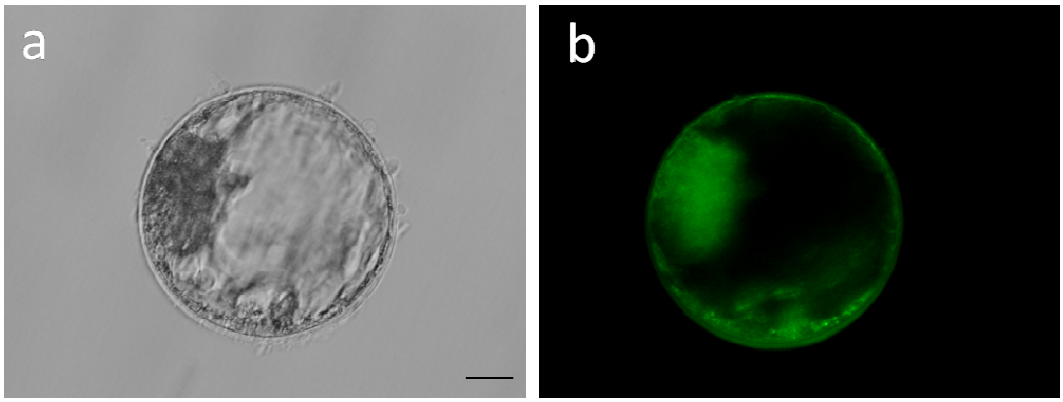


Figure 4.2 The blastocyst of HD rhesus monkey (a) and expression of GFP (b). Scale bar: 20 µm.

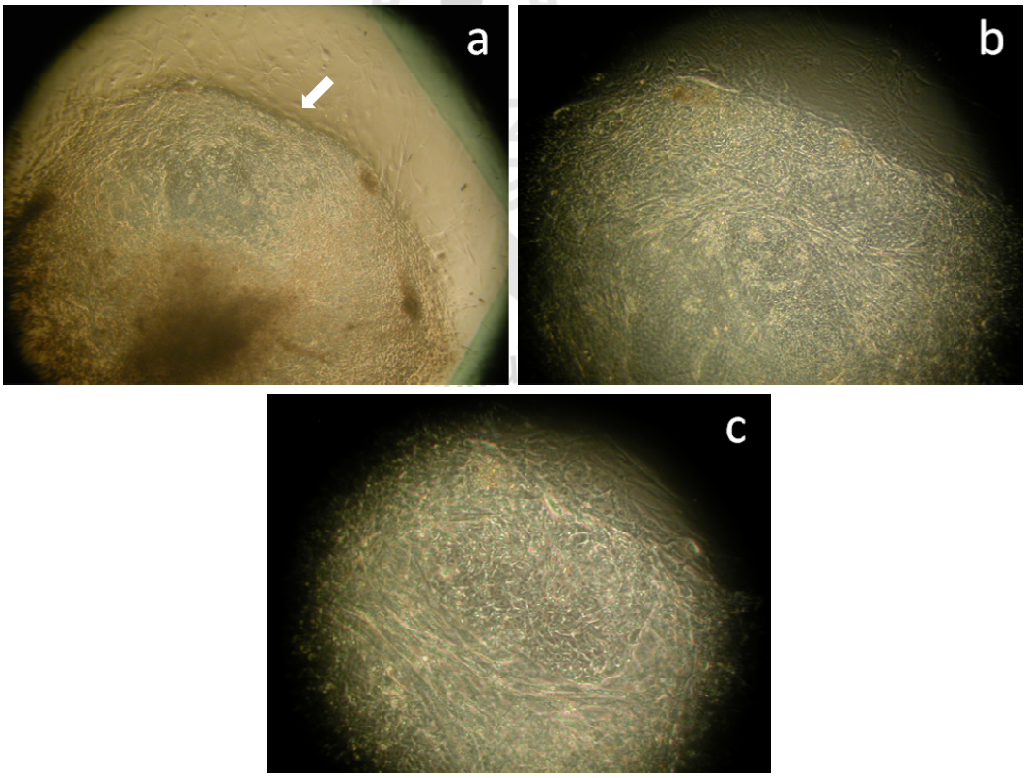


Figure 4.3 ES like cell outgrowths derived from ICMs cultured. (a) The arrow indicate outgrowth of ICMs after culture on feeder cells for 15 days. (b) 20X magnification. (c) 40X magnification.

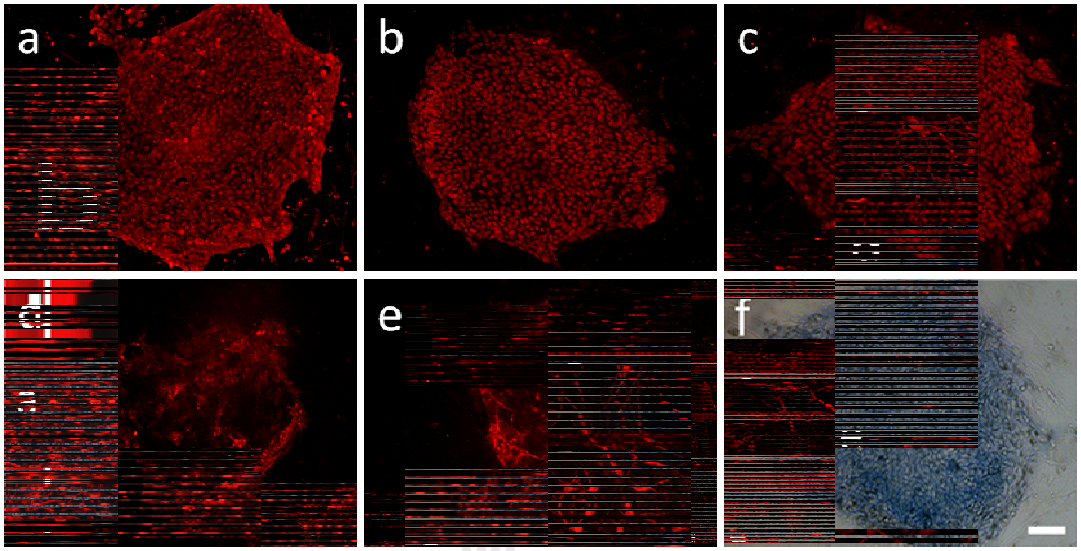


Figure 4.4 Expression of stem cell markers. (a-e) Immunostaining of undifferentiated rHD-ESCs with antibodies which recognize stem cell specific proteins including; Nanog (a), Oct4 (b), SOX-2 (c), SSEA-4 (d), TRA-1-60 (e) and alkaline phosphatase staining (f). Scale bar = 50 μm.

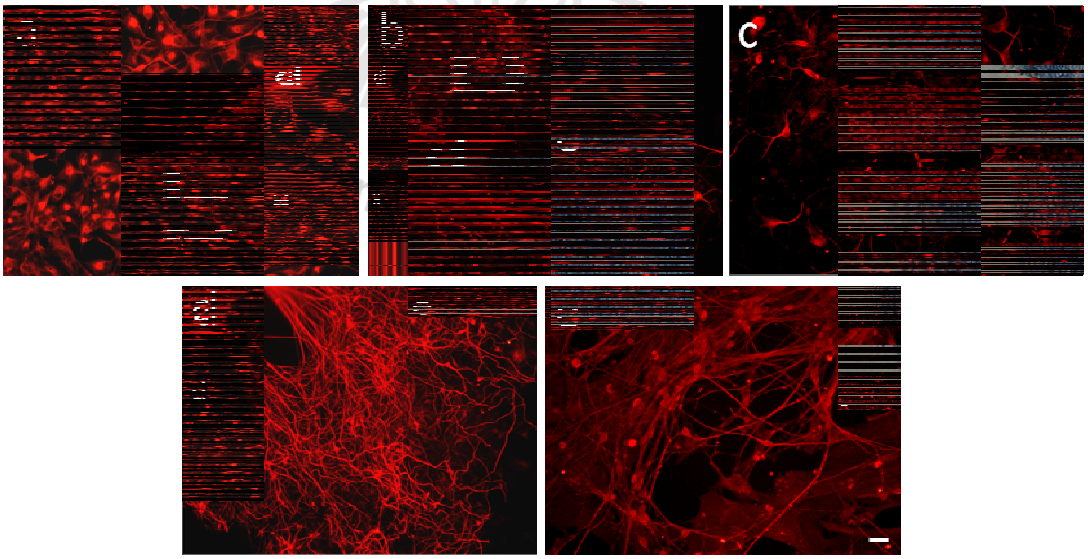


Figure 4.5 The *in vitro* differentiation. (a-e) Immunostaining of neural differentiated from rHD-ESCs with antibodies which recognize neural specific proteins including; Nestin (a), MAP2 (b), GFAP (c), βIII tubulin (d), and CHAT (e). Bar (a-f) = 10 μm.

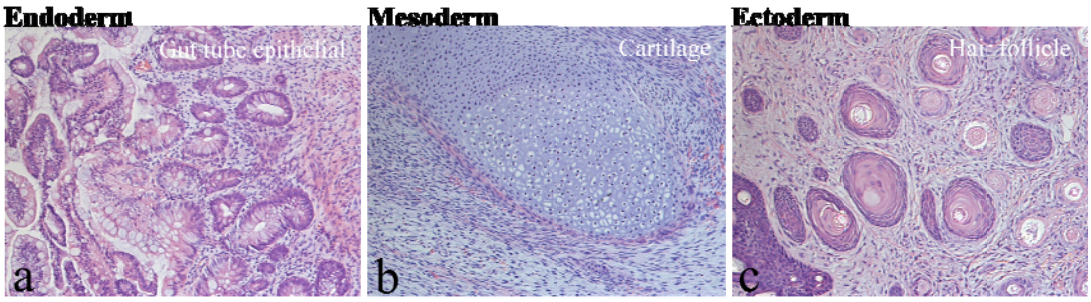


Figure 4.6 Teratoma formations of SCID mice after graft of undifferentiated ESCs cells into kidney capsule. Teratoma had differentiated into different tissue layers, (a) gut tube epithelial (endoderm), (b) cartilage (mesoderm) and (c) hair follicle (ectoderm). Samples were stained with hematoxylin and eosin. Bar = 50 μm.

Figure 4.7 Cytogenetic analysis of rHD-ES1 shows the normal diploid chromosome (42; XY).

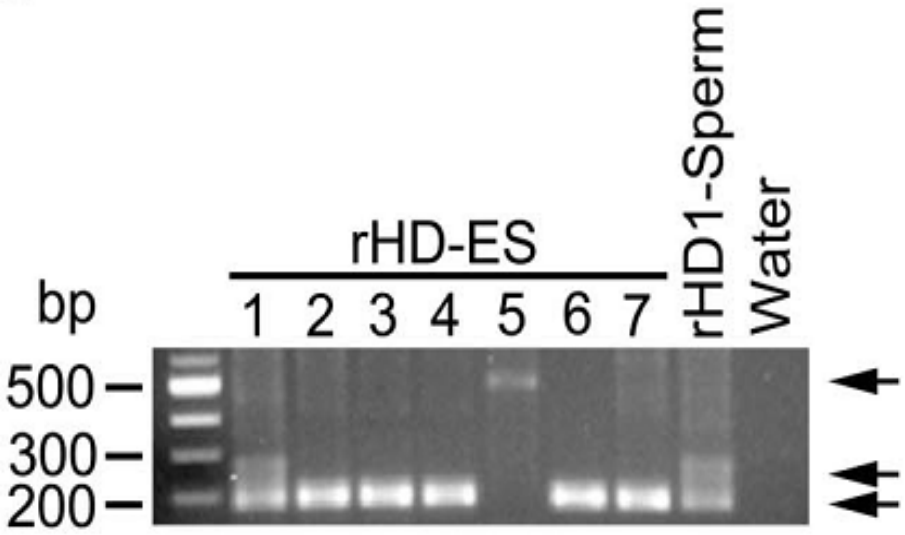


Figure 4.8 Genotyping of rHD-ESCs by PCR analysis.

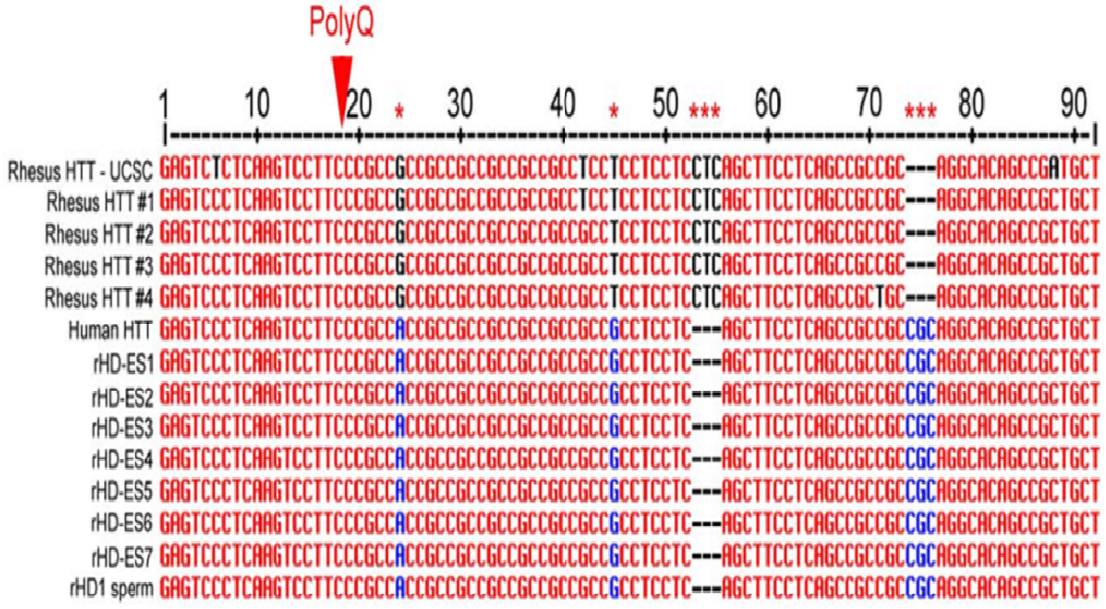


Figure 4.9 Sequence analysis of the PCR amplicon flanking the polyQ region compared with the following genes; *HTT* sequences of four control rhesus monkeys, rhesus sequence from the University of California at Santa Cruz (UCSC) genome database, human *HTT*, and rhesus *HTT* from the 7 rHD-ESC cell lines.

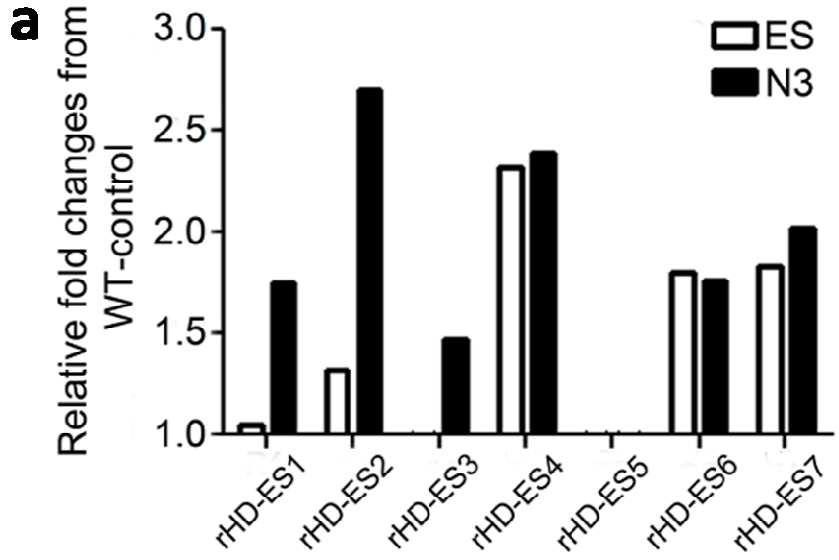
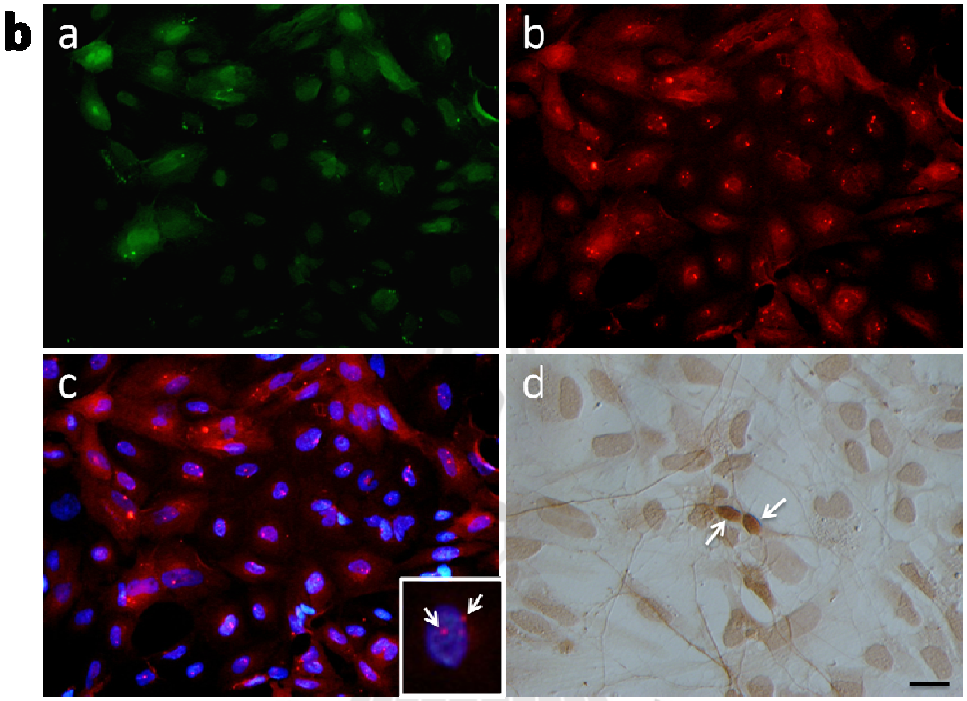


Figure 4.10 Expression of mutant *HTT*. (a) Expression level of mutant *HTT* in rHD-ESCs cells was determined by q-PCR and was normalized to endogenous *HTT* and WT control monkey *HTT* expression. Undifferentiated rHD-ES cells (ES) and *in vitro* neural differentiated rHD-ES cells (N3) were collected, followed by RNA extraction and real-time PCR (qPCR) analysis with *HTT* specific Taqman assays. All qPCR results were normalized with the geometric mean of two endogenous controls, beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydroge *(GAPDH)*, and then normalized to WT control; (b) The inmmunostaining mutant *HTT* specific marker in rHD-ESCs cells derived neural differentiation; (a) Epifluorescent image of *in vitro* neural differentiated rHD-ESCs cells; (b) Immunstaining of *in vitro* neural differentiated rHD-ESCs cells using antibody specific to mutant *HTT* aggregates; (c) Overlay images of (b) and DAPI stained nucleus (arrow-

nuclear inclusion); (d) Immunostaining of mutant *HTT* aggregates (arrow-cytoplasmic mutant *HTT* aggregate); Bar = 10 μm.



4.7 References

Ambrose, C.M., Duyao, M.P., and Barnes, G. (1994). Evidence against simple inactivation due to an expanded CAG repeat. **Somat Cell Molec Genet** 20: 27-38.

Bates, G.P., Wanker, E.E., and Davies, W.W. (1998). Murine models of Huntington's disease. In Genetics instabilities and hereditary ne urological diseases. S.T. Warren, ed. **San Diego, Academic Press** 8: 355-370.

Bavister, B.D., Leibfried, M.L., and Lieberman, G. (1983). Development of preimplantation embryos of the golden hamster in a defined culture medium. **Biol Reprod** 28: 235-247.

Chan, A.W., Chong, K. Y., Martinovich, C., Simerly, C., and Schatten, G. (2001). Transgenic monkeys produced by retroviral gene transfer into mature oocytes. **Science** 291: 309-312.

Chan, A.W.S. (2004). **Reprod Biol Endocrinol** 2: 39.

Copp, A.J. (1995). Death before birth: cluess from gene knockouts and mutations. **Trends Genet** 11: 87-93.

Courtine, G., Harkema, S.J., Dy, C.J., Gerasimenko, Y.P., and Dyhre-Poulsen, P. (2007). Modulation of multisegmental monosynaptic responses in a variety of leg muscles during walking and running in humans. **J Physiol** 582: 1125-1139.

Davies, S., and Ramsden, D.B. (2001). Huntington's disease. **Clin Pathol Mol Pathol** 54: 409-513.

Dong, Q., Rodenburg, S.E., Huang, C., and VandeVoort, C.A. (2008). Cryopreservation of rhesus monkey (*Macaca mulatta*) epididymal spermatozoa before and after refrigerated storage. **J Androl** 29: 283-292.

Duyao, M., Ambrose, C., Mters, R., Noveletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., and Abbott, M. (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. **Nat Genet** 4: 387-392.

Gusella, J.F., and MacDonald, M.E. (1996). Trinu-cleotide instability: A repeating theme in human inherited disorders. **Ann Rev Med** 47: 201-209.

Gutekunst, C.A., Lee, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M. and Li, X.J. (1999). Nuclear and neuropil aggregates in huntington's disease: Relationship to neuropathology. **J Neurosci** 19: 2522-2534.

HD Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. **Cell** 72: 971-983.

Heng, M.Y., Tallaksen-Greene, S.J., Detloff, P.J., and Albin, R.L. (2007). Longitudinal evaluation of the Hdh (CAG) 150 knock-in murine model of Huntington's disease. **J Neurosci** 27: 8989-8998.

Laowtammathron, C., Cheng, E.C.H, Cheng, P.H., Snyder, B.R., Yang, S.H., Johnson, Z., Lorthongpanich, C., Kuo, H.C, Parnpai, R., and Chan, A.W.S. (2010). Monkey hybrid stem cells develop cellular features of Huntington's disease. **BMC Cell Biol** 11.

Li, H., Li, S.H., Cheng, A.L., Mangiarini, L., Bates, G.P., and Li, X.J. (1999). Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. **Hum Mol Genet** 8: 1227-1236.

Li, S.H., Cheng, A.L., Zhou, H., Lam, S., Rao, M., Li, H., and Li, X.J. (2001). Interaction of Huntington's disease protein with transcriptional activator Sp1. **Mol Cel Biol** 22: 1277-1287.

Lin, C.H., Tallaksen-Greene, S., Chien, W.M., Cearley, J.A., Jackson, W.S., Crouse, A.B., Ren, S., Li, X.J., Albin, R.L., and Detloff, P.J. (2001). Neurological abnormalities in a knock-in mouse model of Huntington's disease. **Hum Mol Genet** 10: 137-144.

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

Menalled, L.B., Sison, J.D., Dragatsis, I., Zeitlin, S., and Chesselet, M.F. (2003) Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. **J Comp Neurol** 465: 11-26.

Metzler, M., Chen, N., Helgason, C.D., Graham, R.K., Nichol, K., McCutcheon, K., Nasir, J., Humphries, R.K., Raymond, L.A., and Hayden, M.R. (1999). Life without huntingtin: normal differentiation into functional neurons. **J Neurochem** 72: 1009-1018.

Myers, R.H., Leavitt J., and Farrer, L.A. (1989). Homozygote for Huntington's Disease. **Am J Hum Genet** 45: 615-618.

Nasir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G., and Hayden, M.R. (1995). Targeted

disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. **Cell** 81: 811-823.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008). Disease-specific induced pluripotent stem cells. **Cell** 134: 877-886.

Persichetti, F., Carlee, L., and Faber, P.W. (1996). Differential expression of normal and mutant Hunt-ington's disease gene alleles. **Neurobiol Dis** 3: 183-190.

Reiner, A., Dragatsis, I., Zeitlin, S., and Goldowitz, D. (2003). Wild-type Huntingtin plays arole in brain development and neironal survival. **Mol Neu** 28(3): 259-275.

Rogers, J., Katze, M., Bumgarner, R., Gibbs, R.A., and Weinstock, G.M. (2002). White paper for complete sequencing of the rhesus macaque (*Macaca mulatta*) genome. **Rhesus Macaque White Paper**.

Rubinsztein, D.C. (2002). Lessons from animal models of Huntington's disease. **Trends in Genetics** 18.

Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A. , Copeland, N.G., Price, D.L., Ross, C.A., and Borchelt, D.R. (1999). Intranuclear inclusions and neur itic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. **Hum Mol Genet** 8: 397-407.

Tabrizi, S.J. (2011). Biological and clinical changes in premanifest and early stage Huntington's disease in the TRACK-HD study: the 12-month longitudinal analysis. **Lancet Neurology** 10: 31-42.

Wang, C.E., Tydlacka, S., Orr, A.L., Yang, S.H., Graham, R.K., Hayden, M.R., Li, S., Chan, A.W., and Li, X.J. (2008). Accumulation of N-terminal mutant huntingtin in mouse and monkey models implicated as a pathogenic mechanism in Huntington's disease. **Hum Mol Genet** 17: 2738-2751.

Wexler, N.S., Young, A.B., and Tanzi, R.E. (1987). Homozygotes for Huntington's Disease. **Nature** 326: 194-197.

Wheeler, V.C., White, J.K., Gutekunst, C.A., Vr banac, V., Weaver, M., Li, X.J., Li, S.H., Yi, H., Vonsattel, J.P., Gusella, J.F., Hersch, S., Auerbach, W., Joyner, A.L., and MacDonald, M.E. (2000). Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny st riatal neurons in HdhQ92 and HdhQ111 knock-in mice. **Hum Mol Genet** 9: 503-513.

Yang, S.H., Cheng, P.H., Banta, H., Piotrows ka-Nitsche, K., Yang, J.J., Cheng, E.C., Snyder, B., Larkin, K., Liu, J., Orkin, J., Fa ng, Z.H., Smith, Y., Bachevalier, J., Zola, S.M., Li, S.H., Li, X.J., and Chan, A.W. (2008). Towards a transgenic model of Huntington's disease in a non-human primate. **Nature** 453: 921-924.

Zeitlin, S., Liu, J.P., Chapman, D.L., Papaioannou, V.E., and Efstratiadis, A. (1955). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. **Nat Genet** 11: 155-163.

Zheng, P., Wang, H., Bavister, B.D., and Ji, W. (2001). Maturation of rhesus monkey oocytes in chemically defined culture media and their functional assessment by IVF and embryo development. **Hum Reprod** 16: 300-305.

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.

Hoechst 33342 (stock)

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

L-glutamine (stock)

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

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 (200 mM)	1 ml.

Monkey ES cell outgrowth

1) Base solution	8.9470 ml.
2) FBS	1 ml.
3) rhbFGF (20 ng/ml)	15 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.

PUBLICATION

Putkhao, K. Kocerha, J. Cho, IK. Yang, JJ. Parnpai, R. and Chan, AWS. (2013). Pathogenic cellular phenotypes are germline transmissible in a transgenic primate model of Huntington's Disease. International Journal of Stem Cells and Development, Vol. 22, doi: 10.1089/scd.2012.0469 (2013 Impact factor = 4.459)

STEM CELLS AND DEVELOPMENT
Volume 22, Number 8, 2013

DOI: 10.1089/scd.2012.0469

Pathogenic Cellular Phenotypes are Germline Transmissible in a Transgenic Primate Model of Huntington's Disease

Kittiphong Putkhao,[1,2] Jannet Kocerha,[1,3] In-Ki Cho,[1,3] Jinjing Yang,[1,3] Rangsun Parnpai,[2] and Anthony W.S. Chan[1,3]

A transgenic primate model for Huntington's Disease (HD) first reported by our group that (HD monkeys) carry the mutant Huntingtin (*HTT*) gene with expanded polyglutamine (CAG) repeats and, develop chorea, dystonia, and other involuntary motor deficiencies similar to HD [1]. More recently, we have found that longitudinal magnetic resonance imaging of the HD monkey brain revealed significant atrophy in regions associated with cognitive deficits symptomatic in HD patients, providing the first animal model which replicates clinical phenotypes of diagnosed humans. Here we report germline transmission of the pathogenic mutant HTT in HD monkey by the production of embryos and subsequent derivation of HD monkey embryonic stem cells (rHD-ESCs) using HD monkey sperm. rHD-ESCs inherit mutant *HTT* and green fluorescent protein (*GFP*) genes through the gametes of HD monkey. rHD-ESCs express mutant HTT and form intranuclear inclusion, a classical cellular feature of HD. Notably, mosaicism of the pathogenic polyQ region in the sperm as well as derived ESCs were also observed, consistent with intraindividual and intergenerational reports of mosaic CAG repeats [2,3]and CAG expansion in HD patients [4–7]. The confirmation of transgene inheritability and development of pathogenic HD phenotype in derived rHD-ESCs reported in this study is a milestone in the pursuit of a transgenic primate model with inherited mutant *HTT* for development of novel disease biomarkers and therapeutics.

Introduction

Huntington's Disease (HD) is a devastating neurodegenerative disorder that leads to motor disability and cognitive deterioration throughout the course of the disease, with a duration of approximately 15 years after clinical symptoms appear [8,9]. HD is a genetic disorder caused by the expansion of the polyQ at the exon 1 of the IT15 gene that encodes the HTT protein. CAG repeat lengths over 39 in humans result in pathological HD. A negative correlation has been shown between repeat length and age of onset [6,7,10,11] and lifespan [12]. Patients with the long CAG repeats exhibit severe symptoms of HD in adolescence [6,7,11]. Although recent advancement in induced pluripotent stem cell technology has opened a new gateway for modeling human diseases using patients own pluripotent stem cells [13–19], the rationale in the derivation of rHD-embryonic stem cells (ESCs) from a transgenic HD monkey are (1) confirming germline transmission of transgenic HD monkeys and (2) predicting expression pattern of the mutant *HTT* transgene in second generation HD monkeys. Nonhuman primates are a unique model for studying human diseases due to the high similarity in physiology and genetics with humans [1,20,21]. A transgenic nonhuman primate which carries a genetic defect leading to a human disease, such as HD will not only recapitulate human conditions but also the pathogenic events that are critical for investigating disease pathogenesis and the development of novel biomarkers and treatments [1,20,21].

Materials and Methods

Generation of transgenic HD monkey [1,21,22]

We developed a transgenic HD rhesus macaque, rHD1, born and raised at the Yerkes National Primate Center of Emory University following the IACUC protocols for animal care. In brief, lentiviruses carrying exon 1 of the *HTT* gene containing 84 CAGs under the control of human polyubiquitin C (*UBC*) promoter was used to infect metaphase II arrested rhesus monkey oocytes followed by fertilization and embryo transfer into surrogate females.

[1]Yerkes National Primate Research Center, Atlanta, Georgia.
[2]Embryo Technology and Stem Cell Research Center, School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.
[3]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia.

Regimen of follicular stimulation [21,23]

Female rhesus monkeys exhibiting regular menstrual cycles were induced with exogenous gonadotropins. On day 1 or 2 of menses, subcutaneous human follicle-stimulating hormone [r-FSH: Organon Inc. 30 IU, intramuscular injection (IM)] was injected twice daily for 6 days. On day 7–10, daily injection of Gonadotropin-releasing hormone (GnRH) antagonist (Acyline 75 mg/kg; NICHD), r-FSH (20 IU, IM, twice daily) and recombinant human-luteinizing hormone (r-hLH; Ares Serono; 30 IU each, IM, twice daily) were administered. Ultrasonography was performed on day 7 of the stimulation to confirm follicular responses. An IM injection of 1,000 IU recombinant human chorionic gonadotropin (r-hCG; Ares Serono) was administered for ovulation at approximately 37 h before oocyte retrieval.

In vitro maturation [21,23]

Oocytes were matured in modified CMRL-1066 containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc.) supplemented with 40 μg/mL Sodium pyruvate, 150 μg/mL Glutamine, 550 μg/mL Calcium lactate, 100 ng/mL estradiol, and 3 μg/mL of Progesterone for up to 36 h in 35-μL drops of medium under mineral oil at 37°C with 5% CO_2, 5% O_2, and 90% N_2.

Production of transgenic HD monkey embryos and establishment of ESCs [21,23–25]

MII arrested oocytes were retrieved from hormone-stimulated female rhesus macaques and used for in vitro fertilization by intracytoplasmic sperm injection (ICSI) followed by in vitro culture using HECM-9 [24]. HD and WT-Control monkeys were chair trained for semen collection using IACUC approved electroejaculation protocol [20,21,23]. Expanded blastocysts were selected for inner cell mass (ICM) isolation by using XYClone laser (Hamilton Throne, Inc.). The isolated ICMs were cultured until attached onto mouse fetal fibroblasts (MFFs; ICR mice) to form an outgrowth. Outgrowths with prominent stem cell morphology were mechanically passaged and continued in culture. Monkey ESCs were cultured in medium composed of knockout-Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen), 1 mM glutamine, 1% nonessential amino acids, and supplemented with 4 ng/mL of human basic fibroblast growth factor (bFGF; Chemicon).

Immunostaining of stem cell markers [24,25]

rHD-ESC culture was fixed in 4% paraformaldehyde (PFA), permeabilized by 1% Triton-X (excluded for cell surface markers), blocked with 2% BSA and 130 mM glycine in a phosphate buffer saline (PBS). After overnight incubation with primary antibodies, Nanog (Santa Cruz Biotechnology Cat# sc-30329 Conc: 1:200), Oct4 (Santa Cruz Biotechnology Cat# sc-5279 Conc: 1:500), SSEA-4 (Chemicon Cat# MB4303 Conc: 1:250), TRA-1-60 (Chemicon MAB4360 1:200) followed by thorough washes, a secondary antibody conjugated with Alexa Red (Molecular Probe Cat# A21203 Conc: 1:1000) was used for detection of the primary antibodies. DNA was counterstained with Hoechst 33342 (5 μg/mL Molecularprobe Conc 1:10000). The specimen was examined using epifluorescent microscope. Alkaline phosphatase assay was performed following the manufacturer's instruction (Vector Lab Cat# SK-5300).

Immunostaining of mutant HTT [24,25]

Differentiated rHD-ESCs were fixed using 4% PFA for 15 min, permeabilized, and blocked. The sample was then incubated with primary antibody mEM48 (1:50) at 4°C overnight. After washing with the PBS, the samples were processed with avidin/biotinylated enzyme complex using the Vectastain Elite ABC kit (Vector Laboratories, Cat# PK-6102), and immediately stained with DAB (Vector Laboratories, Cat# SK-4100) for 30–40 s. Cell samples were examined and captured by CellSens software (Olympus, Inc.). For fluorescent imaging, a secondary antibody conjugated with Alexa Red (Molecular Probe) was used for detection of the primary antibody. DNA was counterstained with Hoechst 33342 (5 μg/mL).

Cytogenetic analysis/G-banding analysis [24,25]

rHD-ESCs were treated with KaryoMax® colcemid (Invitrogen) for 20 min, dislodged with 0.05% Trypsin-EDTA, centrifuged, and resuspended in hypotonic 0.075 M KCl solution for 20 min. After centrifugation, the cells were fixed thrice 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 h. Slides were immersed in 0.5× Trypsin-EDTA (Invitrogen) with two drops of 67 mM Na_2HPO_4 for 20 to 30 s, rinsed in distilled water and stained with Leishman Stain (Sigma) for 90 s. 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). Karyotyping service provided by Cell Line Genetics, LLC.

In vitro differentiation to neuronal lineage [24,25]

rHD-ESC clumps were cultured in suspension for 7 days for the formation of embryoid bodies (EBs) and then allowed to attach onto a gelatin-coated plate. EBs were cultured in N1 medium (selection of neural progenitor cells; KO-DMEM [Invitrogen Cat# 10829-018]) supplemented with minimum essential amino acid (Invitrogen 10370-021), 200 mM of L-glutamine (Invitrogen Cat# 25030-081) and N2 supplement (Invitrogen Cat# 17502048) for 7 days, in N2 medium (neural progenitor cells expansion; N1 medium supplemented with 20 ng/mL bFGF [R&D Systems 233-FB-025]) for 14 days and in N3 medium (maturation of neuronal cells; KO-DMEM supplemented with 1% FBS [Hyclone]) and B27 supplement (Invitrogen 17504-044) for 7 days to allow differentiation into mature neuronal cell types. Successful differentiation of neuronal cell types was confirmed by the expression of Nestin (Chemicon Cat# MAB353,CONC 1:500), microtubule-associated protein (MAP2 Millipore Cat# AB5266 Conc: 1:200), neuron specific βIII tubulin(Chemicon Cat# MAB1637 Conc: 1:500), glial fibrillary acidic protein (GFAP Chemicon Cat# MAB360 Conc:1:500), and choline acetyltransferase

(CHAT; Millipore Cat# AB143,Conc 1:200) by using specific antibodies [24,25].

Formation of teratoma in SCID mice [24,25]

Undifferentiated rHD-ESC clumps were recovered mechanically and implanted under the kidney capsule of severely compromised immune deficient (SCID) mice for 6 to 8 weeks. Animals were then euthanized followed by recovering of the teratoma and histological analysis. All animal procedures were approved by the IACUC at Emory University.

Genomic DNA isolation and genotyping PCR [1]

Genomic DNA (gDNA) was extracted from a cell pellet generated from all cell lines using the Promega Wizard Kit. Additionally, gDNA was extracted from sperm with phenol-chloroform extraction and precipitation with isopropanol. For genotyping of mutant HTT and green fluorescent protein (GFP) transgenes, 50 ng of gDNA was amplified by PCR at an annealing temperature of 68° for 40 cycles. For mutant *HTT*, primer set of HD32-F 5′-CTACGAGTCCCTCAAGTC CTTCCAGC-3′ and MD177-R 5′-GACGCAGCAGCGGCT GTGCCTG-3′ were used with expected amplicon size with 29 CAGs of 185 bp. For *GFP*, primer set of Ubiquitin-F1 5′-GAGGCGTCAGTTTCTTTGGTC-3′ and EGFP-R1 5′-CTG CTGCCCGACAACCACTA-3′ were used with expected amplicon size of 869 bp. All products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide.

Sequencing of polyQ [1]

For polyQ sequencing from the transgenic *HTT* transcripts of all HD cell lines, 500 ng of total RNA was extracted from the cells and reverse-transcribed to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems). The *HTT* transcript was amplified from the cDNA by PCR at an annealing temperature of 67° for 40 cycles with the following primers; HD32-Forward 5′-CTACGAGTCCCT CAAGTCCTTCCAGC-3′ and MD177-Reverse 5′-GACGC AGCAGCGGCTGTGCCTG-3′. All PCR products were electrophoresed on a 1.5% agarose gel and target bands were gel purified, cloned into the pGEM-T easy vector (Promega), and subsequently sequenced at Genewiz Corporation with T7 and SP6 primers. Both the expanded polyQ *mHTT* transgene and the endogenous *HTT* gene were cloned into the pGEM-T easy vector and sequenced.

Real-time PCR quantitation of HTT mRNA expression [1]

To quantitate the expression of *HTT* mRNA from all cell lines, 500 ng of total RNA from all cells was reverse transcribed to cDNA. Quantitation of *HTT* mRNA expression was performed using custom-designed gene-specific Taqman assays (forward sequence - 5′GCCGCTGCTGCCTCA4 ′3; reverse sequence–5′TGCAGCGGCTCCTCAG′3; probe sequence- 5′CCGCCGCCCCCGCC′3) in a 1× final reaction of Taqman Gene Expression Master Mix (Applied Biosystems). All real-time PCR (qPCR) results were first normalized with the geometric mean of 2 endogenous controls, beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase and *(GAPDH)* (custom Taqman assay for *ACTB* forward sequence- *5′GCCGTCTTCCCCTCCAT3′*; reverse sequence–5′CTGACCCATGCCCACCAT′3; probe sequence–5′CACGCCCTGGTGCCTG′3; *GAPDH* measured with commercially available rhesus Taqman assay from ABI). The *ACTB-GAPDH* normalized qPCR results were then further normalized to expression of the endogenous *HTT* transcript via a Taqman assay designed to exon 10–11 of HTT (forward sequence–5′AGCCCTGTCCTTTCAAGAAAACAA′3; reverse sequence–5′CATCCTCCAAGGCTTCTTCTTCT′3; probe sequence - 5′CCTAAGAGCACTTTGCCTTT′3).

Results

A male transgenic HD monkey, rHD1, carrying exon 1 of the human *HTT (hHTT)* gene with 29 CAG repeats and a *GFP* gene under the regulation of human polyubiquitin C (*UBC*) promoter was generated by lentiviral transfection of a mature oocyte followed by in vitro fertilization, culture, and embryo transfer into a surrogate female as described previously [1]. To determine successful germline transmission of the *mHTT* and *GFP* transgenes, we performed ICSI using sperm of rHD1 followed by in vitro production of embryos (Fig. 1) and the derivation of ESCs from the ICM of the resulting blastocyst stage embryos (Fig. 2a–c). Development of embryos fertilized with the sperm of rHD1 was similar to that of wild-type nontransgenic rhesus macaque embryos (WT-Control; Fig. 1c). Fertilization rate of rHD1 embryos was 92.5%±2% and 61.3%±4.8% for blastocyst, compared to WT-control group of 82.5%±9.2% and 52.3%±9.4%, respectively (Fig. 1c). A total of seven ESC lines were established from rHD1 derived blastocysts. Three of the rHD-ESC lines are male and four are female with normal karyotype based on a total of 20 cells analyzed in each cell line (Fig. 2d, g). All rHD-ESC lines carry mutant *HTT* and *GFP* transgenes and were confirmed by PCR (Fig. 2e). GFP expression was observed in all cell lines with the various intensity of green fluorescent (Fig. 1b), while all rHD-ESCs carry mutant *HTT* gene (Fig. 2e). Sperm from rHD1 carry 28 to 48 CAG repeats and rHD-ESC lines carry 28–131 CAG repeats suggesting possible germline expansion of polyQ (Fig. 2g). To further confirm if rHD-ESCs carry the human mutant *HTT* transgene, sequence analysis of the PCR amplicon flanking the polyQ region was aligned and compared with the following genes; *HTT* sequences of four control rhesus macaque, rhesus *HTT* sequence from the University of California at Santa Cruz (UCSC) genome database, human *HTT*, and human mutant *HTT* transgene from the 7 rHD-ESC cell lines (Fig. 2f). All rHD-ESC lines carry the human specific *HTT* sequence with variations in the number of pathogenic CAG repeats (Fig. 2e–g).

Each of the rHD-ESCs expresses stem cell markers, including Nanog, Oct4, Sox2, SSEA4, TRA-1-60, and alkaline phosphatase by immunocytochemistry (Fig. 3a–f). In addition to the expression of stem cell markers, rHD-ESC lines can differentiate into neural cells in vitro and were confirmed by immunocytochemistry using specific antibodies that recognize Nestin (NES), microtubule-associated protein (MAP2), neuron specific βIII tubulin (TUBB3), GFAP, and CHAT (Fig. 3g–k). Teratoma assay in immune compromised mice further demonstrated the pluripotency of rHD-ESC lines and their capability to differentiate into different lineages (Fig. 2g and Fig. 3l–q).

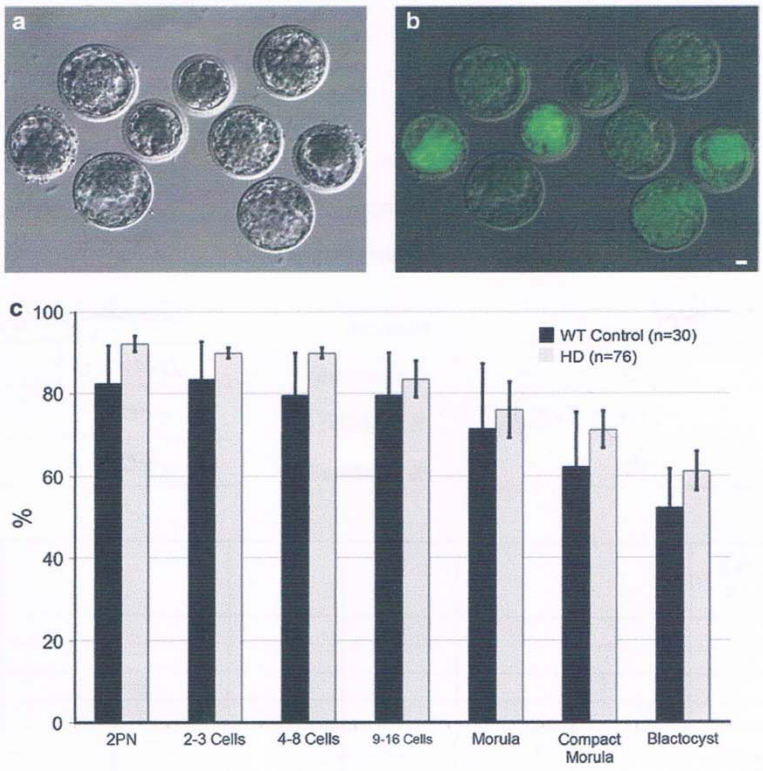
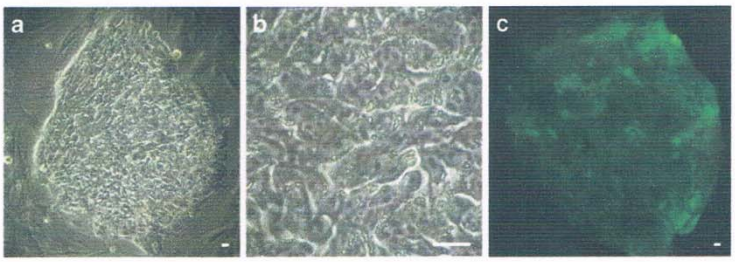


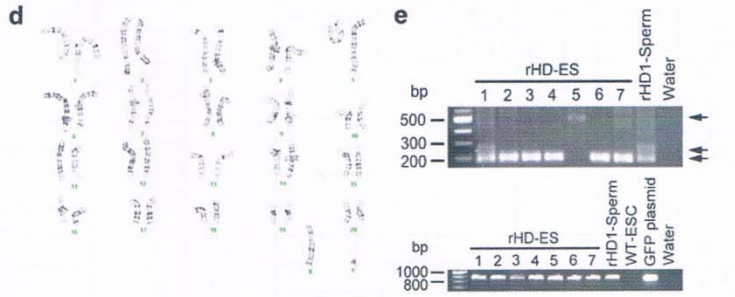
FIG. 1. Development of transgenic Huntington's Disease (HD) monkey embryos. **(a)** Transmission light image of HD monkey blastocyst; **(b)** epifluorescent image of HD monkey blastocyst expressing green fluorescent protein (GFP); **(c)** development rate of HD monkey embryos. Bar = 20 μm.

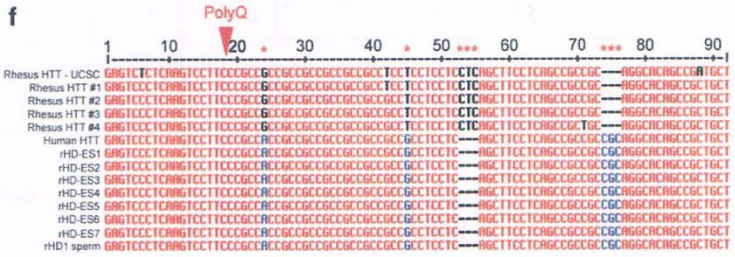
Expression of the GFP transgene was observed in embryos and rHD-ESCs (Fig. 1b and Fig. 2c) by fluorescent microscopy. The expression level of mutant HTT in the rHD-ESC lines was evaluated by Q-PCR analysis (Fig. 4a) and immunostaining using an antibody specifically recognizing mutant HTT (Fig. 4b–e). The expression of mutant HTT was detected in undifferentiated rHD-ESCs with increased expression as neural differentiation progresses in culture of rHD-ES1, 2, 3, and 7 when compared with endogenous HTT and WT-control (Fig. 4a). The expression level of HTT in rHD-ES5 was not upregulated compared to control ESC levels (Fig. 2e). It is not clear whether the expression of mutant HTT in rHD-ES5 is impacted by the expanded polyQ (131Q), or if aberrant gene rearrangement occurred. While the protein level of aggregated mutant HTT was too low to be detected by western blot analysis, aggregated mutant HTT and intranuclear inclusions in the neural-differentiated rHD-ESCs was detected by immunostaining in rHD-ES4 and rHD-ES7 (Fig. 4b–e).

Discussion

One of the key rationales in developing a transgenic animal model is the inheritance of the transgene through the germ cells which mimics the inheritable pattern of human genetic disorders [1,20,22,26,27]. Unlike most of the laboratory animal species, rhesus macaque reaches pubertal age at around 4 years old. It is a relatively long physiological event compared to most laboratory species, including small nonhuman primates, such as marmosets [28]. While there is no perfect animal model to embody humans, it is important to identify appropriate model systems to address specific underlying pathogenic mechanisms of inherited neurological disorders, such as HD, that are influenced by aging events [29,30]. A nonhuman primate model is particularly important in modeling diseases, such as HD since various systems in the body are impacted during the course of the disease, which include motor functions, psychiatric, and metabolic disturbance [9,21,31]. To capture the systemic impact of disease, such as HD, nonhuman primates can effectively model the progression of HD through longitudinal analysis with similar clinical measurements evaluated in human patients, such as magnetic resonance imaging. Rhesus macaques also share a similar motor repertoire that allows the evaluation of fine movement control that most of the currently available model systems cannot offer [29]. Most importantly, psychiatric impact can be evaluated with a sophisticated battery of cognitive behavioral tests developed







g

	Gender	Karyotype	Maximum Passage #	Karyotype Passage #	Neural Diff Passage #	Mutant HTT	GFP	Endogenous rhesus HTT CAG#	Mutant HTT CAG #	mHTT aggregate & intranuclear inclusion	Teratoma
rHD-ES1	M	42,XY	>70	70	35	+	+	10	30-48	ND	+
rHD-ES2	F	42,XX	>25	21	36	+	+	11	28	ND	+
rHD-ES3	F	42,XX	>60	60	24	+	+	10	30	ND	+
rHD-ES4	F	42,XX	>55	55	13	+	+	10	29-30	+++	+
rHD-ES5	M	42,XY	>56	56	22	+	+	10	131	ND	+
rHD-ES6	F	42,XX	>53	53	15	+	+	10	29-30	ND	+
rHD-ES7	M	42,XY	>51	51	10	+	+	10	28-29	+	+
rHD1 Sperm	M	N/A	N/A	N/A	N/A	+	+	10	28-48	N/A	N/A

ND – Not Determined
N/A – Not Applied

FIG. 2. Establishment and characterization of rHD-embryonic stem cells (ESCs). **(a)** Transmission light image of rHD-ESC colony (low magnification); **(b)** transmission light image of rHD-ESC colony (high magnification); **(c)** epifluorescent image of rHD-ESC colony expressing GFP; **(d)** karyotype of rHD-ESC; **(e)** genotyping of rHD-ESCs by PCR analysis. Top panel-genotype of mutant HTT transgene. Lower panel-genotype of GFP transgene; **(f)** alignment of flanking regions of the CAG repeats of PCR amplicon of human *HTT*, rhesus macaque *HTT*, *rHD1 sperm*, and rHD-ESC mutant *HTT* genes; **(g)** summary of rHD-ESCs. Bar = 10 μm.

for macaques that cannot be examined in other species [32–34].

rHD1, one of the first transgenic HD monkeys, has reached pubertal age and germline transmission was confirmed by fertilization of mature macaque oocytes with HD monkey sperm followed by the production of transgenic embryos (Fig. 1a, b) and subsequent derivation of rHD-ESCs (Fig. 2). Our result suggests that sperms carrying mutant *HTT* and *GFP* transgene are not impacted, and the development competence of the resulted embryos was similar to wild-type nontransgenic monkey embryos. rHD-ESCs inherit and express both the mutant *HTT* and *GFP* transgenes (Fig. 2c, e, f), and develop unique HD cellular features, including intranuclear inclusions, as they differentiate toward neuronal lineage (Fig. 4b–e). It is interesting that all rHD-ESC lines carry both mutant *HTT* and *GFP* transgenes. rHD1 carry a single copy of mutant *HTT* transgene and at least six copies of the *GFP* transgenes [1]. The two transgenes are expected to segregate independently because independent lentiviral integration event is expected [35]. As a result, each rHD-ESC line may carry different copy of GFP transgene which may result in the variation in GFP expression level (Fig. 1b). Furthermore, we found instability of the pathogenic CAG repeat in the gametes of the HD monkey as well in the ESCs, which is an underlying feature of not only HD [6,7,11] but other triplet nucleotide repeat diseases as well, including

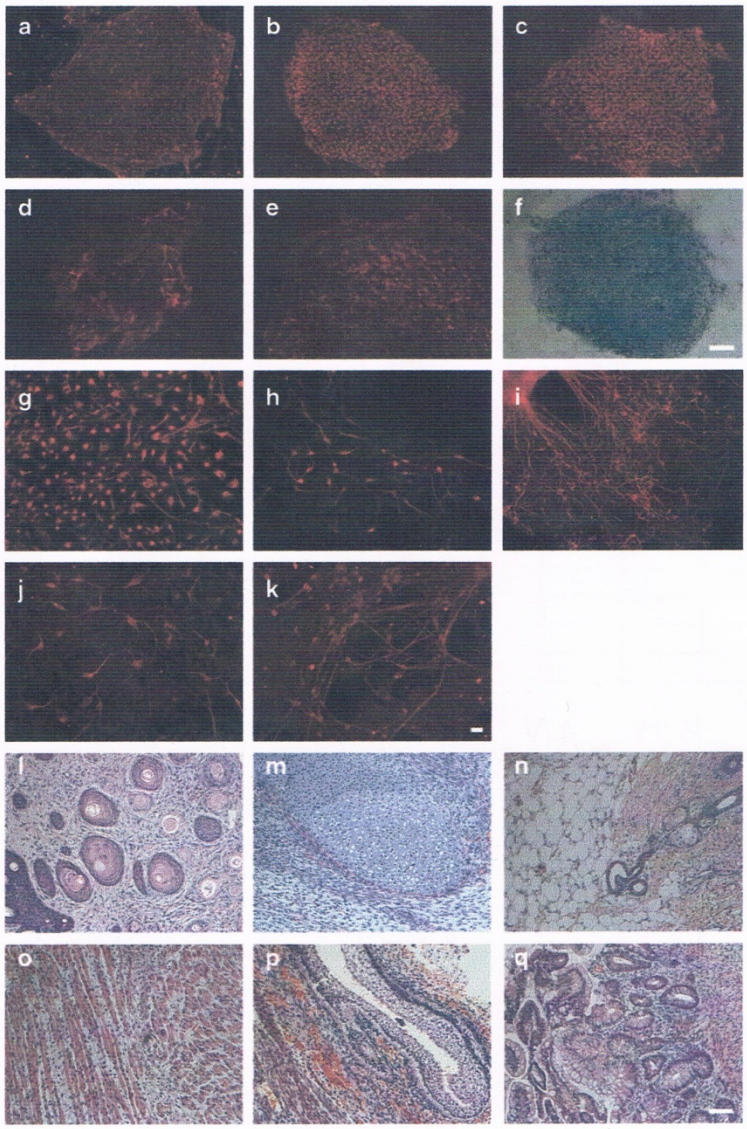


FIG. 3. Expression of stem cell and differentiation markers, and the formation of teratoma. **(a–e)** Immunostaining of undifferentiated rHD-ESCs with antibodies which recognize stem cell specific proteins (A-Nanog, B-Oct4, C-Sox2, D-SSEA4 and E-TRA-1-60); **(f)** alkaline phosphatase staining; **(g–k)** immunostaining of in vitro neural-differentiated rHD-ESCs with antibodies which recognize neural-specific proteins (G-Nestin, H-Map2, I-βIII tubulin, J-GFAP, and K-CHAT). **(l–q)** Teratoma derived from rHD-ESC grafts under the kidney capsule of immune compromised mice (**l**: ectoderm; **m–o**: mesoderm; **p–q**: endoderm). Teratoma composed of tissues originated from different germ-layers. Bar **(a–f)** = 50 μm; Bar **(g–k)** = 10 μm; Bar **(l–q)** = 50 μm.

spinocerebellar ataxia type 1 [36,37] and dentatorubral-pallidoluysian atrophy [38]. Notably, this is the first report, to the best of our knowledge, of CAG mosaicism in a primate animal model for HD, and could, therefore, serve as a unique tool for further evaluation of this pathogenic mechanism. While rHD-ESCs could be a useful stem cell model for studying HD, the confirmation of germline transmission through the gametes is an important and critical step in the development of transgenic primate models of human inherited genetic diseases. Overall, the successful inheritability of the mutant *HTT* transgene in our nonhuman primate model can now lead to the establishment of second generation HD monkeys for studies of HD pathogenic trajectories and much needed development of novel treatments.

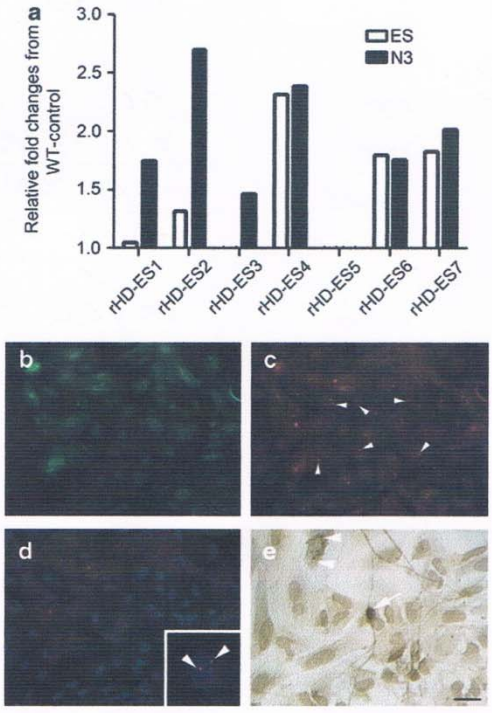


FIG. 4. Expression of mutant HTT. **(a)** Expression level of mHTT in rHD-ESCs was determined by Q-PCR and was normalized to endogenous HTT and WT-control monkey HTT expression. Undifferentiated rHD-ESCs (ES) and in vitro neural-differentiated rHD-ESCs (N3) were collected, followed by RNA extraction and real-time PCR (qPCR) analysis with *HTT* specific Taqman assays (Applied Biosystems). All qPCR results were normalized with the geometric mean of two endogenous controls, beta-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*, and then normalized to WT-Control; **(b)** epifluorescent image of GFP expression in neural-differentiated rHD-ESCs; **(c)** immunstaining of in vitro neural-differentiated rHD-ESCs using antibody specific to mutant HTT aggregates; **(d)** a montage of epifluorescent images of DNA staining (*blue*) and **(c)**; **(e)** immunohistochemistry of mutant HTT aggregates using antibody specific to mutant HTT aggregates and detected by avidin/biotinylated enzyme complex followed by staining with DAB (*arrow*-cytoplasmic mutant HTT aggregate; *arrowhead*-nuclear inclusion). Bar = 10 μm.

Acknowledgments

All protocols involving animal care and handling were approved by Emory University's IACUC. We thank Tim Chi and Adam Neumann for assisted reproduction in rhesus macaque and Dr. X.J.Li for providing mEM48 antibody. Acyline was provided by NICHD. We thank Yerkes National Primate Research Center (YNPRC) veterinarian and animal care staff for providing outstanding services. KP and RP are supported by the Royal Golden Jubilee PhD program of Thailand Research Fund and Suranaree University of Technology. YNPRC is supported by the National Center for Research Resources P51RR165 and is currently supported by the Office of Research and Infrastructure Program (ORIP)/OD P51OD11132. This study is supported by a grant awarded by the ORIP/NIH (RR018827) to AWSC.

Author Disclosure Statement

The authors have no commercial association that might create a conflict of interest in connection with the submitted manuscript.

References

1. Yang SH, PH Cheng, H Banta, K Piotrowska-Nitsche, JJ Yang, EC Cheng, B Snyder, K Larkin, J Liu, et al. (2008). Towards a transgenic model of Huntington's disease in a non-human primate. Nature 453:921–924.
2. Telenius H, B Kremer, YP Goldberg, J Theilmann, SE Andrew, J Zeisler, S Adam, C Greenberg, EJ Ives, LA Clarke and MR Hayden. (1994). Somatic and gonadal mosaicism of the Huntington disease gene CAG repeat in brain and sperm. Nat Genet 6:409–414.
3. Telenius H, HP Kremer, J Theilmann, SE Andrew, E Almqvist, M Anvret, C Greenberg, J Greenberg, G Lucotte, et al. (1993). Molecular analysis of juvenile Huntington disease: the major influence on (CAG)n repeat length is the sex of the affected parent. Hum Mol Genet 2:1535–1540.
4. Nicolas G, D Devys, A Goldenberg, D Maltete, C Herve, D Hannequin and L Guyant-Marechal. (2011). Juvenile Huntington disease in an 18-month-old boy revealed by global developmental delay and reduced cerebellar volume. Am J Med Genet 155A:815–818.
5. Ribai P, K Nguyen, V Hahn-Barma, I Gourfinkel-An, M Vidailhet, A Legout, C Dode, A Brice and A Durr. (2007). Psychiatric and cognitive difficulties as indicators of juvenile huntington disease onset in 29 patients. Arch Neurol 64:813–819.
6. Langbehn DR, MR Hayden and JS Paulsen. (2010). CAG-repeat length and the age of onset in Huntington disease (HD): a review and validation study of statistical approaches Am J Med Genet B Neuropsychiatr Genet 153B:397–408.
7. Lee JM, EM Ramos, JH Lee, T Gillis, JS Mysore, MR Hayden, SC Warby, P Morrison, M Nance, et al. (2012). CAG repeat expansion in Huntington disease determines age at onset in a fully dominant fashion. Neurology 78:690–695.
8. RI Scahill, NZ Hobbs, MJ Say, N Bechtel, Henley SM, H Hyare, DR Langbehn, R Jones, BR Leavitt, et al. (2011). Clinical impairment in premanifest and early Huntington's disease is associated with regionally specific atrophy. Hum Brain Mapp. [Epub ahead of print]; DOI 10.1002/hbm.21449.
9. SJ Tabrizi, R Reilmann, RA Roos, A Durr, B Leavitt, G Owen, R Jones, H Johnson, D Craufurd, et al. (2012). Potential endpoints for clinical trials in premanifest and early Huntington's disease in the TRACK-HD study: analysis of 24 month observational data. Lancet Neurol 11:42–53.
10. RG Snell, JC MacMillan, JP Cheadle, I Fenton, LP Lazarou, P Davies, ME MacDonald, JF Gusella, PS Harper and DJ Shaw D. (1993). Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. Nat Genet 4:393–397.
11. Geevasinga N, FH Richards, KJ Jones and MM Ryan. (2006). Juvenile Huntington disease. J Paediatr Child Health 42: 552–554.

12. Andrew SE, YP Goldberg, B Kremer, H Telenius, J Theilmann, S Adam, E Starr, F Squitieri, B Lin, et al. (1993). The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. Nat Genet 4: 398–403.
13. HD iPSC Consortium. (2012). Induced pluripotent stem cells from patients with Huntington's disease show CAG-repeat-expansion-associated phenotypes. Cell Stem Cell 11:264–278.
14. An MC, N Zhang, G Scott, D Montoro, T Wittkop, S Mooney, S Melov and LM Ellerby. (2012). Genetic correction of Huntington's disease phenotypes in induced pluripotent stem cells. Cell Stem Cell 11:253–263.
15. Dimos JT, KT Rodolfa, KK Niakan, LM Weisenthal, H Mitsumoto, W Chung, GF Croft, G Saphier, R Leibel, et al. (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321:1218–1221.
16. Ma L, B Hu, Y Liu, SC Vermilyea, H Liu, L Gao, Y Sun, X Zhang, SC Zhang. (2012). Human embryonic stem cell-derived GABA neurons correct locomotion deficits in quinolinic acid-lesioned mice. Cell Stem Cell 10:455–464.
17. Park IH, N Arora, H Huo, N Maherali, T Ahfeldt, A Shimamura, MW Lensch, C Cowan, K Hochedlinger and GQ Daley. (2008). Disease-specific induced pluripotent stem cells. Cell 134:877–886.
18. Perrier A and M Peschanski. (2012). How can human pluripotent stem cells help decipher and cure Huntington's disease? Cell Stem Cell 11:153–161.
19. Carter RL and AW Chan. (2012). Pluripotent stem cells models for Huntington's disease: prospects and challenges. J Genet Genomics 39:253–259.
20. Chan AWS. (2004). Transgenic nonhuman primates of neurodegenerative diseases. Reprod Biol Endocrinol 2:39.
21. Yang SH and AW Chan. (2011). Transgenic animal models of Huntington's disease. Curr Top Behav Neurosci 7:61–85.
22. Chan AW, KY Chong, C Martinovich, C Simerly and G Schatten. (2001). Transgenic monkeys produced by retroviral gene transfer into mature oocytes. Science 291:309–312.
23. Chan AWS, KY Chong and G Schatten. (2002). Production of transgenic primates. In: *Transgenic Animal Technology*. Pinkert CA, ed. Academic Press, San Diego, pp. 359–394.
24. Chan AW, PH Cheng, A Neumann and JJ Yang. (2010). Reprogramming Huntington monkey skin cells into pluripotent stem cell. Cell Reprogram 12:509–517.
25. Laowtammathron C, EC Cheng, PH Cheng, BR Snyder, SH Yang, Z Johnson, C Lorthongpanich, HC Kuo, R Parnpai and AW Chan. (2010). Monkey hybrid stem cells develop cellular features of Huntington's disease. BMC Cell Biol 11:12.
26. Bates GP, L Mangiarini and SW Davies. (1998). Transgenic mice in the study of polyglutamine repeat expansion diseases. Brain Pathol 8:699–714.
27. Schilling G, MW Becher, AH Sharp, HA Jinnah, K Duan, JA Kotzuk, HH Slunt, T Ratovitski, JK Cooper, et al. (1999). Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum Mol Genet 8:397–407.
28. Sasaki E, H Suemizu, A Shimada, K Hanazawa, R Oiwa, M Kamioka, I Tomioka, Y Sotomaru, R Hirakawa, et al. (2009). Generation of transgenic non-human primates with germline transmission. Nature 459:523–527.
29. Courtine G, MB Bunge, JW Fawcett, RG Grossman, JH Kaas, R Lemon, I Maier, J Martin, RJ Nudo, et al. (2007). Can experiments in nonhuman primates expedite the translation of treatments for spinal cord injury in humans? Nat Med 13:561–566.
30. Rice J. (2012). Animal models: Not close enough. Nature 484:S9–S9.
31. Tabrizi SJ, RI Scahill, A Durr, RA Roos, BR Leavitt, R Jones, GB Landwehrmeyer, NC Fox, H Johnson, et al. (2011). Biological and clinical changes in premanifest and early stage Huntington's disease in the TRACK-HD study: the 12-month longitudinal analysis. Lancet Neurol 10:31–42.
32. Bachevalier J, CJ Machado and A Kazama. (2011). Behavioral outcomes of late-onset or early-onset orbital frontal cortex (areas 11/13) lesions in rhesus monkeys. Ann N Y Acad Sci 1239:71–86.
33. Bachevalier J, L Malkova and M Mishkin. (2001). Effects of selective neonatal temporal lobe lesions on socioemotional behavior in infant rhesus monkeys (Macaca mulatta). Behav Neurosci 115:545–559.
34. Ewing-Cobbs L, MR Prasad, P Swank, L Kramer, D Mendez, A Treble, C Payne and J Bachevalier. (2012). Social communication in young children with traumatic brain injury: relations with corpus callosum morphometry. Int J Dev Neurosci 30:247–254.
35. Yang SH, PH Cheng, RT Sullivan, JW Thomas and AW Chan. (2008). Lentiviral integration preferences in transgenic mice. Genesis 46:711–718.
36. Chung MY, LP Ranum, LA Duvick, A Servadio, HY Zoghbi and HT Orr. (1993). Evidence for a mechanism predisposing to intergenerational CAG repeat instability in spinocerebellar ataxia type I. Nat Genet 5:254–258.
37. Orr HT, MY Chung, S Banfi, TJ Kwiatkowski, Jr., A Servadio, AL Beaudet, AE McCall, LA Duvick, LP Ranum and HY Zoghbi. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nat Genet 4:221–226.
38. Takano H, O Onodera, H Takahashi, S Igarashi, M Yamada, M Oyake, T Ikeuchi, R Koide, H Tanaka, K Iwabuchi and S Tsuji. (1996). Somatic mosaicism of expanded CAG repeats in brains of patients with dentatorubral-pallidoluysian atrophy: cellular population-dependent dynamics of mitotic instability. Am J Hum Genet 58:1212–1222.

Address correspondence to:
Dr. Anthony W.S. Chan
Yerkes National Primate Research Center
Emory University
954 Gatewood Road, North East
Atlanta, GA 30329

E-mail: awchan@emory.edu

Received for publication August 28, 2012
Accepted after revision November 28, 2012
Prepublished on Liebert Instant Online November 29, 2012

BIOGRAPHY

Kittiphong Putkhao was born in Nakhon Ratchasima, Thailand on Thursday April 24th, 1986. He finished his high school at Srisukwittaya School in Nakhon Ratchasima. In 2007, he received Bachelor's Degree (B.Sc.) in Animal Production Technology from Institute of Agricultural Technology, Suranaree University of Technology. Then, he pursued his Ph.D. study in Animal Biotechnology at School of Biotechnology, Institute of Agricultural Technology. During the study he also received a scholarship from Royal Golden Jubilee (RGJ) Ph.D. Program of Thailand Research Fund. His Ph.D. thesis title was Production of Transgenic Huntington's Blastocyst from Transgenic Huntington's Rhesus Monkey Sperm and Derivation of Embryonic Stem Cell Lines. Part of this work was presented as poster presentation in the RGJ-Ph.D. Congress XII on April 6-8, 2012 at Jomtien Palm Beach Hotel and Resort, Pattaya, Chonburi, Thailand.