IN VITRO SPERMATOGENESIS OF RHESUS MONKEY PLURIPOTENT STEM CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2019

การสร้างเซลล์สืบพันธุ์ลิงวอกเพศผู้ในหลอดทดลองจากเซลล์ต้นกำเนิดพลูริโพเทน

นางสาวสุจิตรา คำผาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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IN VITRO SPERMATOGENESIS OF RHESUS MONKEY PLURIPOTENT STEM CELLS

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

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สุจิตรา คำผาง : การสร้างเซลล์สืบพันธุ์ลิงวอกเพศผู้ในหลอดทดลองจากเซลล์ต้นกำเนิดพลูริโพเทน (*IN VITRO* SPERMATOGENESIS OF RHESUS MONKEY PLURIPOTENT STEM CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 128 หน้า.

เซลล์ต้นกำเนิดชนิดพลูริโพเทนของลิงวอก เป็นแหล่งวัตถุดิบที่สำคัญสำหรับการศึกษาวิจัยทางชีววิทยาการแพทย์ในมนุษย์ เนื่องจากเซลล์พลูริโพเทนของลิงวอก มีคุณสมบัติที่คล้ายคลึงกันกับเซลล์ต้นกำเนิดตัวอ่อนมนุษย์ และมีความสามารถเหนี่ยวนำไปเป็นเซลล์ชนิดอื่นได้อย่างไม่จำกัด อีกทั้งยังมีข้อจำกัดด้านจริยธรรมน้อยกว่าการศึกษาวิจัยในมนุษย์ ดังนั้น เซลล์ที่ได้จากการเหนี่ยวนำเซลล์พลูริโพเทน ของลิงวอก จึงเป็นองค์ประกอบที่สำคัญในการศึกษา กลไก กระบวนการเปลี่ยนแปลงและชีววิทยาของโรคที่มีข้อจำกัดในการศึกษาวิจัยในมนุษย์ การศึกษาในครั้งนี้แสดงให้เห็นถึงความสามารถในการเหนี่ยวนำ เซลล์พลูริโพเทนของลิงวอก ไปเป็นเซลล์ของระบบสืบพันธุ์เพศชาย (Spermatogenic cells; SSCs) โดยวิธี Direct differentiation โดยที่ไม่มีการดัดแปลงพันธุกรรมของเซลล์ เซลล์SSCs ที่ได้จากการเหนี่ยวนำภายนอกร่างกายสามารถจำลองถึงกระบวนการสร้างเซลล์สืบพันธุ์เพศชายเช่นเดียวกันกับที่เกิดในร่างกาย โดยการแสดงออกของตัวบ่งชี้ทางชีวภาพที่จำเพาะของเซลล์สืบพันธุ์เพศชาย ได้แก่ การแสดงออกของยีน VASA ซึ่งเป็นตัวบ่งชี้ที่สำคัญของเซลล์สืบพันธุ์ในสัตว์ชั้นสูง, SALL4 และ PLZF ตัวบ่งชี้ของเซลล์ต้นกำเนิดเซลล์สืบพันธุ์ชนิด Spermatogonia, cKit ตัวบ่งชี้ของ Spermatogonia ที่มีการแบ่งตัว และ Piwil1 เซลล์ที่เจริญพัฒนาขึ้นของ Spermatocyte ไปจนถึงเซลล์ Spermatid ที่มีสารพันธุกรรมลดลงครึ่งหนึ่งเหมือนในสเปิร์ม ในขณะเดียวกันการตรวจสอบด้วยวิธี Fluorescence activated cells sorting โดยใช้องค์ประกอบของสารพันธุกรรม DNA เป็นตัวแยกเซลล์ ช่วยยืนยันว่าการเหนี่ยวนำเซลล์พลูริโพเทนของลิงวอกในหลอดทดลอง สามารถสร้างเซลล์สืบพันธุ์ที่มีสารพันธุกรรมลดลงครึ่งหนึ่ง (Haploid spermatid cells) ซึ่งเป็นระยะก่อนสร้างสเปิร์มที่สมบูรณ์ได้ ดังนั้น เราจึงใช้กระบวนการเหนี่ยวนำแบบเดียวกันเพื่อใช้เป็นแบบจำลองในอนาคต ในการสร้างเซลล์สืบพันธุ์เพศชายขึ้นใหม่จากเซลล์ผู้ป่วยเอง (Patient specific SSCs) โดยใช้เซลล์ต้นกำเนิดของลิงวอกที่เป็นโรคฮันติงทัน (Transgenic rhesus monkey HD ESCs; rHD ESCs) เหนี่ยวนำให้เป็น rHD SSCs แล้วนำไปตรวจสอบพยาธิสถาพของโรค ผลจากการศึกษาเป็นที่น่าสนใจว่า เราสามารถตรวจสอบกระบวนการ mutation ในลำดับเบสของยีนฮันติงทันที่มีผลต่อกระบวนการเซลล์สืบพันธุ์เพศชายในหลอดทดลองได้ และแสดงให้เห็นถึงการถ่ายทอดของยีนผิดปกตินี้ ผ่านเซลล์สืบพันธุ์จากรุ่นสู่รุ่น ความไม่สเถียรของลำดับเบสใน rHD SSCs แสดงออกโดยการเพิ่มขึ้นของลำดับเบส CAG ที่ซ้ำกันในอัลลีนที่ก่อโรค (Disease allele) โดยพบการเพิ่มขนาดเบสซ้ำของ CAG ขนาดเล็กกลาง และขนาดใหญ่ใน rHD SSCsเปรียบเทียบกับเซลล์ก่อนการเหนี่ยวนำ ยิ่งไปกว่านั้นพบว่ายีน PLZF และ Piwil1 มีการแสดงออกที่ตรงกันข้ามจากกลุ่มควบคุม (Wild type control) โดย PLZF มีการแสดงออกที่คงที่จากวันที่ห้าไปจนกระทั่งสิ้นสุดการเหนี่ยวนำในวันที่สิบเซลล์สืบพันธุ์ลดลงใน

กระบวนการเหนี่ยวนำเซลล์พลูริโพเทนท์ของลิงวอกและเซลล์ที่เป็นโรคฮันติงทันไปเป็นเซลล์สืบพันธุ์เพศชายในครั้งนี้เป็นแหล่งวัตถุดิบที่สำคัญสำหรับการศึกษาวิจัยขั้นพื้นฐานไปถึงขั้นสูงของโรคฮันติงตัน การเข้าใจกระบวนการเกิดของโรคฮันติงทันที่มีผลต่อเซลล์สืบพันธุ์ จะนำไปสู่กระบวนการคิดค้นวิธีการและพัฒนาการรักษาที่มีประสิทธิภาพ นอกจากนั้นการสร้างเซลล์สืบพันธุ์ในหลอดทดลองในครั้งนี้ สามารถนำไปสู่กระบวนการทางเลือกในอนาคตสำหรับการรักษาโรคที่มีผลต่อเซลล์สืบพันธุ์ในเพศชายและยังสามารถเพื่อแก้ปัญหาการลดลงของจำนวนสเปิร์มที่กำลังเกิดขึ้นอย่างต่อเนื่องทั่วโลก

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา ปริญญา ศรีพาง
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

SUJITTRA KHAMPANG : *IN VITRO* SPERMATOGENESIS OF RHESUS MONKEY PLURIPOTENT STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 128 PP.

SPERMATOGENESIS/RHESUS MONKEY/HUNTINGTON DISEASE

Rhesus monkey pluripotent stem cells (rPSCs) are the great promising source for basic and advanced regenerative biomedical research in human. Since rPSCs share the same number of properties similar to human pluripotent stem cells (hPSCs) and an unlimited differentiation capacity with less ethical uses in advance stem cell research, thus, the desired cells derived from rPSCs differentiation are valuable materials for developmental and biological study of human disease. In this study, we demonstrated differentiation ability of rPSCs into advance male germ cells lineages by *in vitro* direct differentiation and without genetic manipulation. Derived rhesus monkey spermatogenic cells (rSSCs) form rPSCs recapitulated *in vivo* spermatogenesis by expressing the molecular phenotype of specific male germ cells including VASA, a predominant germ cells marker of primate SSCs, SALL4, PLZF positive of spermatogonia (Spg) population, cKit and Piwil1 expression showed the marker of differentiating Spg, and more mature spermatocytes to haploid spermatid cells respectively. Taken together, fluorescence activated cells sorting based on DNA content of the cells revealing haploid population are derived from rPSCs induction *in vitro*. Moreover, using this platform we created a model for study of patient specific SSCs production. Transgenic rhesus monkey Huntington disease PSCs (rHD PSCs) were induced to rHD SSCs, and subsequently were used for pathogenic cellular phenotype investigation. We captured CAG repeats mutation during *in vitro* spermatogenesis recapitulating paternal germ line transmission of *HTT* transgene *in vivo*. Instability of trinucleotide repeats (TNR) in rHD SSCs was observed during *in vitro* spermatogenesis by increased small, intermediate and

persistently expressed throughout the 10 days differentiation, whereas Piwil1 mature SSCs marker declined in rHD SSCs by day10. Taken together, reduction of germ cells apoptotic cell deaths against normal SSC apoptosis, are observed in rHD SSCs suggesting pathogenic effects of *HTT* mutation on spermatogenesis progression and male germ cells development. This reveals a potential way to induce rPSCs as well as a pathogenic mode for changing rHD PSCs into male germ cells lineage, thus providing material for basic and advance research of HD and better understanding of the molecular phenotype of SSCs under disease condition as part of a therapeutic strategy. Additionally, generating male germ cells *in vitro* presents an alternative source for male fertility preservation and studies of worldwide sperm count decline.

School of Biotechnology

Academic Year 2019

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ACKNOWLEDGEMENT

This work was supported by a SUT-PhD scholarship, Suranaree University of Technology Thailand. Part of the research was carried out and supported by Yerkes National Primate Research Center, Emory University School of Medicine, Atlanta, USA.

First, I would like to give sincere thanks to my advisor, Assoc. Prof., Dr. Rangsun Parnpai who provided me a great opportunity for my Ph.D. study at Suranaree Univerisity of Technology. Without his support, none of this would be possible. I have learned so much from his patience, thoughtful guidance, planning and problem solving with my study and research. I greatly value his advice, encouragement and belief in me. I am deeply indebted to him for his fundamental role in the success of my doctoral work. Next, I would like to express my deeply felt appreciation to Professor, Dr. Anthony W.S. Chan, my co-advisor, who gave my Ph.D. research his help and support, and for his confidence in me throughout my two years research work in his laboratories. I would not have my passion for stem cells research today without all of his support. In addition to suggestions, advice and encouragement, he taught me great practical laboratories skills and the way to walk through any problem. Besides academic work, he greatly supported me personally throughout the time in USA. I am so thankful to know him and will walk in his steps, as I follow in the way of a great scientist. I would also like to thank all of my committee members, Prof. Dr. Montarop Yamabhai, Assoc. Prof. Dr. Mariena Ketudat-Cairns and Dr. Chuti Loawthumathorn for their valuable comment and suggestions that helped me make the best account of this research.

I would like to thank all the people who were involved to my two-year research work in Atlanta, Georgia. First, I would like to especially thank Dr. In Ki Cho, who influenced

my research work at Yerkes. His great advice, information, suggestion and research skills practice made me a better scientist. My appreciation also to Charles Easely IV and Alyse Steves for their kind collaboration, support and suggestions that provided me material and many ideas during my work. I also offer a sincere thanks to my greatest and warm friend, Leslee Sinclair, who supported me in both sides of my life in Atlanta. She provided kind support in editing of this research work. Beside my study, Leslee is the one who gave me the experience in foreign cultural, life and fun. It never feels lonely in her house.

I wish to acknowledge all of Dr. Chan's lab members as a part of my research work since they provided great suggestion, discussion, technical support and beautiful friendship; Dr. Kanchana Punyawai, Siran Tianliu, Jin-Jing Yang, Cari Emerson Hunter, Alexandra Vanilla Goodnight, Ezana Assefa and Terell Hamlet at Yerkes National Primate Research Center. I would also like to acknowledge all ESRC's Lab members for providing their support in my documentation, as well as encouragement during my research work. I am so thankful to my kind colleagues; Suthatip Jirundon, Monsikarn Chaiyakit, Natchanok Puangjit, Thanya Pranomphon, Pattamon Meaungchan, Prapot Thunthaisong and Whachira Panta for their help, beneficial suggestions and support that helped me fulfill my research.

Most importantly, I wish to express my deepest feelings of thanks to my parents, my older sister and her family for their warm love and unconditional support during my life.

To complete my acknowledgements, I wish to express my love to all of my experimental stem cells and samples that were patiently growing and reached our experimental goals. Your valuable small unit is building up a large- scale benefit for human well-being.

Sujittra Khampang

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LIST OF ABBREVIATIONS

SSCs	=	Spermatogenic cells
PGCs	=	Primordial germ cells
GCs	=	Gonocyte cells
Spgc	=	Spermatogonia stem cells (Undifferentiated spermatogonia)
Spg	=	Spermatogonia cells (Undifferentiated and differentiated spermatogonia)
Adark	=	Type A spermatogonia with condense nuclear staining
Apale	=	Type A spermatogonia with lighten nuclear staining
B	=	Type B Differentiating spermatogonia
PSCs	=	Pluripotent stem cells
ESCs	=	Embryonic stem cells
r	=	Rhesus monkey
NHP	=	Non-human primate
HD	=	Huntington disease
HTT	=	*Huntingtin* gene
mHtt	=	Mutant Huntingtin protein
CAG	=	Cytosine-Adenine-Guanine
TNR	=	Trinucleotide repeat
VASA	=	RNA binding protein with an ATP-dependent RNA helicase
PLZF	=	Promyelocytic leukemia zinc finger (ZBTB16)

LIST OF ABBREVIATIONS (Continued)

SALL4	=	Sal-like protein 4
Piwil1	=	Piwi-like protein 1, a member of Piwi-interacting RNAs
c-Kit	=	KIT proto-oncogene, receptor tyrosine kinase
ml	=	milliliter
µm	=	micron
rpm	=	round per minute
ng	=	nanogram
%	=	percentage
°C	=	degree Celsius
mM	=	millimolar
µM	=	micromolar
nM	=	nanomolar

CHAPTER I

INTRODUCTION

1.1 Introduction

The current global phenomenal of persistently reduced sperm count over the past decade has become a concerning issue, and research on the cause of this continuing decline is urgently needed (Jørgensen et al., 2006; Mascarenhas et al., 2012; Levine et al., 2017). Infertility is now affecting couples' lives worldwide, and around 40-50% of the cases are caused by male factors infertility including low number of sperm counts and defective semen quality (Sharpe, 2010; Dada et al., 2012; Ibtisham et al., 2017; Durairajanayagam, 2018). A wide range of factors have assumed to cause infertility in man, such as genetic, epigenetic, environmental and lifestyle conditions. Genetic defects, especially in male germ line transmissible genes can play a major role in male fertility, which is maintained by a healthy pregnancy and determines the health progression of progenies (Bisht et al., 2017). Gender has been an influence to trinucleotide repeat (CAG) mutation and instability which is represented as a cellular pathogenic phenotype of Huntington disease (HD). The expanded CAG repeat in HD can occur dominantly during germ line transmission and spermatogenesis (Kovtun et al., 2000; Wheeler et al., 2007; McMurray, 2010; Putkhao et al., 2013). Although, HD is represented as a progressive neurodegenerative disorder, testicular degeneration and progressive loss of spermatogenic cells which were observed in HD patients (Van Raamsdonk et al., 2007), supporting that this genetic defect disorder is impactin male gametogenesis. The underlying mechanism of HD pathogenic cellular phenotype on male germ line progression will help elucidate disease progression in patient as well as fertility in HD.

Spermatogenesis and spermiogenesis are complex structures and an organized process of surrounding cells in seminiferous tubule working together to generate mature sperm. Presently, in the field of reproductive biotechnology, we are trying to study male germ cells development to understand and improve infertility problems. *In vitro* spermatogenesis demonstrates a powerful tool and a potential model to study male germ cells development especially in the field of patient specific stem cells production (Ibtisham et al., 2017). Completely mature sperm productions *in vitro* haven't been achieved, however evaluation of *in vitro* spermatogenesis in various models have been developed to clarify the mechanism of *in vivo* spermatogenesis (Yamauchi et al., 2009; Sato et al., 2011; Easley et al., 2012; Zhou et al., 2016; Sosa et al., 2018; Zhao et al., 2018) Notably, it hasn't been reported that *in vitro* differentiation of pathogenic SSC under disease condition is a term of patient specific SSCs production. Moreover, human SSC derived *in vitro* would be a critical challenge of *in vitro* and *in vivo* functional assay to prove that SSC derived *ex vivo* has a potential in advanced application because of ethical concerns.

Assessment in nonhuman primate (NHP) has been important for human pathological and biomedical research because of their genetic and physiological similarity to humans (Chan, 2013). Rhesus monkeys (*Macaca mulatta*) are the most widely used throughout NHP studies for basic and applied biomedical research (Gibbs et al., 2007). Moreover, rhesus studies are even more important because they are the basic foundation for the inaccessible experiments in human due to ethical concerns. Monkey pluripotent stem cells (PSCs) share a number of properties with human ESCs proved to have less tumorigenic risks by autologous transplantation, showing an efficient and safe model for anticipated human research (Hong et al., 2014). Moreover, monkey PSCs shows the ability to differentiate to various cell types in the body including germ cells. Thus, study of *in vitro* spermatogenesis of rhesus monkey PSCs, as well as if rPSCs can cooperate with genetic defect and producing pathogenic SSCs will be advantageous to study human genetic disorders and powerful stem cells-based

material for therapeutic investigation and male fertility preservation.

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CHAPTER II

REVIEW OF LITERATURE

2.1 Spermatogenesis

2.1.1 Prenatal germ cells

Primordial germ cells (PGCs) are the founder cells of eggs and sperm. Formation of PGCs marks the initiation of the life cycle of germ cell lineage in all species, thus PGCs progression determine the inception point of germ cells developmental process. During embryogenesis, fertilized oocytes undergo sequential change from morula, blastocyst to implantation state. At post implantation state, PGC precursors translocate form epiblast cells by the inducing of extraembryonic ectoderm signal, then subsequently migrate toward primitive streak and segregate into PGCs (Zhao and Garbers, 2002). PGCs become a cluster adjacent to a posterior epiblast of post implantation embryos around embryonic day (E) 6.5 to 7.25 and continue to migrate individually through the hindgut endoderm and mesentery to colonize in the embryonic gonad around E10.5 (Saitou and Miyauchi, 2016). By E12.5-13.5, 25,000 to 30,000 PGCs stop mitotic proliferation, but continue apoptosis. During this quiescence period, PGCs are referred to as gonocytes (GCs), which undergo sex specific determination and progress forward to gametogenesis. In males, inactive GCs are located in the center of seminiferous tubule called **pro-spermatogonia** which remain arrested during fetal period (Figure 2.1). Two days after birth, pro-spermatogonia start to proliferate then continue to migrate into the basement membrane of seminiferous tubule and are later differentiated into undifferentiated spermatogonia which are consisting of **spermatogonia stem cells** (Spgs) and **spermatogonia progenitor cells** (SPCs), as the precursor of

spermatogenesis. In postnatal day 10 (P10) in mice and pubertal testis in men, major populations of Spgs enter into the first wave of spermatogenesis, generating the first population of haploid sperm cells, whereas small Spgs generate Spgs themselves to provide spermatogonia (Spg) that enter the next round of spermatogenesis and produce sperm throughout adulthood (Spradling et al., 2011; Kanatsu-Shinohara and Shinohara, 2013).

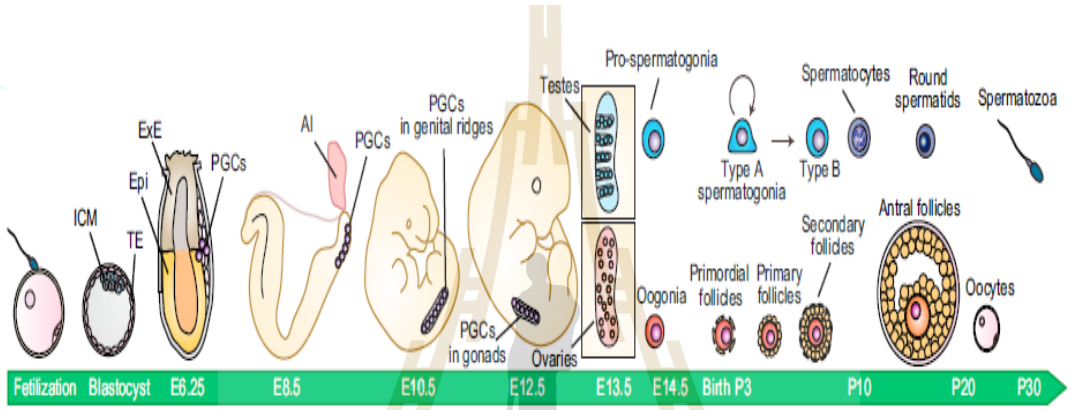

Figure 2.1 Schematic of male mouse germ cell development timeline and germ cells differentiation. The outer layer of blastocyst gives rise to the trophectoderm (TE) while inner cell mass (ICM) contains ESCs. During gastrulation the blastocyst cavitates and develops the three germ layers and the epiblast (Epi). The primordial germ cells (PGCs) are localized near the extra-embryonic ectoderm at the base of the allantois (Al). Once PGCs are specified, they migrate to the fetal gonads becoming Gonocytes (GCs) and undergo sex-specific development to male and female gametogenesis after birth (Adapted from; Saitou and Miyauchi 2016; Ibtisham et al., 2017).

2.1.2 Spermatogenic pools in adult testes

Stem cells of spermatogenesis are called **spermatogonia stem cells** (Spgs) that have the ability of self-renewing to sustain themselves and generate progenitors of Spg that will continue to differentiate into spermatozoa and maintain male fertility (Fayomi and Orwig, 2018). In mice, the population of Spg is located on a basement membrane called

A_{single} Spg (As) which divides into a pair of Spg (Apaired; Apr) that will either develop by continuing to produce a chain of four $A_{aligned}$ Spg (A_{al1-4}) and two new A_S Spg to sustain stem cells niches for the next round of spermatogenesis. Thus, As Spg are considered to be Spgs since they are the only self-renewing germ cells which reveal both regenerative and functional reserve in rodent testes (Clermont and Bustos-Obregon, 1968; Huckins and Oakberg, 1978; Hess, 1990; Ehmcke et al., 2006a). A_{al1-4} Spg mitotically divides into a series of differentiating Spg composed of A1 - A4 Spg, intermediate (I) and B Spg, which next divide into primary spermatocytes. Primary spermatocyte undergoes meiotic division twice that gives rise to secondary spermatocytes and a round spermatid with containing half of the DNA content, thus they are called **haploid spermatids**. Immature haploid cells continue to develop into mature sperm by the process of spermiogenesis, which does not involve cell division but undergo morphological and epigenetic change. Therefore, through the dynamic change of cell division, rodents produce approximately 40 million sperm per gram of testis parenchyma in each day (Fayomi and Orwig, 2018) and take 34.5 days for one round since Spgs enter spermatogenesis.

In human, Spgs exhibit two morphologically distinguishable instances of nuclear staining referred to as A_{dark} and A_{pale} Spg. A_{dark} represents testicular stem cells or **true stem cells** as they show high-proliferation during prepubertal testicular cells development (de Rooij et al., 1986; Ehmcke et al., 2005), thus representing the regenerative reserve of testicular germ cells. On the otherhand, A_{pale} is a spermatogenic progenitor that shows both self-renewing ability to maintain the cells for the next cycle and enter into the differentiation process of spermatogenesis (Ehmcke et al., 2006). In contrast with rodents, number of A_{dark} and A_{pale} Spg in human are present equally and constitute a high population of 22% of germ cells in human testes (Paniagua et al., 1987). A_{pale} mitotically divides into differentiating Spg called **B Spg**, subsequently into primary spermatocytes and with continuing meiotic division give rise to secondary spermatocytes and haploid round spermatids. Human spermatogenesis

takes only two mitotic steps to achieve primary spermatocytes, since the dividing process of Spg progenitor passes through only one differentiating B Spg, while other species have many more types of B Spg. Thus, human spermatogenesis could produce four spermatocytes and 16 spermatozoa from each progenitor (Figure 2.2), which takes approximately 64 days for one round of spermatogenesis and producing about 4.4 million sperm per gram of testis parenchyma in each day (Fayomi and Orwig, 2018).

In rhesus monkey, Spg type A is defined based on the nuclear architecture of hematoxylin staining and biological functions similar with humans. A_{dark} Spg, definite a true as they show inactive proliferation during normal spermatogenic activity while becoming active after Spg depletion by toxicity irradiation (de Rooij et al., 1986; van Alphen and de Rooij, 1986) or during pubertal expansion (Simorangkir et al., 2005). A_{pale} Spg, a self-renewing progenitor continuously proliferate in each cycle of spermatogenesis to maintain sperm production (Clermont and Leblond, 1959; Clermont, 1969). The differentiating B Spg is composed of four types including B_1, B_2, B_3 and B_4 that undergo mitotic division to produce primary and secondary spermatocytes, and then subsequently with meiotic activity produce haploid spermatid and spermatozoa. Thus, rhesus spermatogenesis requires five mitotic divisions to produce primary spermatocytes from a progenitor A_{pale} Spg division (Ehmcke et al., 2005; Ehmcke et al., 2006b). In contrast to humans, rhesus clonally produce 32 spermatocytes and 128 haploid spermatids from any of an A_{pale}, which takes 10.5 days for one cycle (12 stages) of seminiferous epithelium and complete duration of spermatogenesis are taking about 36 days (Barr, 1973). Thus, monkey produces sperm closely approximate to rodents, which is estimated to be 41 million sperm per gram of testis parenchyma in each day (Fayomi and Orwig, 2018).

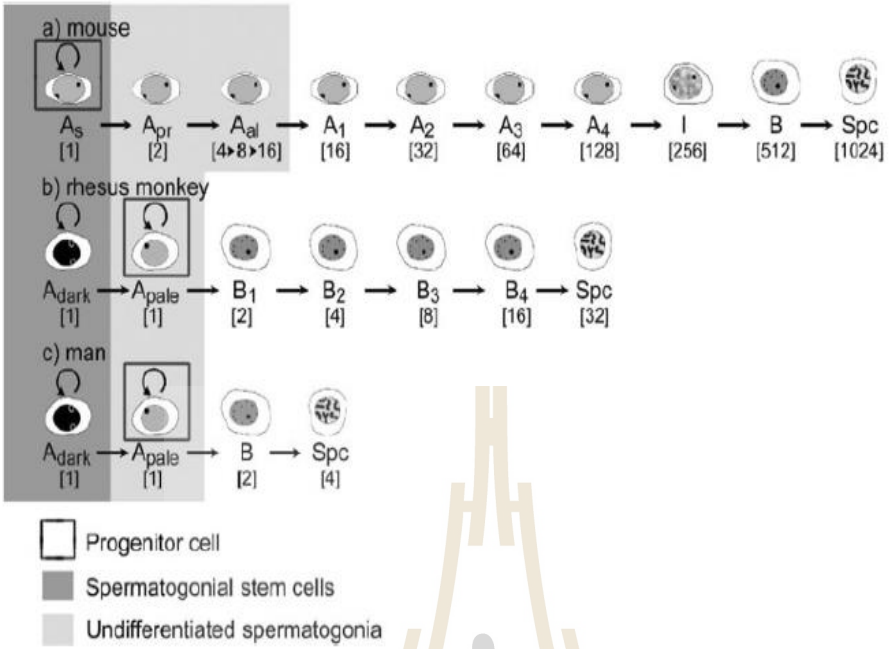

Figure 2.2 The clonal expansion in premeiotic steps during spermatogenesis in mouse, monkey and human. The number given in brackets underneath the cells indicates the total number of daughter cells derived from any one progenitor cell that enters one spermatogenic cycle (From; Ehmcke et al., 2006).

2.1.3 Spermatogenesis and spermiogenesis

The process of adult spermatogenesis is started in pubertal men by the regulation of hormonal control and somatic cellular association. Spermatogenesis together with spermiogenesis are driving male germline stem cells develop and progress forward to mature sperm in male reproduction (Figure 2.3). The stages of spermatogenesis have been defined as a series of mitotic, meiotic and spermiogenesis actions along a length of seminiferous tubule, producing mature cells translocated from basement membrane through the luminal compartment of the seminiferous tubule. Distinguished types of spermatogenic cells are able to be observed in seminiferous cross section base on nuclear DNA and morphological transition (Clermont and Leblond, 1959).

1) Mitotic phase

Diploid Spgs (2N) which are located in the basement membrane of the seminiferous tubule start to duplicate their number by undergoing mitotic proliferation that produce daughter Spg or differentiating Spg type B that contain the same DNA and chromosomal content (2N). Taken together, mitotic division of Spgs is generating others Spgs for replenishment of the Spgs themselves as their purpose is to support sperm cells production for an entire lifetime. Mitotically division of differentiating Spg type B continues along different types of B Spg completely and ends when B Spg yields two primary spermatocytes (2N). Thus, progression of given cells during this stage of spermatogenesis can be called **mitotic** or **pre-meiotic spermatogenic cells**.

2) Meiotic phase

A diploid primary spermatocyte is transformed into two secondary spermatocytes during first meiotically division, resulting in the half reduction of nuclear content in the given secondary spermatocytes. The secondary spermatocytes undergo a second meiotic division producing four daughter haploid (1N) round spermatid cells. Conversion of secondary spermatocytes to spermatid cells is rapid, thus rarely are secondary spermatocytes observed in a testis cross section examination (Barr, 1973). Spermatogenic cells progressed in this stage onward and then were called **meiotic** or **post-meiotic spermatogenic cells**, defined as a more mature state with only one-half of genetic material remaining in given germ cells.

3) Spermiogenesis

A haploid round spermatid undergoes the metamorphosis from a typical round cell to a highly specialized, elongated and compacted structure for traversing in the reproductive tract of male and female to achieve further fertilization. No additional cell division occurs during spermiogenesis as the streamlined process involves nuclear condensation, acrosomal cap formation and development of the tail. Changing of round

spermatid morphology to spermatozoa is defined as a particular series. Higher primate as human spermatid undergoes 12 series of steps and takes about five weeks to complete spermiogenesis (Dadoune et al., 2004). The major event that occurs during spermiogenesis is epigenetic modification of nucleosomal chromatin contents. Nuclear DNA is transformed into compact chromatin fiber by function of transition protein to replace histone by protamine results in nuclear condensation and turns into inactive transcription and shuts off the RNA synthesis. As nuclear DNA is compacted, it results in cessation of active gene transcription, which is a conservation mechanism of male gametogenesis to keep genetic materials from paternal genome throughout the next generation. Spermitation is the last process to release spermatozoa into the tubular lumen by Sertoli cells, which then move through epididymis and begin motility during their migration time.

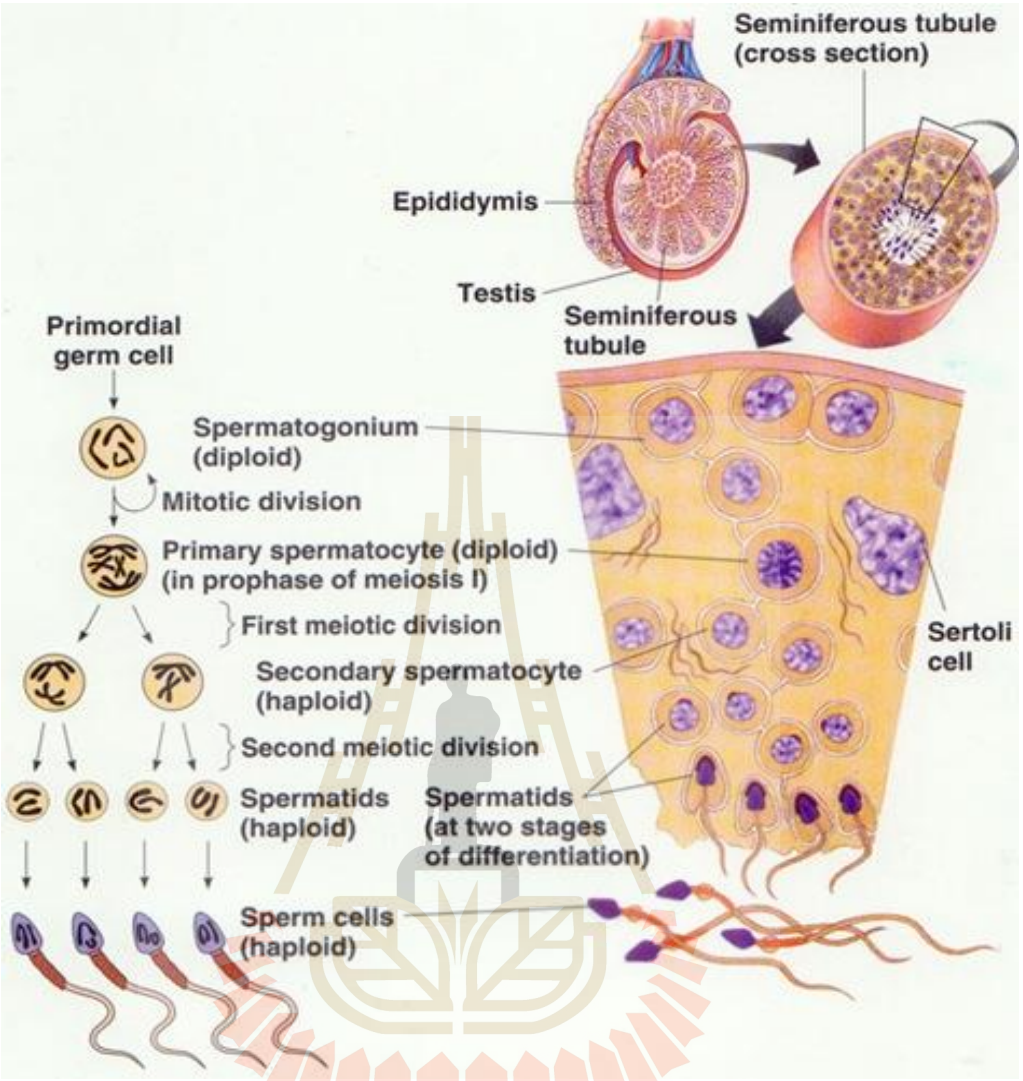

Figure 2.3 Schematic of spermatogenesis in adult testis occurs in seminiferous tubules of the testes. Spgs located in basement membrane where the origin of spermatogenesis occurs; SSCs then being developed until entering spermiogenesis after completion of last meiotic division. Transformation of spermatids into spermatozoa, called spermiogenesis, occurs in the final step before mature spermatozoa is motile when it is released into epididymis and secreted with seminal fluid throughout male adulthood (From: www.majordifferences.com/2013/06/difference-betweenspermatogenesis and. html#. XMm6kegzbIU).

2.1.4 Kinetics of spermatogenesis (Rhesus monkey)

Duration of spermatogenesis in rhesus monkey has been identified by tritiated thymidine-methyl H technique, a tracer incorporated with nuclei of given germ cells and periodic acid-Schiff (PAS) staining (Clermont and Leblond, 1959; Barr, 1973) to determined stage of the cycle of seminiferous epithelium. In adult testes, a specific type of Spg is associated with a given type of spermatocyte and spermatid, forming the cellular grouping of constant composition in seminiferous tubule specifically in each species called **stage of cycle of seminiferous epithelium**, which shows the dynamic change of spermatogenic cells during spermatogenesis. Clemont (1959) has identified 12 stages (I-XII) in rhesus testes as representing the cellular complement observed over time in a particular area, forming one cycle of SSCs progression. The determination of the stage of the cycle during spermatogenesis underlying mechanism of *in vivo* germ cells differentiation and expansion as well producing valuable data of germ cell count is important (Ehmcke et al., 2005). Duration of one cycle of seminiferous epithelium in rhesus monkeys has first been determined as 9.5 ± 0.3 days (Clermont and Leblond, 1959; Barr, 1973) while a later study defines it as 10.5 ± 0.2 days (de Rooij et al., 1986). According to Clemont and Arnold's earlier studies, the total duration of rhesus spermatogenesis is 36 days (Figure 2.4). The first appearance of the preleptotene primary spermatocyte in Stage 7 until Stage 6 late spermatid production which takes three cycles of seminiferous epithelium, represents a total duration of spermatogenesis in rhesus monkey (Barr, 1973; Clermont and Leblond, 1959). In contrast, the linear pattern in the stages of cycle of seminiferous epithelium in men is less ordered than rhesus, man needs six stage of cycle and four cycles of 16 days (64 days) to complete spermatogenesis (Fayomi and Orwig, 2018).

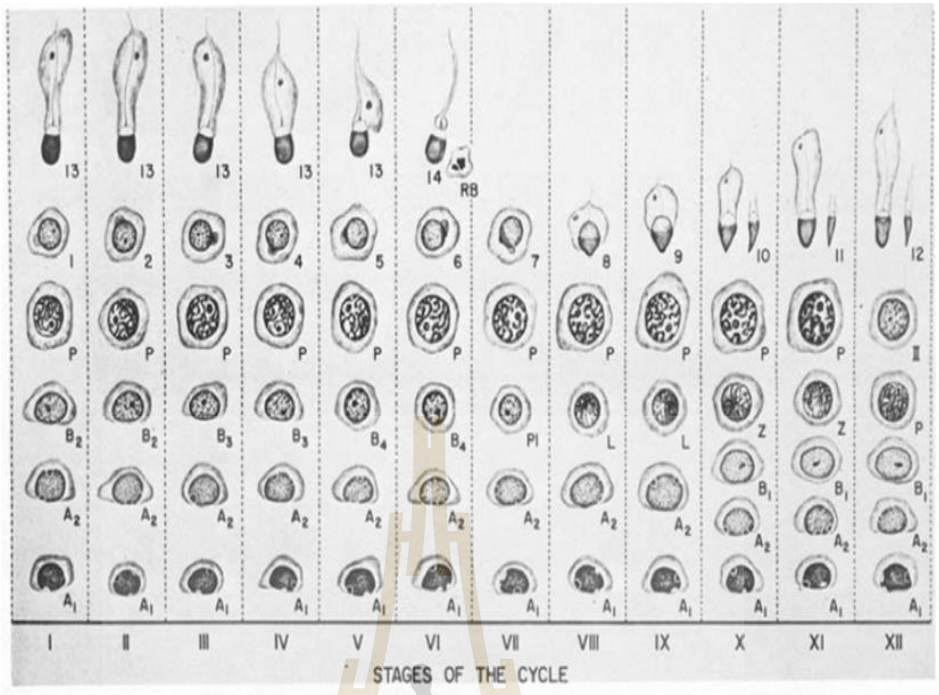

Figure 2.4 Illustration of **cycle of seminiferous epithelium** in rhesus monkey seminiferous tubules cross section. Twelve stages of the cycle represented by Roman numeral were identified by first 12 steps of spermiogenesis (spermiogenesis consist of 1-14 steps) based on changes observed in the nucleus and in the acrosomic structure with PAS techniques. The stages of cycle in each column demonstrated the cell types in one of the **stage of cycle** found in cross-sections. Stage of cycles are succeeded by each other in time in any given area of seminiferous tubule according to the sequence indicated from left to right in figure, thus after completing stage XII, stage I reappears again and the sequence starts over again. A1, A2 = spermatogonia type A1 and 2, B1-B4 = spermatogonia type B1-4, PI = preleptotene primary spermatocyte, L = leptotene spermatocyte, Z = zygotene spermatocyte, P = pachytene spermatocyte, II = secondary spermatocyte and RB = residual body (Barr, 1973).

2.1.5 Regulation of germline stem niches

Spermatogenesis is a highly organized process between spermatogenic cells in seminiferous epithelium associated together with somatic cells and interstitial tissue. The association of testicular cells in basement membrane are presented as **stem cell niches** to support and promote SSCs function and differentiation going forward in the cycle. Within seminiferous epithelium, SSCs are associated closely together with testicular somatic Sertoli cells, which playing the major rule for support quantitatively in normal spermatogenesis to produce functional sperm, thus Sertoli cell is referred to as **nurse cells** of spermatogenesis. Sertoli cells form tight junctions with each other are called a **blood testis barrier** and separates seminiferous epithelium into basal and luminal compartment in testicular lumen. Spgs are located in basal compartment while other differentiating germ cells and mature germ cells develop into the luminal compartment within the cytoplasmic folds of Sertoli cells (Figure 2.3). As the closest proximity of Sertoli cells and germ cells is at the basement membrane of the tubules, thus the basal compartment is defined as the localize area of SSC niches. Sertoli cells paracrine signaling are dominant in processing and promoting and controlling spermatogenic cells progression.

1) GDNF signaling

Glial cell line-derived neurotrophic factor (GDNF) is critical to Spgs self-renewal and maintenance, as this soluble factor is secreted by Sertoli cells and plays a role as paracrine regulate the Spgs population. The signaling of GDNF start via RET tyrosine kinase receptor which is present on type A Spgs and requires GFRα1, a ligand-specific co-receptor interaction, that subsequently activates PI3K/AKT-dependent or SRC family kinase (SFK) intercellular signaling pathway to promote Spgs self-renewal transcripts including that ID4, T, POU3F1 and BCL6B (Chen and Liu, 2015). Moreover, previous study showed that GDNF overexpressing mice demonstrated overproduction of undifferentiated Spg and lack of later differentiating cells (Meng et al., 2000). In addition, GDNF could be maintained in

a long-term culture of Spgs (Hofmann, 2008; Kubota et al., 2004) thus it provided the Spgs for further molecular analysis of *in vitro* culture. Similar to rodents, humans need the GDNF factor to maintain Spgs and growth *in vitro* (Wu et al., 2009).

2) Fibroblast growth factor signaling

Basic fibroblast growth factor (bFGF), also known as FGF2, is expressed and secreted by Sertoli cells and regulates Spgs self-renewal. The study showed that co-supplementation of FGF2 together with GDNF promotes long term self-renewing expansion of Spgs in mouse (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). *In vitro* mouse germline stem cells expansion revealed that FGF2 relies on MAP2K1 activation to upregulate Etv5, Bcl6b and Lhx1 self-renewal transcripts in Spgs. In addition, FGF2 may function in mouse SSC proliferation and stem cells activity by AKT and ERK1/2 signaling pathway (Chen and Liu, 2015).

In addition to the Sertoli cells paracrine signaling, interstitial tissue composed of Leydig cells, macrophages, mesenchymal and capillaries networks are located in between each seminiferous tubule, which underlie the Sertoli cells tight junction. Their function is defined to regulate and influence spermatogenesis, thus may provide key components of SSCs niche (Oatley and Brinster, 2012). Leydig cells have a purpose as a testosterone product which is consequently utilized by Sertoli cells, thus it regulates self-renewal of Spgs (Oatley et al., 2009). Together, the vascular network in interstitial space has influence in the localization as well as development of Spg progenitor cells in rodent (Yoshida et al., 2007), however the vascular patterning affects of Spgs function or differentiating Spg has not been investigated. In order to continue to progress into mature germ cells, Spgs require a small signaling molecule including growth factors to support Spgs differentiation. Bone morphogenic protein 4 (BMP4) and Activin A have been defined to promote Spgs differentiation, as the supplement of BMP4 results to reduce the Spgs number while promoting the expression of c-kit, a differentiating germ cells hallmark (Nagano et al., 2003;

Pellegrini et al., 2003). Neuregulin1 promotes formation of Spg chains, which reveal the activity as a differentiation inducer (Hamra et al., 2007).

2.1.6 Hormonal control of SSC differentiation

At the pubertal state, spermatogenesis is regulated by endocrine activity that referred to **Hypothalamus-Pituitary-Testiscular Circuit**. When the pubertal initiation in men, a high pulse of Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus to transmembrane GnRH receptor. In response, two major endocrine signals including follicle stimulating hormone (FSH) and luteinizing hormone (LH) are secreted from pituitary gonadotropes via peripheral circulation and function on SSC niches. Within testicular tissue, FSH and LH mediate their action by specific transmembrane receptors (R), FSH-R acts on Sertoli cells and LH-R on interstitial Leydig cells, respectively. The regulation of both gonadotropins actions on spermatogenesis is mainly activated from Sertoli cell. In response to FSH signaling, Sertoli cells secreted the non-steroidal hormone inhibin to control normal spermatogenesis by maintaining Spg production and differentiation further spermatocytes (Ramaswamy and Weinbauer, 2014), while Leydig cells response to LH by secreted a testosterone, presenting as a key factor for normal spermatogenesis and production of sperm. Testosterone is a critical endocrine that stimulate secondary sexual characteristic developed during adolescence in men and drives sperm production via spermatogenesis during adulthood. In addition, when sperm count is too high, Sertoli cells produce inhibin to regulate testosterone function. Increasing of testosterone level has an effect on the hypothalamus and anterior pituitary to inhibit the releasing of GnRH, resulting in FSH and LH reduction and spermatogenesis slowing down, which is referred to as negative feedback signals to maintain homeostasis of spermatogenesis.

2.1.7 Primate germ cells and spermatogenic cells expression profile

1) Prenatal stage

Cellular expression profiles during germ cells migration and spermatogenic differentiation in monkey and man germ cells production had long been investigated. First, study of pluripotent markers at several embryonic days (E) in pre-implantation embryos demonstrated the reliability of PGCs identification. SALL4 and LIN28 are the earliest expression in E50. Later at E65 to E95. Oct4, Nanog, Sall4 and LIN28 are observed in PGCs that are located in extra-gonadal sites and translocate into GCs in gonad (Aeckerle et al., 2015). However, the expression of Oct4 and Nanog were downregulated during the time that Sertoli cells were intact to PGCs in testicular cord (Shamblott et al., 1998).

2) Postnatal stage

Male germ cells expression of 11-week old marmoset's testicular tissue has been used to investigate the early post-natal state and fetal state at the time of birth, which resembled human fetal testicular tissue structure (Gaskell et al., 2004; Mitchell et al., 2008) At the early developmental state, large numbers of germ cells were detected with Oct4, Nanog and TFA2C expression. Oct4 and TFAP2C positive cells were observed until six weeks after birth, while Nanog was consistent to neonatal period (Mitchell et al., 2008). In monkey, Oct4 are presented in testicular germ cells at birth with decrease in the positive proportion in eight weeks-old testes by threefold extension of seminiferous tubule. Results in the small number of Oct4 positive cells in cross section show not only resizing of seminiferous cord (Albert et al., 2010) but also proposed that Oct4+ cells entering apoptosis during proliferation process (McKinnell et al., 2013). VASA presented as distinctive marker of developmental germ cells lineage as revealed by their significantly higher expression than Oct4 from the time of birth till a later state of testicular development (Mitchell et al., 2008, Albert et al., 2010; McKinnell et al., 2013). Thus, the evaluating of germ cells progression during proliferation process is represented by upregulation of VASA expression while a loss

of Oct4+ cells progression. In human, VASA are detectable in second trimester but not in germ cells of gonadal rides (Anderson et al., 2007; Mitchell et al., 2008). The VASA and Oct4 positive cells are observed to be co-localized with c-kit differentiated germ cells marker in PGCs of early developmental state (Gkountela et al., 2013). The pre-migratory germ cells consist of VASA+/OCT4+ cells, while VASA-/Oct4+ or VASA+/Oct4- showed in late post-migratory stage (Sasaki et al., 2016) or were even present until a few weeks after birth (Sharma et al., 2017).

3) Mitotic spermatogenic cells

SALL4, the zing finger transcription factor, is one of the pluripotency regulation markers and plays a crucial role in early embryo development. The study shows that loss of SALL4 results in death of mouse preimplantation embryos (Elling et al., 2006; Sakaki-Yumoto et al., 2006; Tsubooka et al., 2009). SALL4 expression is observed in nucleus of late PGCs, gonocytes and Spg, SALL4 positive germ cells were decreased in pubertal testes as the same was reported regarding the downregulation of Oct4+ cells. A limitation of work with immature human testicular tissues is that SALL4 expression only demonstrated in gonocytes in fetal human testes and type A Spg of one- year-old boy. In adult testes, SALL4 showing a strong expression in a subpopulation of Spg was only observed in Spgs type A (A_{dark} and A_{pale}) (Eildermann et al., 2012).

PLZF and GFRα are the markers of undifferentiated Spg in adult rhesus testicular cells (Meng et al., 2000; Buaas et al., 2004; Costoya et al., 2004; Buageaw et al., 2005; Ryu et al., 2005). PLZF+ cells per cross section shows almost the same amount of A_{dark} and A_{pale} combining number, suggesting that PLZF is an expression hallmark of undifferentiated Spg in rhesus spermatogenesis (Hermann et al., 2007; Hermann et al., 2009). However, two populations of adult Spg determined, based on co-localization of molecular phenotype into two classes, that the mitotically quiescent of A_{dark} Spg represented SALL4+/PLZF+/LIN28+/DPPA4+/DAZL+, while A_{pale} Spg demonstrated mitotic proliferation

proliferation marker of DAZL+/c-Kit+/KI67+ (Lin et al., 2015).

SSEA4, TRA-1-81, TRA-1-60 are the markers that were observed on Spg in rhesus monkey, however it is only a small proportion of immunopositive cells that were detected on testicular tissue, suggesting that the former markers maybe more specific for spermatogenic cells (Sharma et al., 2017). In addition, Thy-1, GFRα, PLZF, NGN3 and c-Kit were observed in juvenile and adult rhesus testicular tissue, co-staining of these molecular markers indicated the presence of Spg type A subpopulations (Hermann et al., 2009).

4) Post mitotic or meiotic spermatogenic marker

Piwi-interacting RNAs (piRNAs) are small RNA molecules required for the regulation of gene expression at the transcriptional process. The Piwi family gene including Piwi, Hiwi, Miwi, Miwi2 and Mili, have shown to play crucial roles in stem cells division and gametogenesis and their expression are detected in mammalian testes during spermatogenesis (Bak et al., 2011). Piwi being localized to the nucleus after PGCs formation which was expressed in both germline and somatic cells of male and female (Cox et al., 1998). At early state of embryo development, Piwi is present in cytoplasm which is important for establishment of germ cells lineage (Lin and Spradling, 1997; Cox et al., 1998; Megosh et al., 2006). During meiotic phase of spermatogenesis, Miwi or Piwil1 (a homolog of Piwi) is presented as a testis-specific gene that demonstrated specifically in spermatocytes and round spermatids (Deng and Lin, 2002; S Tushir et al., 2009; Thomson and Lin, 2009; Bak et al., 2011). The expression of Piwi1 is first detected at 14 days post protium, a state that related to the appearance of pachytene spermatocytes. In adult testis, Piwil1 starts to express in midpachytene state spermatocytes to round spermatid during stage V-VII of the cycle of seminiferous epithelium (Russell et al., 1993). In mice, immunostaining shows more abundance of Piwil1+ cells in diplotene spermatocytes during stage XI throughout stage XII and in early round spermatids, which had reduced abundantly, however it is still detectable

up to step 13 (XIII) of elongating spermatids (Deng and Lin, 2002). Suggesting that the Piwi proteins family is necessary for spermatogenesis, spermiogenesis and male germline stem cells maintenance.

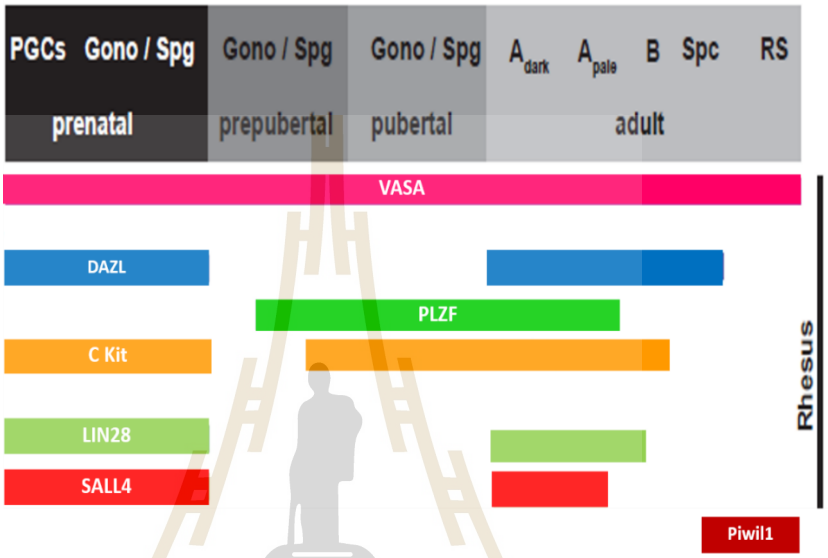

Figure 2.5 Expression profile during germ cells developmental stages in rhesus monkey is presented by colored bars. Prenatally expressed markers constitute PGCs, Gonocytes (Gono) or Prospermatogonia. Spermatogonia stem cells further mature into more differentiated cells including differentiating type B spermatogonia (B), spermatocytes (Spc), round (RS) as well as elongated spermatids, and finally into spermatozoa (Modified from; Sharma et al, 2017).

2.2 *In vitro* spermatogenesis from pluripotent stem cells

Spermatogenesis represents a dynamic series of SSCs proliferating to terminal maturate spermatozoa in a complex structure of seminiferous tubule and testicular somatic cells. Regarding the rare population of *in vivo* male germ cells, for example adult rodents represent very rare germ cells consisting about 0.03% in testis (Tegelenbosch and de Rooij, 1993) or although more germ cells production (22%) has been observed in higher primates, the sperm output per day is lower than mice around 16-fold and 128-fold in monkey and human respectively (Fayomi and Orwig, 2018). Thus, enrichment of SSCs or mature germ cells will

facilitate germ cells biological research and fertility preservation in declining of sperm counts of current male factor infertility issues worldwide (Jørgensen et al., 2006; Mascarenhas et al., 2012; Ibtisham et al., 2017; Nosrati et al., 2017). Induction of male germ cells development *in vitro* outside of SSCs niche is essential to understand biological events of spermatogenesis whether the SSCs can form and expansion outside the body. Various techniques of *in vitro* spermatogenesis are reports in the terms of organs, tissue, as well as stem cells culture, which provides useful materials for study of germ cell biology, toxicology, fertility preservation, generation of transgenic germ cells and therapeutic potential for male infertility (Dissanayake, 2018). Stem cells based *in vitro* culture system of SSCs have been investigated in different studies. Isolation of SSC from testicular tissue and induction into mature haploid germ cells demonstrate the potential model of *ex vivo* germ cells production. Dissociation of testicular tissue and culture *in vitro* has first shown in rodent using serum free, hormone and growth factor supplement medium results to more maturing cells production (Tres and Kierszenbaum, 1983). Derivation of mouse haploid spermatid form *in vitro* testicular cell culture was subsequently reported and improved efficiency of *in vitro* culture has been shown by varying techniques (Ibtisham et al., 2017). In higher primate, prolonged *in vitro* culture of human Spgs was first reported in 2009 from orchidectomy of prostate cancer men and revealed the potential model for restoration of fertility in chemotherapy patient (Sadri-Ardekani et al., 2009). However, in the case of lack of germ cells formation such as azoospermia patients (Chiba et al., 2016), testicular biopsy and SSCs culture would not be a benefit for germ cells production *in vitro*. A novel PSCs-based approach including ESCs and iPSCs to create male germ cells and functional gamete has become of interest to study germ cells biology of spermatogenesis as well as to apply in clinical research. Generation of male gametes from PSCs would further allow patients with no germ cells to be father genetically related children (Valli et al., 2014). The technique will cover patient specific germ cells production under disease condition that destroys testicular

SSCs. First reports of *in vitro* male germ cells establishing from PSCs was presented in mESCs (Toyooka et al., 2003) by genetically knock in endogenous mouse VASA homolog (MVH) of differentiating germ cells, formation of embryoid body expressed MVH positive and enhanced expression by BMP4. Toyooka study participated in spermatogenesis after transplantation of germ cells derived into testicular tubule and germ cell reconstruction, however functional germ cell fertilization has not been reported. Subsequently, PGCs were isolated from embryoid bodies derived mESCs (Geijsen et al., 2004) and continuously grew lines of embryonic germ cells to haploid male gametes as demonstrative of the embryos development after injection into oocytes. *In vitro* induction of mPSCs derived male gametes have been proven over time to generated functional haploid germ cells (Nayernia et al., 2006), as the breakthrough reports producing healthy offspring from mESC derived spermatid revealing that *in vitro* male gametogenesis from mPSCs shows the potential model of functional germ cells production (Zhou et al., 2016).

Investigating the details in particular steps of spermatogenesis between primate and mice, for examples, Spgs and SSCs pools and kinetic of germ cells proliferation resulting in the huge difference of spermatozoa production between two species (Hermann et al., 2010; Behr, 2017; Zhao et al., 2018). Studies built on the success of functional spermatid production in mice, thus accepting the challenge to translate these small animal models to primates, especially in human research. Human *in vitro* PGCs production from hESCs has being reported since 2006 (Kee et al., 2006), BMPs promote the expression of germ cell-specific makers including VASA and SYCP3 in the differentiating embryoid bodies. In 2009, Park group demonstrated *in vitro* PGCs derived from hPSCs by co culture with fetal gonadal stromal cells correspond to the first trimester germ cells formation, which have initiated the imprinting process (Park et al., 2009). In the same year, Kee group (Kee et al., 2009) genetically modified germ cells DAZL reporter to isolate PGCs derived from both male and female hESCs, suggesting human DAZL modulated human germ cell formation.

Taken together, induction of adults and fetal somatic reprogramed cells into PGCs demonstrated a germ cells-specific reporter, specific protein and mRNA. Moreover, they observed meiotic haloid Acrosin positive cells as similar pattern of human spermatid staining (Panula et al., 2011). In 2012, haploid round spermatid like cells directly derived from a 10 days *in vitro* differentiation of male hPSCs without germ cells formation and genetic manipulation, as the results showed specific SSCs were generated in each state during spermatogenesis, suggesting male PSCs have the ability to differentiate directly into advanced germ cells lineages *in vitro* (Easley IV et al., 2012). Recently, Zhao group developed a feeder-free and xeno-free (animal free supplement cocktails) culture system induced hiPSCs into Spg like cells which observed that a small fraction went through meiosis and developed into haploid germ cells, suggesting an experimental platform to investigate human germ cells development in advance research (Zhao et al., 2018). The revolutionary and exciting results of human germ cells progression from PSCs follow scientists' interest in using the resulted germ cells in clinical and reproductive research. However, in order to generate a reliable model to apply to *in vitro* derived male germ cells from PSCs in the field of patient fertility preserve or human clinical trial, there must be time to get through the downstream process of SSCs characterization such as analysis functional SSCs to fertilize with oocytes, study development of resulting embryos, possibility to establish a pregnancy and giving birth. Inability to test germ cells functioning of the production of germ cells in human system must focus on the problems from an ethical perspective (Condic and Rao, 2010; Valli et al., 2014; Rombaut et al., 2017). Thus, safety and feasibility of using *in vitro* germ cells for advanced human research needs to be first established in close relationship with animal such as a nonhuman primate.

Non-human primate PSCs (NHP-PSCs) induction into SSCs has progressed in research since 2007. First, *in vitro* derivation of primate PGCs are isolated from spontaneous differentiation of cynomologus monkey ESCs via EB formation, producing germ cells that

correspond to early gonocytes at the postmigration stage which demonstrated the expression of VASA specific germ cells throughout differentiation time as well as showing some meiosis specific gene expression (Teramura et al., 2007). Subsequently, work from Yamauchi group reported that VASA is a suitable marker for detection of germ cells differentiation from cynomologus monkey ESCs, VASA protein as well as mRNA expression were upregulated in differentiated EB throughout induction period by using mouse gonadal cell-conditional medium and growth factor of which no ESCs were expressed VASA (Yamauchi et al., 2009). Study in NHP holds great potential for research, highlighting an alternative source of SSCs *in vitro* production from higher primate and unlimited application for future investigate of SSCs model. However, the practical maintenance to handle monkey ESCs is complicated in undifferentiated state and to regulate differentiation into desired functional cells, which is the critical challenge for NHP large-scale experiment (Ono et al., 2014). Thus, it may be the reason why only few studies have access to monkey germ cells production from PSCs *in vitro* (Table 2.1). Nine years later, a recent study generated rhesus macaque primordial germ like cells (rPGCLCs) *in vitro* from rhesus monkey iPSCs (riPSCs). Induction of rPGCLCs was carried out through a two-step differentiation protocol by forming of mesoderm like cells (iMeLCs) following with a three-dimensional aggregate germ cell culture. Resulting rPGCLCs from riPSCs *in vitro* differentiation corresponded to bona fide embryonic rPGCs younger than 28 days of embryo development post-fertilization, based on immunofluorescence characteristic. Moreover, xenotransplantation (monkey to mouse) and homologous transplantation (monkey to monkey) revealed that rPGCLCs have ability to colonize and differentiate into further mature spermatogenic cells *in vivo*, suggesting that seminiferous tubule environment support promotes the maturation of rPGCLC derived *in vitro* differentiation (Sosa et al., 2018). Generating of PGCLCs *in vitro* followed by *in vivo* maturation would be beneficial in avoidance of teratoma formation rather than using mature *in vitro* SSCs in transplantation,

which can be applied in cases of fertility treatment in chemotherapy patient. Whereas, in field of assistant reproductive technology (ART) generating of more mature spermatogenic cells *in vitro* would provide good results of *in vitro* fertilization with oocytes and generating embryos. In addition, downstream process of *in vitro* derived male germ cells in NHP need to have extensive further investigation in order to ensure the highest safety standards for human clinical scale (Rombaut et al., 2017). For example, the producing embryos should be screened for genetic, epigenetic and transcriptomic profiles for the possibility of healthy offspring production. Beside the fertility function, the *in vitro* spermatogenesis model in nonhuman primate represents a valuable model to study human genetic defects disorders that affects germ cells biology and testicular germ cell developments (Pearson, 2003) which are limited or unable to studies in some case of male patient. Thus, highlighting that evolution of *in vitro* germ cells production not only presents the concept to restore male fertility, but also provides useful material for a therapeutic strategy of human disease.

Table 2.1 *In vitro* germ cells production form primate PSCs.

Sources	Method	Germ cells production	Results Expression markers				Outcomes	References
			Germ cells	Mitotic	Meiotic	Haploid		
hESCs	EBs formation +BMP4/+BMP7 and BMP8b	hPGCs	+ = VASA		+ = SYCP3		BMPs increases differentiation of human germ cells from hESCs	Keejoanna et al., 2007
hESCs hiPSCs	co-differentiation of hPSCs on human fetal gonadal stromal cells	*in vitro* derived PGCs (iPGCs)	+ = VASA, SSEA1/cKIT/PLAP				iPGCs derived from PSCs correspond to committed first trimester germ cells (before 9 weeks)	Park et al., 2009
hESCs (XX), hESCs (XY)	used a germ-cell reporter to quantify and isolate PGCs derived from both male and female hESCs	hPGCs progressed to haploid gamete	+ = DAZL		+ = DAZ and BOULE		- Human DAZL function in PGC formation - DAZ and BOULE promote later stage of meiosis which developed to haploid cells	Kee et al.,2009
Adult iPSCs, Fetal iPSCs	used germ cells reporter, induction with (BMP)	hPGCs, meiotic germ cells and post-meiotic haploid cells	+ = VASA, DAZL, IFITM1, PELOTA, PRDM1AS		+ = SCP3, CENP-A	+ = Acrosin	Adult iPSCs can form germline cells	Panula et al., 2010
hESCs (XX), hiPSCs (XX)	Direct differentiation into SSCs, supplemented GDNF, bFGF	Spg, Spermatocytes, Haploid spermatid like cells		+ = UTF1, PLZF, CDH1	+ = HIWI, HILI	+ = Acrosin, TNP1, Protamine1	male PSCs have the ability to differentiate directly into advanced germ cell lineages	Easley IV et al., 2012

Table 2.1 (Continued).

Sources	Method	Germ cells production	Results Expression markers				Outcomes	References
			Germ cells	Mitotic	Meiotic	Haploid		
hESCs, hiPSCs	A feeder-free and xeno-free induction system + vitamin C	Spermatogonium-like cells (SLCs) to meiosis and haploid	+ = MVH, DAZL, GFRα1, NANOs3	+ = PLZF, GPR125, CD90	+ = Acrosin, PRM1, TNP1, SPATA19		*In vitro* platform to investigate human germ cell development and pathology related to male infertility	Zhao et al., 2018
Cynomolgus monkey ESCs (CyESCs)	*In vitro* differentiation via EBs	PGCs derived corresponded to early gonocytes at the postmigration stage	+ = VASA				cyESCs can differentiate to developing germ cells such as PGCs or more developed cells as GCs	Teramura et al., 2007
CyESCs	EB formation with mouse gonadal cell-conditioned medium+ BMP4	PGCs	+ = VASA, SSEA1, NANOS, PIWIL1		+ = SCP1		VASA is a valuable gene for the detection of germ cells differentiated from monkey ESCs	Yamauchi et al., 2009
Rhesus monkey iPSCs	-Two step differentiations; 1) forming iMeLCs, 2) three-dimensional aggregate germ cell culture -Xeno-and Homologous-transplantation	rhesus macaque primordial germ like cells (rPGCLCs) developed to SSCs after transplantation	+ = VASA after transplantation	+ = MAGEA4, VASA, putative prospermatogonia			Adult gonadal niche promoted immature rPGCLCs differentiated toward late rPGCs that initiate epigenetic reprogramming process	Sosa et al., 2018

2.3 Huntington disease

Huntington disease (HD) was first described medically by George Huntington in 1872. HD is an autosomal dominant neurodegenerative disorder affecting an average of 5.70 individuals per 100,000 in European, North American and Australia populations (Pringsheim et al., 2012), while ten times less prevalence of 0.40-0.52 per 100,000 was observed in Asian populations (Pringsheim et al., 2012; Baig et al., 2016). Clinical features of HD referred to chorea as the involuntary dancing movement which can further progress into slow movements with rigidity and dystonia of bradykinesia, cognitive impairment, neuropsychiatric deficits including depression and apathy and causing death after 15-20 years of onset (Déglon, 2017). HD is lethal and serious, in juvenile cases about 4.92% were referred at age of onset below 20 years, and it contributed to more severe clinical characteristics in rigidity, dystonia and bradykinesia rather than chorea (Quarrell et al., 2012). Clinical phenotype of early onset is progressive twice as rapid as in adult form, whose demonstration of progressive cerebellar symptoms include ataxia, cerebellar and cortical atrophy, with progression to death within two years after diagnosis (Latimer et al., 2017). Genetical phenotype of HD is defined as the presentation of trinucleotide repeats (TNR) mutation including Cytosine Adenine Guanine (CAG) that encodes for Glutamic acid or Huntington (Htt) protein. HD mutation is presented in Exon1 of human *Huntington* (*HTT*) gene (IT15), which is located on the short (p) arm of chromosome 4 at position 16.3 (Chr4p16.3), resulting in classified HD subtypes into wildtype HD or non-HD (9-35 CAG), intermediate or lower penetrance (36-39 CAG), onset of HD (> 40 CAG). Adults HD presents its clinical onset from 40 repeat size, whereas juvenile is more than 75 CAGs (Saudou and Humbert, 2016), presenting that CAG repeat size and severity of disease inversely proportional with age at onset. Taken together, homozygous patients represented more severe disease progression than heterozygous, even though the same age of onset (Lee et al., 2012).

HD pathogenesis is presented by the accumulation of mutant HTT protein resulting from CAG mutation as described above. The misfolding expanded polyglutamine (Poly Q) stretch close to N-terminus of the HTT protein leads to protein aggregate and accumulates in nuclear or even cytosolic region which is referred to as inclusion body (Nicole, 2017) in the cells, resulting in cellular disfunction and toxicity to the given cells or affecting tissue. HTT is a ubiquitous protein, low expression of mRNA is observed in early development and become higher in adults CNS neuron and testis (Strong et al., 1993), as the highest level of HTT have been seen mainly in several brain regions including cerebellum, hippocampus, cerebral cortex, substantia nigra and pontine nuclei (Landwehrmeyer et al., 1995).

2.3.1 HD pathogenic mechanisms

Underlying mechanisms of HD are not fully understood, but HTT is an organelle-associated scaffolding protein which plays a role in various biological intracellular pathway (Nicole, 2017) for example, the traffics transport vesicles in axon and dendrites of neuron by dynein/dynactin/HAP1-dependent transport pathway, coordinates cell division through spindle poles orientation, regulates ciliogenesis, mediates endocytosis, vesicle recycling and endosomal trafficking and is represented as a autophagy-related protein (Saudou and Humbert, 2016). The wild type (WT) HTT is subjected to proteolysis at several sites by proteases. HTT proteolytic activities allow the production of several N-terminal fragments which supports normal biological function. Importantly, increase of protease activity is specifically found in the brain of HD patients, and enhancing of proteolysis in HD leads to small N-terminal fragments with contained poly Q stretch insoluble, accumulation, translocate into the nucleus and developing apoptosis and cell death by interfering the transcription (Saudou and Humbert, 2016). HTT proteolysis also generates the toxicity C-terminal fragments by dysregulating the activity of dynamin, thus loss of HTT function in HD is involved in the molecular pathway link to neuronal dysfunction, including mitochondrial dysfunction, transcription dysregulation, impairment of protein homeostasis

and degradation, and numerous activation of stress response (Labbadia and Morimoto, 2013).

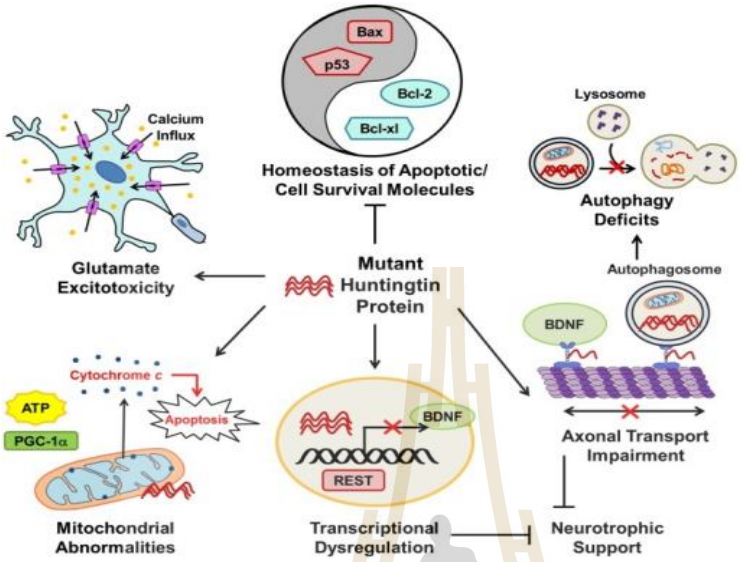

Figure 2.6 Illustration of major pathophysiological process in Huntington's disease (HD). Mutant huntingtin (mHtt) protein disrupts many normal physiological processes and leads to unbalanced homeostasis of apoptotic molecules, deficits in autophagy, axonal transport impairment, transcriptional dysregulation, reduced cellular neurotrophic support, mitochondrial abnormalities and glutamate excitotoxicity (Scheuing et al., 2014).

2.3.2 Effect of gender on *HTT* mutation

One influence factor effecting mutation of *HTT* was reported through the gender and germline dependent. TNR mutation can undergo dynamic change in which the alteration of CAG repeats number (instability) can occur when a gene is transmitted from parent to offspring. CAG instability in HD represented both expansion and contraction repeats in both sexes. Males predominantly transmit the expansion repeats from father to son as its referred to as **paternal-of-origin CAG repeats expansion**, whereas female predominantly transmits contract repeats (Leeflang et al., 1999; Kovtun et al., 2000). In 1993, study from Goldberg and his group revealed that CAG expansion of HD has shown to occur only during transmission through the male germline by observing existence of intermediate range (30-

38 CAG) and meiotically unstable in parental alleles in sporadic case of HD compared to normal population (Goldberg et al., 1993). Taken together, subsequent study demonstrated that the progression of HD CAG mutation from a pre-mutation length (29-35CAG) to disease range (>35 CAG) and the large expansion of more than seven repeats are observed almost specifically in paternal transmission (Kremer et al., 1995; Norremolle et al., 1995; Wheeler et al., 2007). In male, premutation allele can increase small repeat size or losses over a series of generations, but after allele inherited to HD disease allele, the expansion is favored three to 175-fold over contraction (McMurray, 2010). CAG instability in HD can occur both during post fertilization in dividing cells and throughout adulthood with contributions to somatic instability. The study from Kovtun group in mice showed the same mutation concepts with human, as the CAG repeats expansion was dominantly inherited through male progeny, whereas female progeny showed contraction repeat even if HD allele is inherited from same father. Moreover, the study showed that CAG mutation in HD can is influenced by X- or Y-encoded factors of DNA repair or replication process in embryos, thus gender of embryo contributes to CAG expansion, supporting that paternal bias CAG expansions appear to arise during early state of embryogenesis (Kovtun et al., 2000). However, expansion repeat length during transmission in mice is not the result of sperm selection and independent from germ cells of founding father, since both contraction and expansion are presented in progeny of identical father, thus there are no selections that occur during gametogenesis as the contracted and expanded alleles exist in sperm population (Kovtun et al., 2000). In 2013, report of cellular pathogenic of CAG repeat mutation in NHP model by *in vitro* fertilization of transgenic monkey sperm to oocyte and establishment of ESCs, similar results to human and mice, a rhesus monkey transmitted HD disease allele from same transgenic founding sperm to both XY and XX ESCs, but CAG expansion is predominantly presented through the derived male ESCs (XY). Representing that the germline transmissible in rhesus monkey HD can occur during embryo development and suggested HD NHP model for further step

of biomarkers development for HD (Putkhao et al., 2012). In addition, paternal bias mutation is critical in TNR disease, which not only presents in HD, but also others disease, including spinocerebellar ataxia type 1 (SCA1) (Koefoed et al., 1998) SMBA (Xiao et al., 2012) and dentaturbral-pallidoluysian (DRPLA) (Sato et al., 1999). However, underlying mechanism of CAG instability during germline transmission currently has not been well understood, thus it is important to have more detail and better understanding of CAG mutation during male germline development for further therapeutic strategy of HD.

2.3.3 CAG repeats during spermatogenesis

As described above, mutation in HD most occurred in males than females. As the reports of paternal-of-origin-CAG repeat expansion have shown in sperm, embryogenesis or rESCs derived from transgenic embryo leads to more focusing study of pathogenic HD on male gametogenesis including both testicular gem cells during spermatogenesis and mature functional germ cell in sperm. In 2007, Raamsdonk and colleagues revealed testicular degeneration and progressive loss of spermatogenic cells have been observed in HD patient, supporting that *HTT* influence on males and impacts on spermatogenesis in human (Van Raamsdonk et al., 2007). Taken together, recent study suggests that *HTT* is a spermatogenic gene, disrupts normal function of WT *HTT* that results in pathogenesis in testes, including testicular degeneration and abnormal germ cells progression which leads to infertility in mice (Yan et al., 2016). Underlying mechanism of CAG repeat mutation during stage of HD gametogenesis (germ cells progresses to mature sperm) is crucial to understanding this cellular pathogenesis of the mutation and its transmission in affected families. Mutation time point during HD male line development is not clear, but it seems to occur in multiple stages. Notably, CAG instability in HD in both human and mice does not correlate with age of father, which expecting that if mutation occurred in Spg would be impacts to renewing germ cells throughout adulthood (McMurray, 2010).

Early study in mice testicular germ cells demonstrated that CAG mutation is most restricted to post meiotic haploid spermatid or spermatozoa (Kovtun et al., 2001). In contrast, a study of testicular germ cells in HD patients demonstrates TNR expansion mutation presents during mitotic division of spermatogonia (Leeflang et al., 1999; Yoon et al., 2003) as well as before meiotic (dividing Spg) and continues until after meiosis is complete (Spermatocytes to Spermatozoa) and fewer disease length expansion has been found in post meiotic spermatid or spermatozoa, suggesting that CAG repeat expansion are not only restricted to late state of mature germ cells but also presented earlier in spermatogenic dividing germ cells (Yoon et al., 2003). This shows that resulting inter-species differences between mouse and human distinguish pathogenic cellular phenotypes on HD spermatogenesis between two species. For examples, humans have longer divisions steps of spermatogenesis; therefore, increased chances of mutation during SSCs expansion more than mice as well as the difference of DNA metabolism during spermatogenesis between two species, revealing the challenge to assess translated pathogenic CAGs expansion in mouse model to human (Pearson, 2003). Therefore, study of HD CAG mutation during male germ cells progression in larger animal model especially non-human primate would provide valuable results, which can translate to HD patient gametogenesis. Our group previously reports transgenic HD of NHP model shares human HD pathogenic phenotype (Yang et al., 2008) and manifests cellular pathogenic phenotype of HD on male germ line transmission (Putkhao et al., 2013), suggesting potential model for further biomarker development for HD.

2.4 Rhesus monkeys and transgenic HD model

Rhesus macaques (Macaca mulata) are the old-world monkey that have been considered the best animal model for a new therapy in many diseases, especially complex disorders that correlate with aging, cognitive behavior, mental development, psychiatric dysfunction, biomedical research including assistant reproductive technologies and vaccines development

showed more detail in previous review (Chan, 2013). Close genetics, physiology, neuronal as well as biological development of rhesus to human leads undoubtedly to use of a rhesus as the valuable NHP model for basic and applied biomedical research in human. Thus, rhesus macaques are the most frequently displayed and thoroughly studied of all NHP (Gibbs et al., 2007). Rhesus shares a last common ancestor with human from about 25 million years ago, and the genome of rhesus has diverged farther from humans with an average identity around 93% of human-macaque sequence. Two species have shown close living association and rhesus exhibit complex social behavioral repertoires, which have broadly geographic distribution from Afghanistan, India across Asia to the Chinese shore of Pacific Ocean (Gibbs et al., 2007).

Establishment of transgenic NHP model has become an important model of human genetic disorder for basic research and translation in preclinical studies. Recapitulation of human disease conditions thoroughly transgenic NHP provided basic and advance knowledges in physiological, genetical, pathological process as well as guiding to new therapeutics for human disease. The first transgenic NHP was reported in 2001, using moloney retroviral vector that carried the GFP gene under the control of either the cytomegalovirus early promoter (CMV) or of the human elongation factor-1 alpha promoter, creating a transgenic ANDi rhesus monkey which genetical and phenotypical GFP transgene were represented. The outcome from this study provided the ideal model to bridge the scientific gap between human and transgenic mice (Chan et al., 2001). Seven years later, generation of first transgenic Huntington rhesus monkeys (rHD) opened a feasible way to generate valuable NHP model of human genetic disease, raising up the model for better understanding of HD biology and therapeutic development of this irreversible damaging disease (Yang et al., 2008). The transgenic rHD monkey shared physiological, pathological or clinical phenotypes with HD patient, as well genetically characteristic with 27-88 CAG repeats in exon1 of *HTT* transgene, suggesting the potential model of human HD.

Subsequence studies involving HD mechanism from transgenic rHD PSCs including establishment of rHD iPSCs and successful establishment of rHD ESCs, provide better understanding of HD mutation and showing potential applications of stem cells-based studies of HD biology which will provide the possibility to use the unlimited source of PSCs induction for further advanced research (Chan et al., 2010; Putkhao et al., 2013). Importantly, showing potential applications of stem cells-based therapy in regenerative medicine must be pioneered in NHP model prior to or in parallel with human scale research for both scientific and ethical reasons (Mitalipov et al., 2006), thus using rPSCs or transgenic rPSCs for human clinical purposes will benefit in advanced study of *in vitro* and *in vivo* experiments, which will be underlying human developmental biology that could will then be accessed in human scale.

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CHAPTER III

IN VITRO SPERMATOGENESIS OF RHESUS MONKEY PLURIPOTENT STEM CELLS

3.1 Abstract

In the past decade, decline in sperm quality, specifically sperm count. has been the major challenge in male infertility. *In vitro* production of spermatogenic cells (SSCs) from pluripotent stem cells (PSCs) in mice has suggested the possibility to produce functional sperm by *in vitro* fertilization. However, human *in vitro* derived spermatid has not been functionally assessed and will be ethically challenging to produce offspring for ultimate proof due to ethical concerns. Thus, assessment in nonhuman primates (NHP) that are closely related to humans genetically and physiological will be important toward clinical translation of technology. Here, we report our progress in generating rhesus monkey male germ cells from pluripotent stem cells. A 10 days direct differentiation generate SSCs and haploid spermatids based on the expression of cell type specific markers. Differentiated cells express SALL4+ and PLZF+, markers of PSCs and spermatogonia stem cells (Spgs) respectively. Gene expression analysis demonstrates male germ cell progression toward spermiogenesis. VASA, the prominent germ cell marker, increases slightly throughout the course on *in vitro* differentiation. The expression of PLZF peaks on Day 5 with reduced expression as differentiation progresses *in vitro*. Down regulation of SALL4 and c-kit as differentiation progresses further suggest the development toward spermatogenic lineage. taken together, Piwill is a specific marker of more mature SSCs in spermatocyte and a round spermatid, elevated expression until Day-10. Moreover, FACS analysis demonstrates half of the DNA

content population (haploid cells) and the expression of round spermatid markers, TNP1, PRM1 and Acrosin. The present study demonstrates the generation of SSCs from rhesus PSCs based on the expression of cell types specific markers. Our *in vitro* platform for producing male germ cells from PSCs provides a new model system for investigating male germ cells biology and developmental model of alternative treatments for male infertility.

Keywords: Spermatogenic cells, Spermatogenesis, *In vitro* differentiation, Pluripotent Stem Cells

3.2 Introduction

Infertility is now a progressive global health issue affecting the couples worldwide. Forty to 50% of infertility cases are caused by male factors infertility (Ibtisham et al., 2017). The current global phenomenon of reduced sperm counts and quality has been reported over the past decade (Jørgensen et al., 2006; Mascarenhas et al., 2012, Levine et al., 2017, Sengupta et al., 2018). Sperm counts in both fertile and infertile men were reduced by 1.5% per year in the USA and by 3% per year in Europe and Australia (Nosrati et al., 2017). A wide range of factors has been reported that induce infertility in man (Sharpe, 2010; Dada et al., 2012; Durairajanayagam, 2018; Krausz and Riera-Escamilla, 2018), and the effect of male infertility is evident not only on pregnancy, but also affects the health progression of the progeny such as genetic disorder of autism, childhood cancers and neuropsychiatric disorders (Bisht et al., 2017). Because of this current urgent issue worldwide, researchers in reproductive biotechnology are searching for the causes, trying to have better understanding of male germ cells progression and provide the alternative source of male germ cells for further preservation of male fertility.

Nonhuman primates (NHPs), especially rhesus macaques (*Macaca mulatta*) are valuable animal models that are used in basic and applied biomedical research because they show a close relationship and a similarity of genetic and physiological development to the human. Thus, rhesus represents the most valuable biomedical study as a model of human

research (Gibbs et al., 2007). rPSCs are unlimited source for functional cells induction and not affected by bioethical concerns in an advancing study (Mitalipova et al., 2003). Using rPSCs as a longitudinal tool in human research would be useful material in a pre-clinical scale study.

Spermatogenesis is highly organized structure *in vivo* that produces functional spermatozoa supporting male fertility throughout adulthood. Association of SSCs niches inside testicular tissue promote germ cells progression and achieve maturation. *In vitro* spermatogenesis demonstrates a powerful tool and a potential model to study the mechanism of male germ cells developments with a more simplified and less organized system. Evolution of *in vitro* SSCs culture technique has been developed including organ, testicular tissues isolation and stem cell culture (Ibtisham et al., 2017). Moreover, stem cells-based SSC production has recent achievement in rodent as the functional SSCs improvement indicated by producing healthy offspring (Zhou et al., 2016). Other studies have revealed male germ cells production from monkey PSCs (Yamauchi et al., 2009; Sosa et al., 2018) and human (Easley IV et al., 2012; Zhao et al., 2018). However, proving potential realizable techniques of SSC derived *in vitro* needs to show functional investigation as well as downstream characterization in higher primates, which remains an ethical challenge in humans. Thus, induction of NHP into more advanced male germ cells would be a suitable model for studying the possibility of functional sperm derived *in vitro* and will be useful material for study of germ cells biology and the fertility preservation.

3.3 Materials and methods

3.3.1 Feeder cells preparation

A small fetus was separated from a pregnant mouse 13-days post coitum. Head, limbs and internal organs were removed and tissues were washed several times in PBS to remove the blood. Tissues were minced into small pieces and incubated with Trypsin/EDTA at 37°C for 15 min by gentle shaking. Absorbed tissues were suspended in culture medium

to stop trypsin activity, then transferred to 50 ml conical tube and leftover tissues settled down to the bottom for a few minutes. Upper layer supernatant was collected and centrifuged at 1,000 rpm for five minutes. Cell pellets were suspended in culture medium containing DMEM (Invitrogen) supplemented with 10% FBS (FBS; Hyclone), 200 mM L-glutamine (Invitrogen) and 1x Penicillin/Steptomycin (Invitrogen) and cultured at 37°C under humidified atmosphere of 5% CO_2 in air. Medium was changed every two days and cells were sub-cultured when reaching 80% confluence by trypsinization. To prepare feeder for rPSCs culture, 80% confluence of mouse embryonic fibroblasts (MEFs) were inactivated with 10 µg/ml mitomycin C (Sigma) for three hours before trypsinization and seeding the cells at 4×10^5 per plate. Good feeder products were used at 48 hours after preparation.

3.3.2 Rhesus monkey pluripotent stem cells culture

Rhesus monkey embryonic stem cells (rESCs) were cultured and maintained on fresh MEF feeders using rESCs culture medium (composed of Knockout DMEM (gibco), 20% Knockout serum replacement, 200mM L-Glutamine and 100x NEAA supplemented with 4ng/ml rbFGF) at 37°C under humidified atmosphere of 5% CO_2 in air. Medium was changed every two days, and the cells were mechanical passaged using capillary hook needle every three to four days into new MEF feeders. Growing colonies around 70-80% confluence was passaged into STO.LIF (Sandos inbred mouse [SIM]-derived 6-thioguanine- and ouabain-resistant with leukemia inhibitory factor) feeders. rESCs were maintained on STO.LIF feeders for two days before the differentiation process start.

3.3.3 Direct differentiation of rPSCs into rSSCs

rESCs were directly differentiated into rhesus monkey spermatogenic cells (rSSCs) using conditioned medium as described in previous protocol (Easley et al., 2012). Briefly, after two days culture on STO.LIF, rESCs culture medium was changed into differentiation medium composed of MEM alpha (Invitrogen), 0.2% Bovine Serum Albumin (MP Biomedical), 5 µg/ml insulin (Sigma), 10 µg/ml transferrin (Sigma), 60 µM putrescine

(Sigma), 2 mM L-glutamine (Invitrogen), 50 µM β-mercaptoethanol (Sigma), 1 ng/ml hbFGF (human basic fibroblast growth factor, PeproTech), 20 ng/ml GDNF (glial-derived neurotrophic factor, PeproTech), 30 nM sodium selenite (Sigma), 2.36 µM palmitic acid (Sigma), 0.21 µM palmitoleic acid (Sigma), 0.88 µM stearic acid (Sigma), 1.02 µM oleic acid (Sigma), 2.71 µM linoleic acid (Sigma), 0.43 µM linolenic acid (Sigma), 10 mM HEPES (Lonza) and 0.5X penicillin/streptomycin (Invitrogen). Differentiation medium was changed every two days and the cells were cultured for 10 days before characterization. Pellets of differentiated cells were collected at different time points for molecular phenotypes expression. Changing of cell morphology was recorded using Olympus bright field microscope.

3.3.4 Immunofluorescent staining of SSC marker

rSSC medium was removed from culture and differentiated cells were washed with phosphate-buffered Saline Solution (PBS) without Ca^{2+} and Mg^{2} several times before fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Fixed cells were blocked with blocking solution containing 1X PBS (Lonza), 0.20% Triton X-100 (Sigma), 2% bovine serum albumin (BSA) (Sigma), 3mM Sodium azide (Sigma), 0.1% Saponin (Sigma) and 5% normal goat serum (NGS) or donkey serum (Sigma) at 4°C overnight. The primary antibody was diluted with blocking buffer at appropriate concentration; PLZF 10ug/ml (R&D), SALL4 1:200, Acrosin 1:200, Protamine1 1:200 and TNP1 1:200 (Santa Cruz Biotechnology) and incubated overnight at 4°C. Secondary antibody was used at 1:1000 dilution with PBS and incubated for 1 hr. at room temperature. DNA of the cell was counterstained with Hoechst for 10 minutes before imaging. For round spermatid marker, cover slide was coated with 0.01% poly L lysine (Sigma) for 15 minutes before seeding the single cells suspension. Acrosin, Protamine1 and TNP1 were incubated overnight before stained as described above.

3.3.5 Immunohistochemistry of testis tissue

Small pieces of adult testis tissues were embedded in optimum cutting temperature solution (Tissue-Tek® O.C.T. Compound, Sakura® Finetek). Cryosection tissues at 30µm were plated on slide and permeabilized with 1% Sodium borohydride (Sigma). The section was fixed with 10% methanol in 0.3% hydrogen peroxide (Sigma) before blocked with 10% normal serum, 1% BSA (sigma), 0.3% Triton X-100 (Sigma) solution. Blocking tissues were stained following ICC method as described above.

3.3.6 Spermatogenic cells genes expression analysis by Quantitative Real Time PCR (qRT PCR)

rESCs (undifferentiated control group) and differentiated cells at Day 3, Day 5, Day 7 and Day 10 were collected for gene expression analysis. RNA was isolated using Trizol method, and 500 ng of each sample was reverse transcribed to cDNA. Quantitative mRNA expression in each stage of spermatogenesis was measured using single tube commercial TaqMan gene expression assays. Different gene markers were used; Oct4 (Rh028430745_gl) for pluripotent state, VASA (Rh02798028_m1) for germ cell, PLZF (Rh02834704_m1) for Spgs and SALL4 (Rh01010838_g1) for ESCs to Spg, C-kit (Hs001474029_m1) for differentiating Spg to spermatocyte and Piwil1 (Rh04256262_m1) for spermatocytes to round spermatid state. 1X final reaction was prepared by Tagman Gene Expression Master Mix (Applied Biosystems). The experiment was run using Biorald qPCR machine, and results were first normalized with glyceraldehyde-3-phosphate dehydrogenase GAPDH endogenous control (Rh02621745_g1) and then normalized with the control of highest expression in each SSC state (ΔΔCT method). Each data set came from the average of three samples which were collected from different batches of differentiation, and each experiment was repeated by providing three replicates of each particular sample.

3.3.7 Cell cycle analysis and haploid cells production

Cell cycle progression at G0/G1, S and G2/M phases, and haploid spermatid cells population was investigated using Muse® Cell Cycle Assay Kit. Differentiated cells at Day10 were collected by trypsinization to make single cell suspension. Cell samples were prepared following manufacturer's instructions. Appropriate cell concentration was fixed and stained to detect differential DNA content of the cells. The samples were analyzed using Muse® bench top flow cytometer (MilliporeSigma, Billerica, MA) generating DNA profile and cell cycles graph represented by haploid peak, G0/G1, S and G2/M phase of cells. Each sample ran three replicates collected from three different batches of differentiation and analyzed with 5,000 acquisition events. In addition, differentiated cells at day10 were then prepared for cells sorting analysis to isolated the presence of a haploid peak, using FACSDiva software (BD Biosciences) as per manufacturer's instructions. SSCs were stained with RedDot™1 Far-Red Nuclear Stain (Biotium) as per manufacturer's instructions in the SSC medium and run on a FACS Aria sorter (BD Biosciences). Sorted haploid cells were then fixed to coverslip prior to immunostaining.

3.3.8 Statistical analysis

Significant changes were measured using 1-way ANOVA for various experiments in this study. Significance different was determined by * is $p < 0.5$, ** is $p < 0.01$ and *** is $p < 0.001$.

3.4 Results

3.4.1 *In vitro* direct differentiation of rPSC showing SSC characteristics

The dynamic proliferation of SSCs during spermatogenesis leads to appearances of particular characteristic in SSCs of each developmental state, for example, meiotically division of Spg results in the changing of DNA content in distinguish state or morphogenesis of spermatid cells during spermiogenesis and results in the changing of cell size and morphology (Dreef et al., 2007; Liu et al., 2011; Kaavya et al., 2017). As a complex sequence

of differentiation events that occur from a basement membrane along a luminal compartment of seminiferous tubule, scientists are able to identify stage of cycles of seminiferous tubule based on cell morphology and distinguish nuclear DNA staining which are important to demonstrate germ cells differentiation and expansion during spermatogenesis *in vivo* (Clemont and Leblond, 1959, Arnold, 1973, Ehmcke et al., 2005). In this study, we have followed the potential model for generating advanced human germ cells *in vitro* as previous report (Easley IV et al.,2012), and resenting bright field characteristic of SSC morphology derived *in vitro* distinctive from PSC state, which will be useful for further practical SSCs culture hasn't provided the details. Here, we observed morphological change of rSSCs derived from PSCs induction based on bright flied imaging and immunofluorescence staining. Differentiated cells showed a high nucleus cytoplasmic ratio at the first couple days after differentiation which are not distinctively changed from PSC state. Corresponding to previous report (Kaavya et al., 2017), size of single mouse Spg is represented similarly with ESC, thus we expected that the first three days of SSC *in vitro* culture are the beginning state of male germ cells progression (Figure 3.1A). Changing of cell morphology was clearly observed at Day 5 of differentiation by showing a distinctive smaller cells cluster with reduced nucleus and cytoplasmic ratio. The tiny homogenous cell appeared in some areas of the culture, whereas other areas showed mix cells population size, and a cluster of tiny cells progressed toward smaller cells later on Day 10 (Figure 3.1A; second and third column). To confirm our observation of dynamic change in rSSC, a specific marker during male germ cells development including PGCs, GCs and Spg state were evaluated. Both ESCs and differentiated cells at Day 10 showed strongly positive for Sal-like protein 4 (SALL4) (Figure 3.1B); a marker that shown differentially expressed in pluripotent state and in the initiation of male germ cells development in PGCs, GCs until Spgs states (Gassei and Orwig, 2013). Thus, our differentiation showing male germ cells progressed onward. PLZF - transcription factor is a distinguished maker of undifferentiated Spg (Adark and Apale) that

was found in testicular cells and required to regulate self-renewal and maintenance of the Spgs pool (Costoya et al., 2004). Our results show that none of PLZF positive cells were detected in ESCs control group while some of PLZF positive areas were found at Day10, similarly with typical PLZF staining patterns as shown in rhesus monkey testis section (Figure 3.1C). Next, we wished to determine SSCs expression in an earlier induction day since we observed a distinctive change of cell morphology start in day 5. Interesting, the widely spread PLZF positive portions were predominantly detected at Day 5 (Figure 3.1D) as well as SALL4 observation (Figure 3.1E), revealing that rPSCs progression forward to the large Spgs population at Day 5, while some Spgs remained expression at later differentiation time as emerging of smaller signal in Day 10. These results suggest that our *in vitro* differentiation showed the efficient and rapid differentiated rPSCs into SSC lineages as transforming of cell morphology and expressing specific markers of SSCs.

3.4.2 rSSC differentiation elevated germ cell marker from PSCs state

To determine whether PSCs converted to germ cell lineage, differentiated cells were assessed at ESCs, Day3, Day5, Day7 and Day10 of differentiation. mRNA of Oct4 pluripotent state and VASA germ line specific genes were used to investigate the progressions of differentiated cells. The results showed Oct4 mRNA was highly expressed at undifferentiated ESCs control, and then expression was downregulated after differentiation started until day10 (Figure 3.2A). Similarly, with the results in previous description, expression of Oct4 decreased significantly in embryoid body (EB) at Day 14 of germ cells differentiation (Yamauchi et al., 2009). On the other hand, VASA, a germ cell specific marker, was reported as the valuable gene to detect germ cell differentiation from monkey ESCs (Castrillon et al., 2000; Yamauchi et al., 2009). Our results demonstrated that mRNA expression of VASA was upregulated throughout 10 days differentiation and highly expressed at Day10, indicating that rapid direct differentiation of rPSCs evaluated germ cell expression along 10 days (Figure 3.2B). Taken together, our results related to human SSCs

VASA evaluation (Easley et al., 2012) that a number of VASA positive cells are highly expressed along 10 days differentiation whereas longer differentiation does not significantly induce the expression, suggesting this 10 days direct differentiation protocol is suitable for primate species both human and rhesus monkey. Transforming of pluripotent states (Oct4) into distinctive germ cell marker (VASA) revealed the capability of germ cells induction from the pluripotent rESCs.

3.4.3 rSSC expressed mRNA hallmark of spermatogenesis

Previously, the study shown that hPSCs differentiation recapitulate developmental state of spermatogenesis based on qualitative immunofluorescence detection (Easley IV et al., 2012). Here, we show the quantitative data of rSSCs mRNA expression supported that rPSCs progressed onward spermatogenesis *in vitro*. As we observed that the expression of SALL4 and PLZF immunopositive PGCs developed to Spg, we next investigated whether rSSCs could represent further state in spermatogenesis or more maturation cells in spermiogenesis.

First, SALL4 the earliest SSCs gene was evaluated. SALL4 was significantly expressed at ESCs state until early at Day3 in differentiation, and then the expression continuously declined from Day 5 through Day10 (Figure 3.2C). Our results supported that SALL4 presented in initiation processed of rSSC in ESCs to day 4 which corresponded to the progression of ESCs, PGCs, GCs and Spgs of *in vivo* germ cells. Subsequently, we evaluated PLZF or ZBTB16 expression, a novel marker that was specifically detected in stem and progenitor of Spg which played an important role in SSC self-renewal and growth. Interesting, our results showed differentiated cell elevated PLZF expression from ESC control group. PLZF showed a great significantly difference at Day 5 compared to other days (Figure 3.2D). Notably, these results support the series of PLZF immunofluorescence expression that we detected by ICC in Day 5 and Day 10 as described above. Taken together with previous report (Ishikura et al., 2016), PGCs in embryonic testis enter mitotic arrest

around E13.5 and differentiate into pro-spermatogonia or Spgs by postnatal Day 5 before developing into mature germ cells population circulated in spermatogenesis.

In more mature germ cells marker, c-Kit is a maker that were observed in GCs, differentiating B Spg and spermatocytes. c-Kit expression has been a crucial role for proliferation, migration, survival and maturation of SSCs (Yoshinaga et al., 1991; Zhang et al., 2013). Our results showed that, rSSCs show increase in c-Kit expression from ESCs to Day 3, with declined expression later in differentiation (Figure 3.2E). Although, they are not significantly different from c-Kit expression during rSSC timeline but changing of c-Kit profile throughout differentiation time revealed the progression of rPSCs to rSSCs, and development of more mature cells at a later day of differentiation as the lowest c-Kit expression were observed in day 10. Taken together, Piwil1 (also known as HIWI) a post-meiotic cells marker showed as a testis-specific gene, encoding cytoplasmic protein present specifically in spermatocytes and round spermatids (Bak et al., 2011; Easley IV et al., 2012). Our results demonstrated that Piwil1 mRNA expression upregulated throughout 10 days differentiation. Highly expressed of Piwil1, mRNA was significantly observed at Day 10 when compared to early Day 3 and Day 5 (Figure 3.2F). The expression of Piwil1 is a confirmation that our rSSCs demonstrated more mature germ cells whether as a spermatocyte or spermatid at the downstream differentiation process (Figure 3.5).

3.4.4 *In vitro* differentiation of rPSC produced haploid spermatid like cells

Presenting the haploid cells production *in vivo* revealed the occurrence of spermiogenesis, a final morphogenesis of mature spermatozoa before function sperm formation. We have determined that differentiation of rESCs into rSSC represented SSC progression from early PGCs to late mature germ cells based on gene expression profiles. We next evaluated the cell population at later differentiation time of day 10 to see whether rSSCs progressed through haploid spermatid like cells *in vitro*. FACS Muse cell cycle assays were performed to determine haploid cells population based on DNA content of cells (Figure

3.3A). rSSCs Day10 showed haploid peak in sub G0/G1 area around 10.87%, and a small cluster of haploid population was detected in DNA content profile (Figure 3.3B). While none of haploid population was detected in undifferentiated ESCs control group (Table 3.1), other cell cycle states including G0/G1 (the cell in resting state (2n) and preparing for later division step), S phase (cell synthesizes a copy of the DNA and centrosome inside the cells generating larger DNA content), and G2/M phase (growing largest cells (4n) preparing for mitosis) have been evaluated. Our result shows a small difference of other cell cycles population in both rSSCs and undifferentiating ESCs (Figure 3.3C). G0/G1, S and G2/M phase were 44.90%, 18.18%, 26.03% in rSSC and 47.79%, 27.03%, 26.02% in ESCs (Table 3.1) respective. This experiment indicated that our rPSC differentiation could produce haploid spermatid cells which represented early spermiogenesis *in vitro* and the 10 days differentiating process does not impact to normal cells cycle progression.

We next confirmed protein expression of derived haploid cell population at Day10. To isolate haploid spermatid like cells from rSSCs, we have performed the single cells sorting from haploid population (P3 in Figure 3.4A) of rSSC Day 10 using BD FACS Aria sorter. Sorted cells were fixed and stained with haploid specific markers. Acrosin is a major proteinase present in the acrosome of round spermatid and mature sperm. Protamine (PRM1) is abundant basic nuclear proteins of mature sperm that play a crucial role in replacing the histone protein in sperm cells. Transition proteins (TNP1) functions promoted histone protamine exchanges to cease transcription of spermatid elongation phase (Steger et al., 1998). Our results show the expression of those markers, Acrosin, PRM1 and TNP1 which were detected in haploid sorted cells from rSSCs Day10 (Figure 3.4B). Confirming that our *in vitro* differentiation could be produced advanced male germ cells as the haploid spermatid like cells from PSCs.

3.5 Discussions

In vitro advance male germ cells production from PSCs showed potential tools for male germ cells developmental research and is a great achievement reported as human haploid germ cells production (Panula et al., 2010, Easley IV et al.,2012, Zhao et al., 2018) or producing offspring from mice germ cells derived *in vitro* (Yu et al., 2009; Nakaki et al., 2013; Zhou et al., 2016). The revolution in artificial germ cells production from *in vitro* stem cells culture comes with scientific interest for patient specific stem cells production and the possibility for fertility preservation (Dissanayake, 2018) such as in chemotherapy patient (Sadri-Ardekani et al., 2009) or azoospermia patients (Chiba et al., 2016). However, the downstream processing of spermatid derived *in vitro* needs to be carefully investigated to confirm that it is a reliable model to apply in clinical trials. Human study would be considered problematic from an ethical perspective (Condic and Rao, 2010; Rombaut et al., 2017; Valli et al., 2014), this study report that the NHP model would be useful in a longitudinal study of human as it well represents human germ cells biology.

rPSCs differentiation into male germ cells in our model recapitulate male germ cells progression from embryonic state of pre-natal germ cells to post-natal spermatogenesis mirroring normal male germ cells development (Saitou and Miyauchi, 2016). Our investigation presents qualitative and quantitative data of the expression profile in rSSCs shows the progression of male germ cells lineage from ESCs as show in Figure 3.5. rESCs were first induced into germ cells based on VASA, a prominent germ cells marker expression. The establishments of primordial germ like cells (PCGLCs) to Gonocyte likes cells (GCLCs) are being observed at early induction around day 3-4, before the peaks of PLZF raised up in day 5. The presentation of PLZF expression in rSSCs determines undifferentiated Spg in spermatogenesis which is a distinctive marker of rodents, human and in adult rhesus testicular tissues (Buaas et al., 2004 Costoya et al., 2004, Easley et al., 2012, Sharma et al., 2017). Transformation of rSSCs into mature spermatocyte population are

observed around day 7-10 based on peaks of Piwil1 that emerge, suggesting that rSSC undergo meiotic or post meiotic cells (pachytene spermatocyte to round spermatid) into more mature germ cells corresponding to the expression of Piwil1 *in vivo* (Deng and Lin, 2002; S Tushir et al., 2009; Thomson and Lin, 2009; Bak et al., 2011). Since, there is a problem of unspecific blinding across species of the primary antibody to monkey tissue, we could not measure ICC staining as well as in rodent and human studies.

In contrast with other NHP studies, we have represented advanced mature germ cells production *in vitro* outside the SSC niche, while cynomologus monkey ESCs (cyESCs) differentiation could be produced cyPGC from cyESCs as the expression of VASA (Yamauchi et al., 2009) or a recent rhesus monkey primordial germ like cells (rPCLC) production from two steps *in vitro* rPSCs induction could presented more mature germ cells but after *in vivo* transplantation by the promoting of seminiferous tubule environment (Sosa et al., 2018).

Total duration of rhesus spermatogenesis is 36 days (Clermont and Leblond, 1959: Barr, 1973), a 10 days differentiation may represent as a cycle of seminiferous epithelium in rhesus spermatogenesis which that take 10.5 days to form one cycle of SSCs progression through 12 stages (Barr, 1973). However, to determined deeply specific cells type and differentiation characteristics in clonal expansion of rhesus spermatogenesis *in vitro* as those observed *in vivo* (Ehmcke et al., 2006), enrichment of large scale rSSCs production needs to optimize, single cells isolation for rSSC staining and reduced the bias of small haploid cells and debris in FACS still needs to be investigated. Production of haploid spermatid like cells and molecular observation as acrosin, PRM1 and the TNP1 demonstrated spermiogenesis (Courtens et al., 1995; Steger et al., 1998; Heidaran et al., 1988; Toshimori K, 2015) in our culture, however we observed only a small portion of haploid cells production similar to previous report (Easley et al.2012). Suggesting that *in vitro* rSSCs induction by our direct differentiation protocols could develop rESC to the advanced germ cells but need further

maintenance by supporting environments to produced more mature spermatozoa.

Complete sperm cells production using PSCs induction *in vitro* has not been achieved, as it would be a challenge to generate *in vitro* environmental factors that mimic natural spermatogenesis in seminiferous tubule such as reconstruction of testicular stem niches (Oatley and Brinster, 2012; Chen and Liu, 2015), providing Sertoli cells and Leydig cells which play the important role for SSCs progression and the supplement of growth factor and hormone in appropriate concentration for maturation state (de Kretser et al., 1998). Supporting that, a recent study suggests that seminiferous tubule environment could promote rPGCLC maturation after homologous transplantation (monkey to monkey), a model purposed for patient SSC cryopreservation and transplantation back in later adulthood (Sosa et al., 2018).

In conclusion, our rhesus monkey spermatogenesis model is mirroring *in vivo* spermatogenesis, showing the potential model for using it as patient specific germ cells production from only PSCs. Moreover, it would be of benefit to molecular study of human disease that affects germ cells production without genetic manipulation. In addition, additional assays such as the genomic imprinting, transcriptome analysis of our *in vitro* platform comparing male germ cells will be further investigate. Achieving of advance male germ cells as spermatid like cells from NHP-PSCs would be a promising forward downstream characterization process to prove the milestone of artificial germ cells production *in vitro* from higher primate and provide the potential source for male fertility.

A.

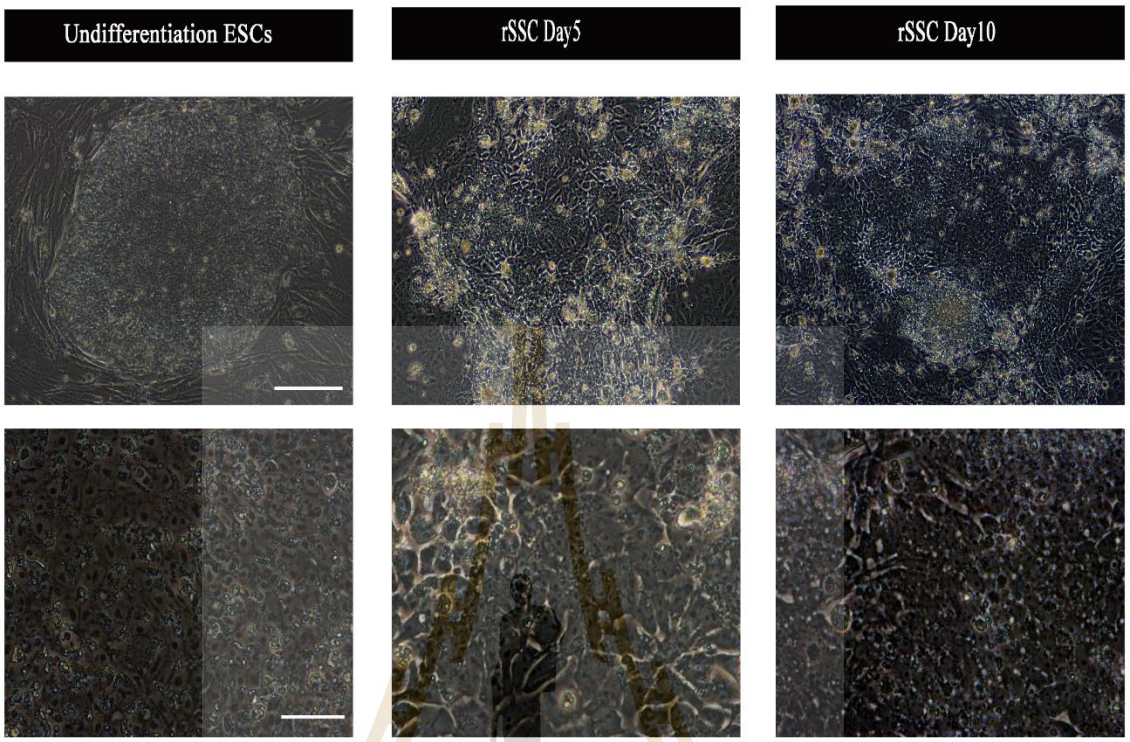
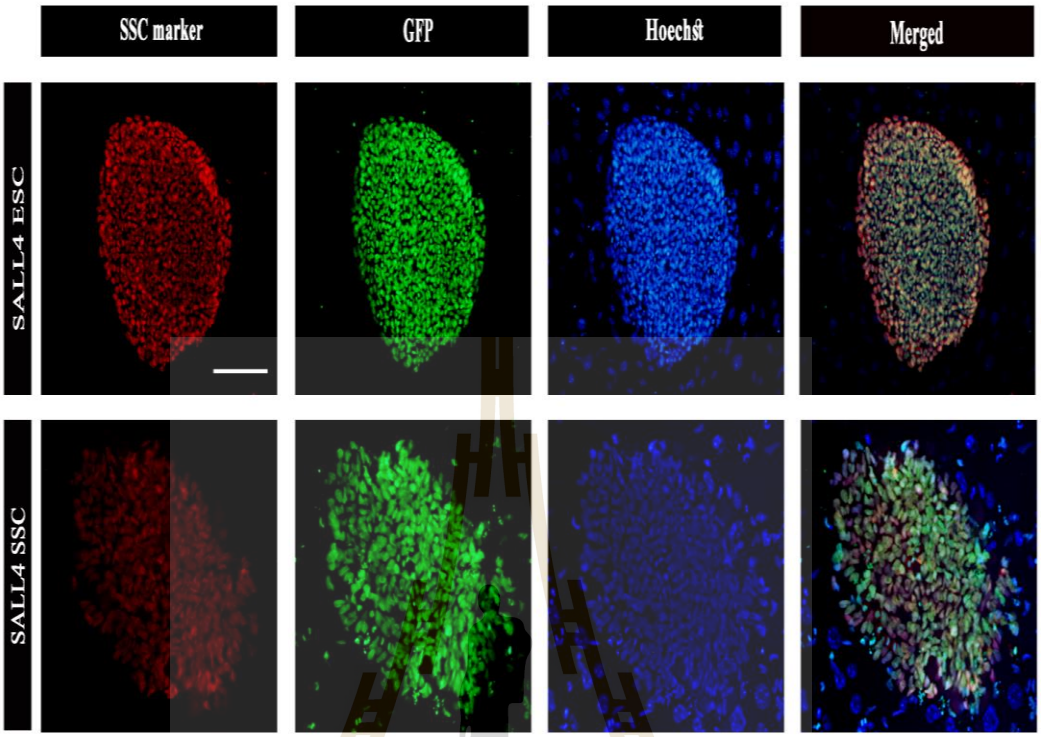


Figure 3.1 Differentiation of rSSC generated SSC characteristics.

(A) Direct differentiation of rPSCs transformed morphologies from highly nucleus cytoplasmic ratio at PSCs state into smaller spermatogenic like cells. At day 5 differentiated cells showed areas of smaller cell size, then progressed forward to homogenous tiny cells areas later at day10 of differentiation. Scale bar = 200 μm in top row and scale bar = 20 μm in bottom row.

(B) Immunofluorescence staining of SALL4; PSCs and SSCs marker. SALL4 (Red) positive were detected in ESCs and SSCs at day10. Endogenous expression of green fluorescent protein (GFP) represented rESCs and rSSCs colonies in culture. DNA stained with Hoechst and scale bar = 200 μm.

B.



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C.

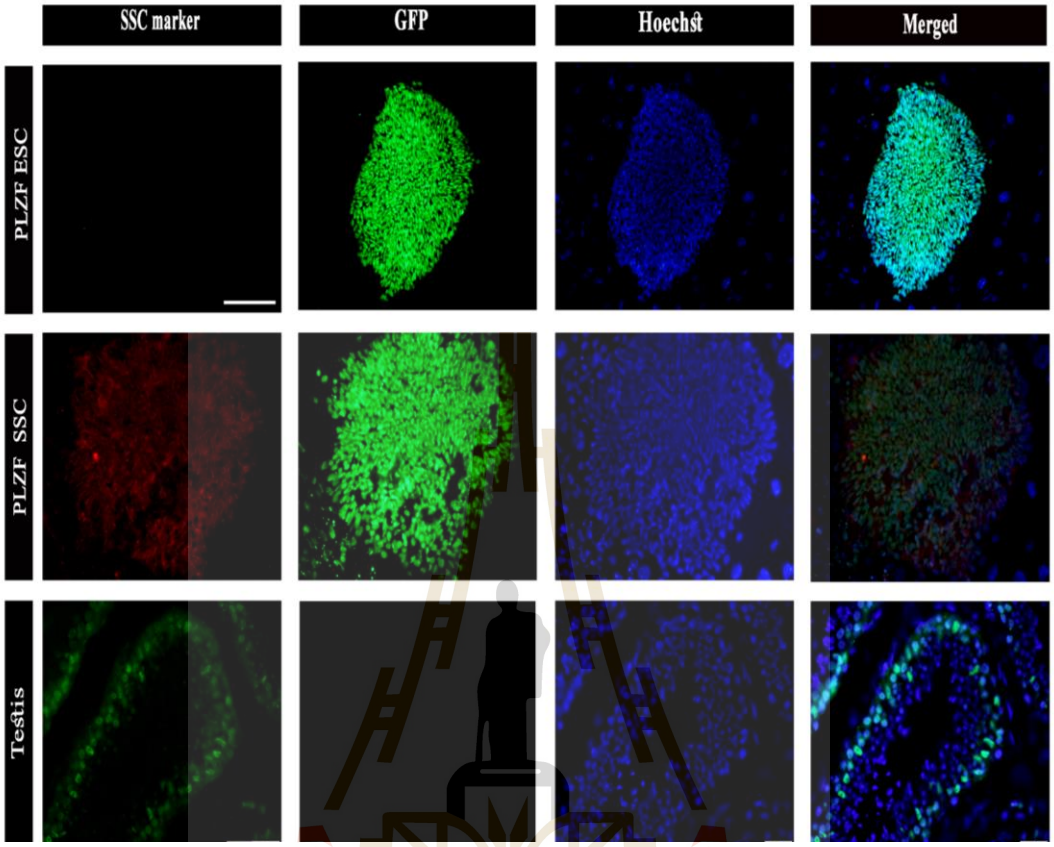

Figure 3.1 Differentiation of rSSC generated SSC characteristics.

(C) Immunofluorescence staining of PLZF; specific marker of undifferentiated Spgs. Differentiated cells at day10 expressed PLZF (Red) signal, while none of PLZF were detected in ESCs. Scale bar = 200 μm. Endogenous expression of green fluorescent protein (GFP) represented rESCs and rSSCs colonies. DNA stained with Hoechst. Row 3 panel represented testis tissue PLZF staining. Scale bar = 20 μm.

D.

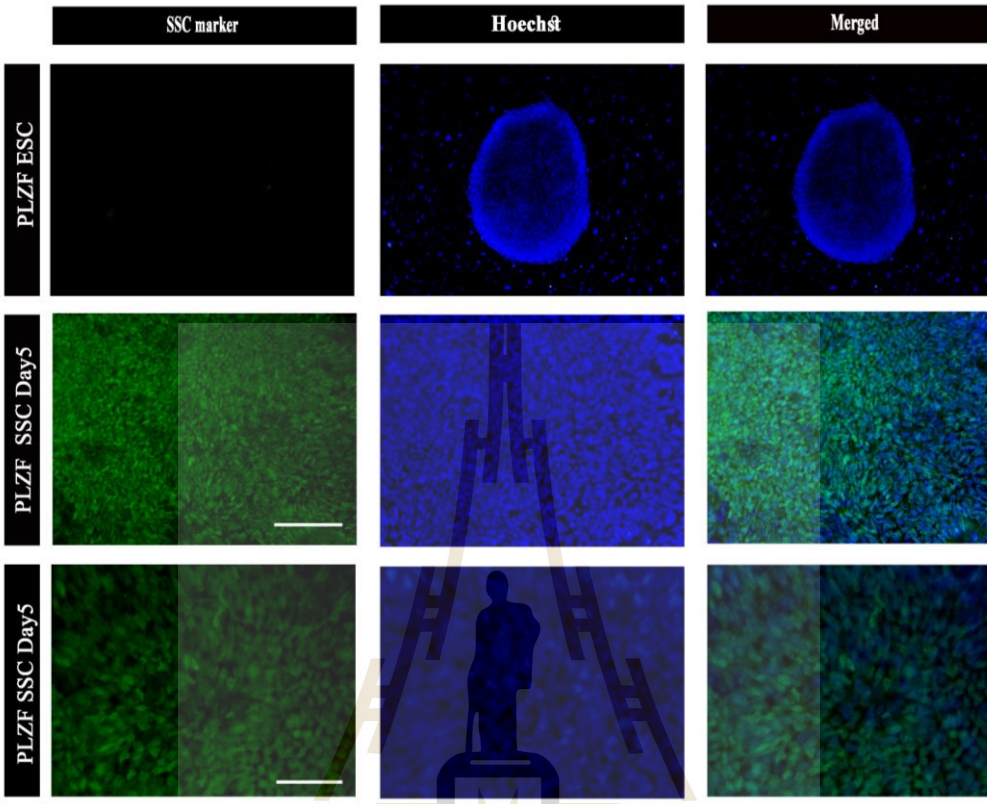

Figure 3.1 Differentiation of rSSC generated SSC characteristics.

(D) Immunofluorescence staining detected the large portion of PLZF (green) cells at day 5. Scale bar = 200 μm in second row panel. Higher magnification of PLZF positive cells third row panel. Scale bar = 100 μm. DNA stained with Hoechst (blue).

E.

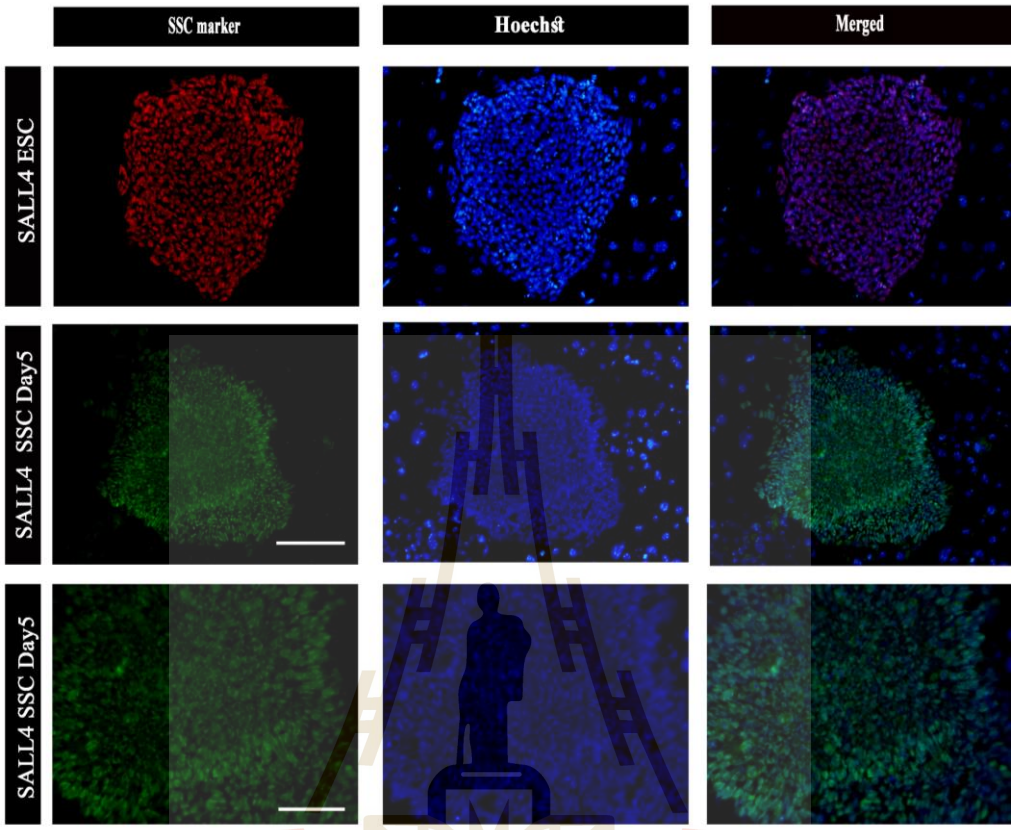

Figure 3.1 Differentiation of rSSC generated SSC characteristics.

(E) Immunofluorescence staining of SALL4 ESCs (red) and rSSCs at day 5 (green) second row. Scale bar = 200 μm. Higher magnification of SALL4 positive cells third row panel. Scale bar =100 μm. DNA stained with Hoechst (blue).

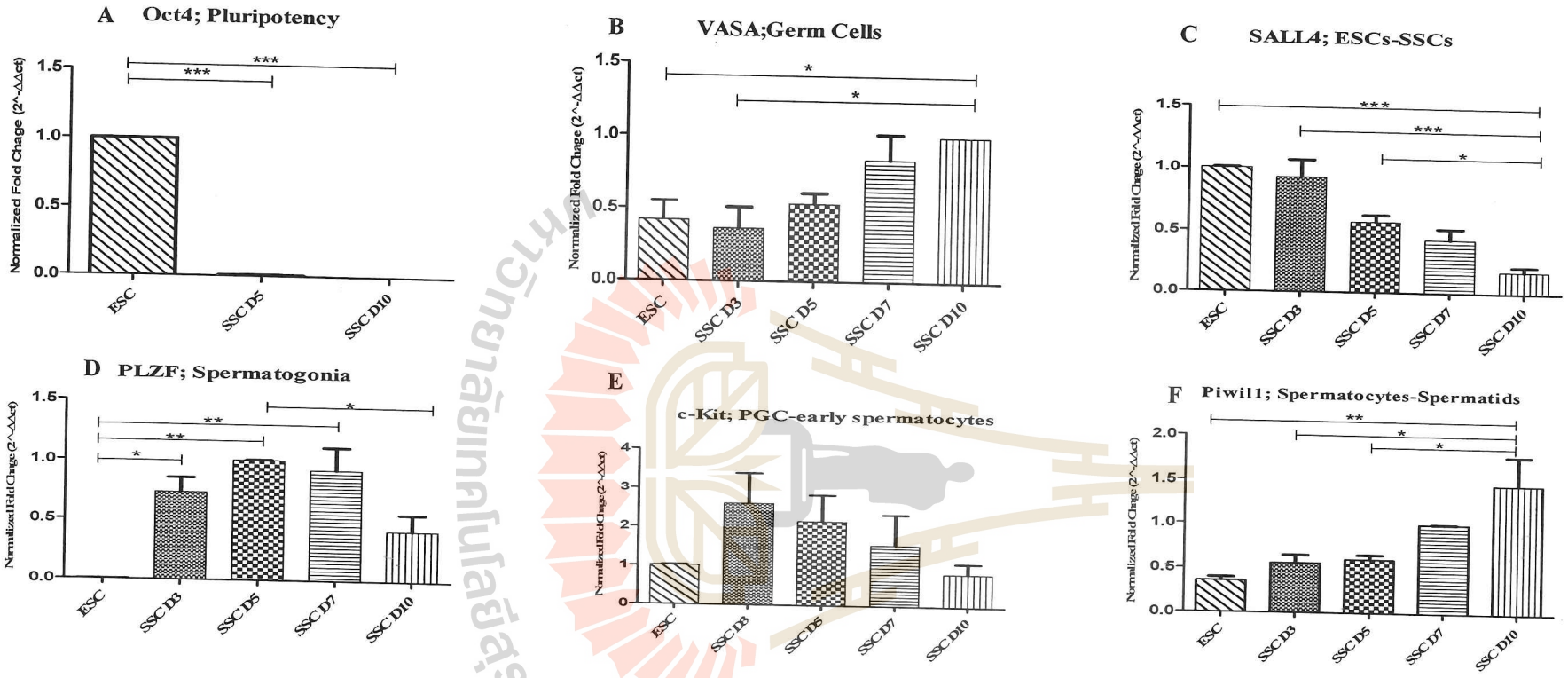

Figure 3.2 Quantitative real time PCR (qRT-PCR). Differentiated cells at different time point Day3, 5, 7, 10 and ESCs undifferentiation were investigated for specific marker of SSCs states. Graph shows mRNA expression of Oct4 (A) and VASA (B), SALL4 (C), PLZF (D), c-Kit (E) and Piwil1(F) genes in different state of spermatogenesis. * indicates significant different compare with other values (*** $p<0.001$), (** $p<0.01$) and (*$p<0.5$).

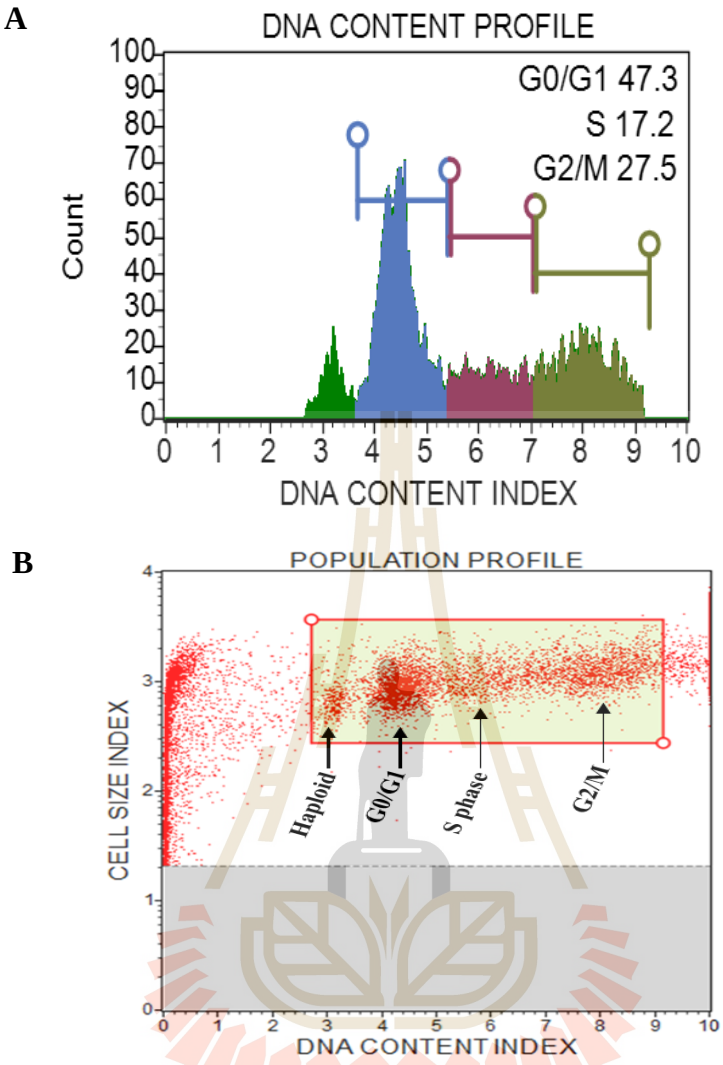

Figure 3.3 Direct differentiation produced haploid spermatid cells. Fluorescent activate cell sorting generated the peaks from DNA content of rSSC at day10. Representative different areas of the peak including haploid peak (green), G0/G1 (blue), S (purple) and G2/M (olive) of rSSC at day10. (A), (B) Indicating population profile in each state of cell cycle from rSSC at day 10.

Table 3.1 Production of haploid cells from rSSCs differentiation and undifferentiation ESCs.

Cells	% Haploid	% G0/G1	% S	% G2/M2	Experiments
rE SCI	-	46.79	27.03	26.62	N=3
rSSCI	10.87	44.90	18.18	26.03	N=3

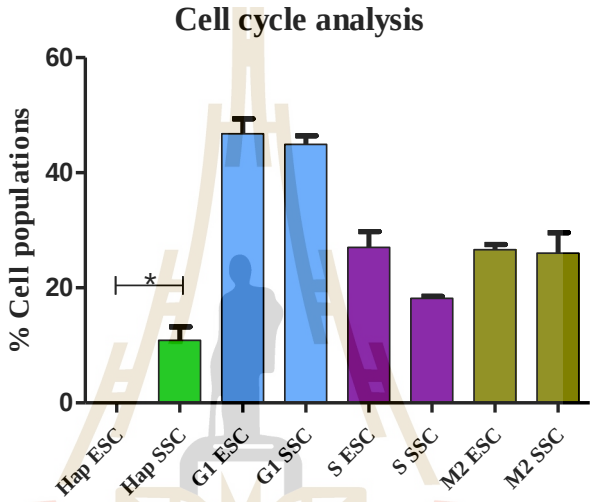

Figure 3.4 Graph represented % of haploid cells population of rSSCs comparing with undifferentiation ESCs. Significant different from * $p<0.05$

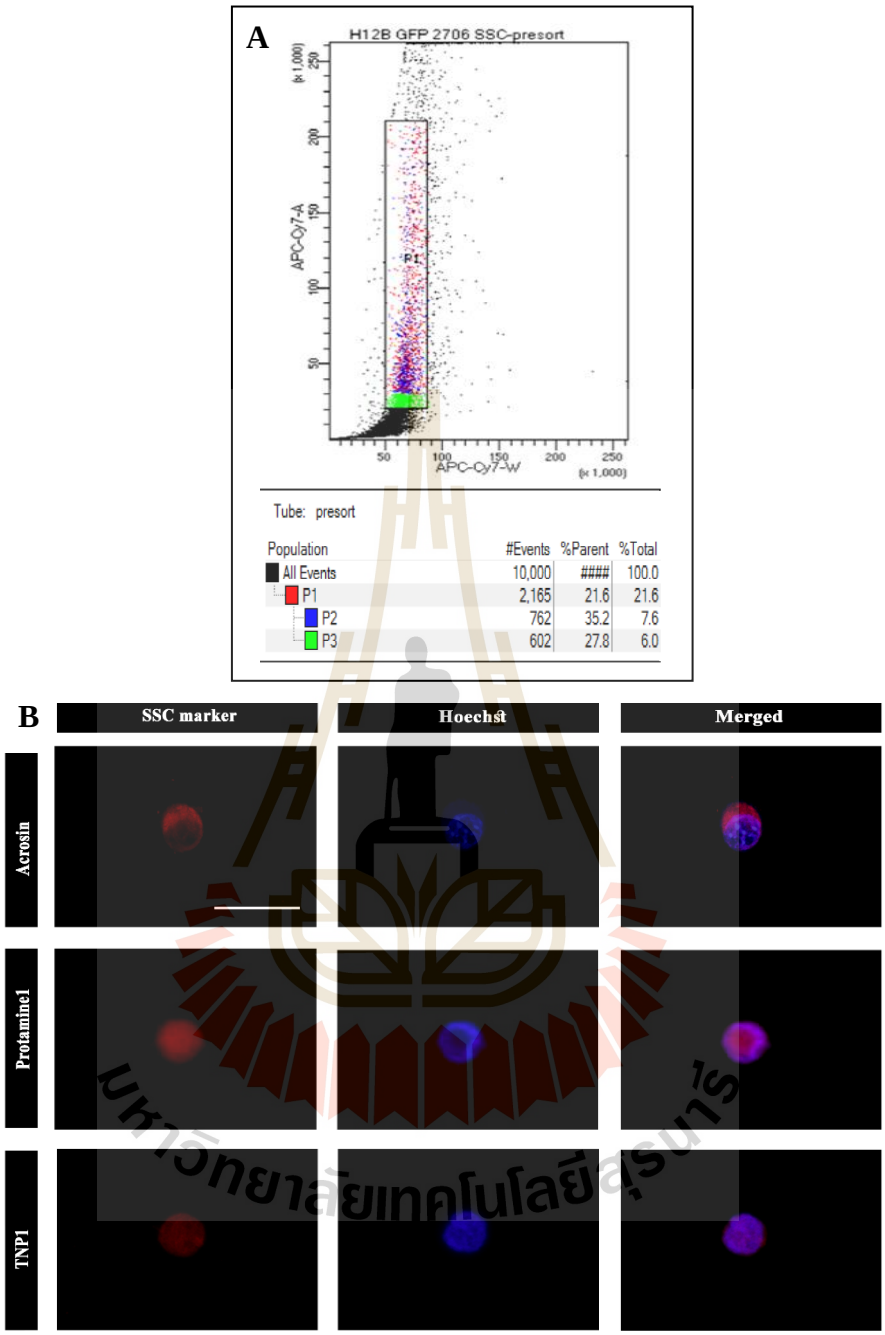

Figure 3.5 Haploid Analysis by FACS and ICC. (A) rSSCs Day10 sorting by BD FACSDiva 8.0.1 revealed haploid cells population (P3). (B) Sorted cells staining with haploid round spermatid markers; Acrosin, PRM1 and TNP1 (Red). DNA stained with Hoechst. Scale bar 10 μm.

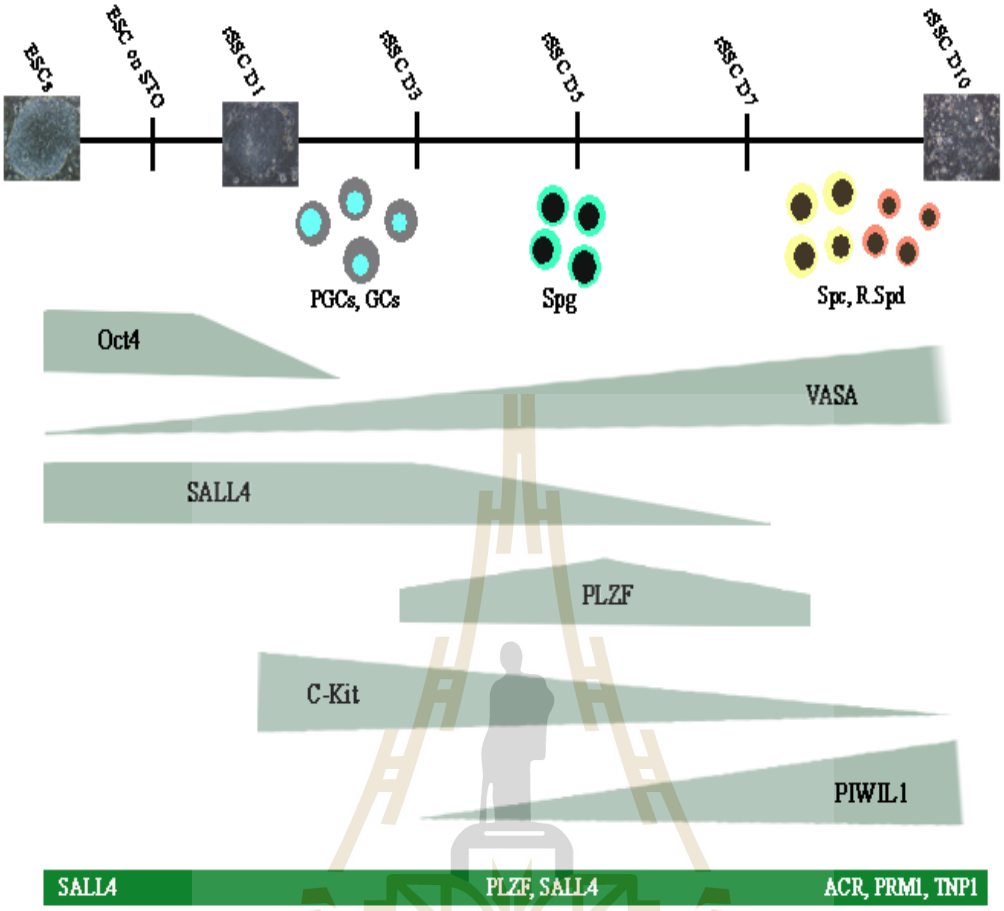

Figure 3.6 Schematic diagram of rhesus monkey *in vitro* PSCs differentiation mirroring *in vivo* germ cells development and spermatogenesis.

Differentiation time line of rSSC during 10 days differentiation. rSSC showed SSCs characteristics by mRNA expression in each state of spermatogenesis (Gray bar). Specific protein expression determined by immunocytochemistry (Green bar) mimicking spermatogenesis *in vivo*. PGCs: Prymodial germ cells, GCs: Gonocytes, Spg: Spermatogonia stem cells, Spc: Spermatocytes, R.Spd : Round spermatid.

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CHAPTER IV

POLYGLUTAMINE INSTABILITY IN *IN VITRO* DERIVED MALE GERM CELLS FROM HD MONKEY PSCS

4.1 Abstract

Gender is an influential factor in trinucleotide repeat (TNR) instability in Huntington disease (HD). Expansion of CAG repeats can occur by germ line transmission during germ cell development dominant in spermatogenesis. CAG instability in testicular cells of adult HD patient demonstrates the expansion during pre to post meiotic dividing cells. However, pathogenic cellular phenotypes in earlier male germ cell stage of HD patients are limited, since usual onset is adulthood. In this study we report on *in vitro* spermatogenesis of transgenic rhesus monkey HD pluripotent stem cells (rHD PSCs) underlying cellular mechanism of pathogenic *Huntingtin* (*HTT*) mutation on male germline development. rHD PSCs including a rHD1-iPSCs and two progenies transgenic rHD ESCs derived from rHD1 sperm, carrying mutant *HTT* transgenes from rHD1, the first transgenic monkey (Yang et al., 2008). Transgenic cells were differentiated into spermatogenic cells (rHD SSC). *In vitro* induction of rHD SSCs elevated VASA specific germ cells expression and the early male germ cell hallmark SALL4 expression, revealed male germ cells lineage progression from rHD PSCs. Moreover, rHD SSCs showed SALL4+ as well as PLZF+ cells that demonstrated spermatogonia stem cells (Spgs) arise after *in vitro* differentiation. Notably, transgenic rHD SSCs showed the impact on Spgs progression, as the consistent expression of PLZF Spgs marker, while a low expression level of Piwil1mature germ cells are observed. A TNR study demonstrates CAG instability during *in vitro* spermatogenesis by the increasing of small,

intermediate and large expansion repeat length in rHD SSCs compared to WT control, suggesting that pathogenic HD CAG expansion of *in vitro* spermatogenesis presents in early state before mitotic or during mitotic division of Spgs. Moreover, we recapitulate male germ line transmissible characteristics of HD as the mutant *HTT* transgene inherited CAG expanded allele from paternal rHD1 sperm and SSCs throughout the progenies rHD SSCs by *in vitro* spermatogenesis. Increasing of mHTT mRNA level after *in vitro* differentiation was observed together with abnormal apoptosis control of male germ cells development, suggesting pathogenic HD on testicular germ cells degeneration.

Our study anticipates pathogenic cellular phenotype of HD on male germ cells progression. Producing rHD SSCs *in vitro* represents valuable tools for evaluating patient's germ cells under testicular degeneration. Determination of pathogenic HD on male germline *in vitro* provided valuable data cover earlier male germ cells development throughout spermatogenesis in HD which will be useful in disease evaluations for therapeutic strategies.

Keywords: Huntington disease, *In vitro* spermatogenesis, CAG expansion, Male germ line transmission

4.2 Introduction

Human huntingtin (*HTT*) encoding particular protein is important to physiological development and growth (Nasir et al., 1995; Auerbach et al., 2001; Yang et al., 2008; Yan et al., 2016; Jimenez-Sanchez et al., 2017). *HTT* mutation leads to increases in trinucleotides repeat (TNR) Cytosine Adenine Guanine (CAG) tract in *Exon1* of *HTT* gene (*IT5*) and causes HD. HD phenotype is characterized by loss of motor function as involuntary movements, dystonia and dementia, apathy and death around 15-20 years after clinical onset (Déglon, 2017). CAG repeat length is related to disease severity while inversely correlated with age of onset (Duyao et al., 1993; Reiner et al., 2011). Clinical phenotype of HD is observed in CAG length of more than 39 repeats, while non-HD range is between 9 to 35 repeats, and mutable HD is between 27-35 CAG repeats (Trottier et al., 1994; Aronin et al., 1995;

Vnencak-Jones, 2003; Saudou and Humbert, 2016).

Gender is an influence factor on CAG repeat transmission, as HD disease allele has a paternal bias which is more likely attributed to inherited expanded allele (pre-mutation to disease range) of large expansion size (>7 CAGs) in males (Kovtun et al., 2000; Wheeler et al., 2007a; McMurray, 2010; Putkhao et al., 2012). Although, dominant effects of HD are found in neuronal cells, testicular degeneration and progressive loss of SSCs have been observed in HD patients (Van Raamsdonk et al., 2007). Given that the underlying mechanism of HD pathogenic cellular phenotype on male germline progression will help elucidate disease progression in HD. CAG repeat mutation of HD patient's testicular germ cells have been observed in pre-meiotic to post meiotic dividing cells (Yoon et al., 2003). In contrast, mice testicular cells only observed CAG expansion in post meiotic haploid spermatid or spermatozoa (Kovtun and T McMurray, 2001). Showing that inter-species differences between mouse and human revealed the challenge to translated pathogenic CAGs expansion in mouse model to human (Pearson, 2003). A transgenic non-human primate model then becomes a valuable model for anticipating pathogenic cellular phenotype of human HD (Yang et al., 2008; Chan et al., 2010; Putkhao et al., 2013).

Progressive loss of SSCs in adults HD testis are a challenge for biological research to understand the overall disease progression on germ cells development (Van Raamsdonk et al., 2007), especially in earlier germ cells state, thus we report in *in vitro* transgenic HD monkey spermatogenesis from transgenic HD PSCs. Present work reveals the cellular phenotype of HD during pluripotent state develops to male germ cells *in vitro*. Interestingly, we can capture CAG expansion in rHD SSC population and likely in undifferentiating Spgs, underlining that CAG expansion arises even before postnatal male germ cells states. This confirms that *in vitro* rHD SSCs would be a potential model for better understand the parent of origin effects in CAG expansion and biomarker development for therapeutic strategies in HD.

4.3 Material and methods

4.3.1 Transgenic HD rhesus monkey PSCs (rHD PSCs)

rHD1, the first transgenic HD rhesus monkey were generated previously (Yang et al., 2008) using lentiviral vector carrying mutant *HTT* with 84 CAG repeats in *Exon 1* of *HTT* gene and GFP under the control of human polyubiquitin C (*UBC*) promoter. It was subsequently introduced into monkey oocytes with sperm injection, producing embryos, then transferred to surrogate mothers. rHD1 was characterized with 29 CAG repeats and carried three insertions of two mutant *HTT* and a GFP transgenes (Yang et al., 2008). RPg12 iPSCs, a reprogrammed rHD1 dental pulp stem cells, were used as paternal cells in our differentiation model. rHD1 sperm carrying mutant *HTT* transgene containing 82-148 CAG repeats were used for intracytoplasmic sperm injection (ICSI) followed by *in vitro* embryo culture. Inner cell mass (ICMs) of producing blastocysts were isolated and maintained as previously described in Putkhao study (Putkhao et al., 2013). Establishments of rHD ESCs5 and 7 (RPg5 and RPg7 ESCs) as well as two wild type controls; rWT ESCs (rZH2 and YRES5 ESCs) were used in this study.

To prepare feeder for rPSCs culture, Mouse embryonic fibroblast (MEF) was isolated from small fetus of pregnant mice 13 days post coitum and cultured as described in chapter III 3.3.1. Two rWT controls, two rHD ESCs and rHD iPSCs were thawed and cultured on freshly prepared feeders by standard rESCs culture. Growing cells at 70-80% confluence was collected and seeded on STO. LIF and two days' period of differentiation followed. Direct differentiation of rHD PSCs into rHD SSCs were carried out by similar procedures with rESCs differentiation as described in chapter III 3.3.3.

4.3.2 Characterization of rHD SSCs derived from *in vitro* spermatogenesis

4.3.2.1 Gene expression analysis by qRT-PCR

The pellets of two rWT and two rHD were collected at undifferentiated ESCs, day 5 and day 10 for SSC gene expression analysis. Total RNA isolation was

performed by Trizol method, 500 ng of each sample was reverse transcribed to cDNA. Quantitative mRNA expression in each stage of spermatogenesis was measured using single tube commercial TaqMan gene expression assays (Thermofisher). Specific SSCs primers were used for gene expression profiles; VASA (Rh02798028_m1) for germ cell, PLZF (Rh02834704_m1) for undifferentiating Spg and SALL4 (Rh01010838_g1) for ESCs to Spg and Piwil1 (Rh04256262_m1) for spermatocytes to round spermatid state, and the 1X final reaction was prepared by Tagman Gene Expression Master Mix (Applied Biosystems). The experiment was run using Biorald qPCR machine, and results were first normalized with glyceraldehyde-3-phosphate dehydrogenase GAPDH endogenous control (Rh02621745_g1). The GAPDH normalized data were then further normalized with the control of each SSC state (ΔΔCT method).

Quantitative expression of mHTT mRNA before and after differentiation at day 10 was performed using ABI Sybergreen assay of mutant *Exon1* (forward sequence; GCGACCCTGGAAAAAGCTGAT and reward sequence; CTGCTGCTGCTGGAAGGACT) versus endogenous *Exon26* transcripts (forward sequence; ACCCTGCTCTCGTCAGCTTGG and reward sequence; AGCAADTTTCCGGCCAAAAT) of *HTT*. Final reactions of ABI Sybergreen qPCR master mix (Applied Biosystems) were run using Biorald qPCR machine. The results of *Exon1* and *Exon26* expression were first normalized with glyceraldehyde-3-phosphate dehydrogenase GAPDH endogenous control (forward sequence; GAAGGTGAAGGTCGGAGTC and reward sequence; CATTGATGGCAACAATATCC), the normalized fold change of mutant *Exon1* then calculated by normalization with endogenous *Exon26* expression (ΔΔCT method). Each sample was collected from three different batches of rSSCs differentiation and three replicates were performed in the particular experiments.

4.3.2.2 Immunocytochemistry of rSSC markers

Immunofluorescent staining of SALL4 (Santa Cruz Biotechnology) 1:200 and PLZF 10ug/ml (R&D) specific marker of the SSCs was performed as the same procedure described in chapter III 3.3.4.

4.3.3 Analysis of trinucleotide repeats instability in *HTT*

The degree of CAG repeat instability in Exon 1 of *HTT* gene was analyzed using polymerase chain reaction (PCR) using GeneScan size standard. Approximately 100 ng of genomic DNA was isolated by using Qiagen DNeasy Blood & Tissue Kits (Qiagen) following the manufacturer's instructions. The quantity of DNA was determined by NanoDrop™ 2000 Spectrophotometers (Thermo Scientific). PCR was carried out using primers specific to the region of CAG repeats in Exon 1 of *HTT*: HD32F (forward sequences 5'-FAM-CTACGAGTCCCTCAAGTCCTTCCAGC-3') and MD177R (reverse sequence 5'-GACGCAGCAGCGGCTGTGCCTG-3'). PCR was performed using 100 ng of genomic DNA, 0.2 µM of forward and reverse primers, 1X PCR Buffer (Takara), 1 mM of deoxyribonucleotide triphosphates (Takara), 0.5 U of Taq polymerase (Takara), 4 µM of Betain (Sigma), and nuclease-free water was added to bring up the total reaction to 50 µL. PCR protocol was set as 98 ºC for 5 minutes, followed with 40 cycles of 96 ºC for 5 minutes, 67 ºC for 45 seconds, 72 ºC for 1.5 minutes and final extension of 72 ºC for 10 minutes. The 10 µL of each PCR product was sent to Emory Integrated Genomics Core for a GeneScan analysis. 1.5 µL of PCR products were mixed with 0.5 µL of GeneScan™ 1000 ROX™ (ThermoFisher, Waltham, MA) and 9.5 µL of Hi-Di™ Formamide (Applied Biosystems). Samples were denatured at 95°C for 5 minutes and ran on 3130xl Genetic Analyser (Applied Biosystems). The data were analyzed using GeneMarker® (SoftGenetics).

4.3.4 GeneScan sizing

In order to define WT and HD allele cluster from the GeneScan analysis, we used the criteria as previously described in Lee publication (Lee et al., 2010). Electrogram and

peak areas derived from GeneMarker® SoftGenetics version 3.0.0 indicated the FAM (6-fluorescein amidite (6-FAM)) labeling samples. Two percent (2%) of the height of the highest peak was set as the threshold baseline for each analysis to eliminate background signal. Standard size GS500 or Rox1000 was applied for the suitable quality of data set. Highest peak of ESCs cluster was determined as the main allele in between each sample calculation. The non-templates control was used to set the background threshold. Mean number of repeat and Instability index was calculated using the formula adopted from a previous publication (Lee et al., 2010).

Mean number of repeat = (∑ (peak heights x base-pair lengths) / ∑ peak heights))

Instability index = ∑ ((peak height / ∑ peak heights) X (ΔTNR number from the main allele))

4.3.5 Pathogenic effects on rHD SSCs derived *in vitro* differentiation

To investigate pathogenic effects of HD on *in vitro* spermatogenesis, cellular apoptosis assay based on cell surface phosphatidylserine (PS) expression was used in our experiment. To generate four cell stages including live, early, late and dead cell population, the samples were investigated using Muse™ Annexin V & Dead Cell kit with Muse® bench top flow cytometer (MilliporeSigma, Billerica, MA). Single cells were collected by trypsinization of the cells before and after differentiation, and then further prepared the samples per manufacture instructions. Cells concentration was adjusted to 1 x 10^5 to 5 x 10^5 cells/ml and stained for 20 minutes at room temperature and under dark area. To eliminate the bias from the time consuming in experiment which causes more apoptotic cells, stained cells were run within one hour after preparation. Each sample was collected from three different batches of rSSCs differentiation and three replicates were performed in the particular experiments.

4.3.6 Statistical analysis

Data in this study were analyzed using one-way AVONA analysis by Tukey's multiple comparison test. Significant differences were generated from comparing two data set with * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$.

4.4 Results

4.4.1 *In vitro* direct differentiation of rHD PSCs generates early male germ cells, a hallmark of spermatogenesis.

Pluripotent stem cells are an unlimited source for *in vitro* differentiation into others cell types in the body, including male germ cells (Yamauchi et al., 2009; Easley et al., 2012; Medrano et al., 2012; Zhou et al., 2016; Sosa et al., 2018). Thus, *in vitro* germ cells production from PSCs are a promising tool in the field of clinical investigation and patient specific germ cells treatment. However, it hasn't been confirmed that pathogenic PSCs under disease condition are able to develop into male germ cells *in vitro*. In this study, we aim to produce *in vitro* male germ cells from the pathogenic rHD PSCs that carries mutant *HTT* transgene as a model study of a serious HD degenerative disorder (Nopoulos, 2016). We first investigated gene expression profile of rHD SSCs derived from *in vitro* culture of PSCs state throughout germ cells production. Oct4 mRNA expression showed that rHD ESCs differentiation has a progressive loss of pluripotent state similar with WT cells throughout 10 days differentiation (Figure 4.1A). To evaluate whether HD PSCs progresses forward in germ cells lineages, a prominent germ cells specification hallmark VASA was measured by qRT-PCR. VASA was presented across germ line development from PGCs, GCs and most abundantly in later stage of post-meiotic spermatocytes cells (Fujiwara et al., 1994; Castrillon et al., 2000). *In vitro* direct differentiation of rHD PSCs elevated VASA mRNA level throughout 10 days as shown by a slight increase at day 5 with highest level at day10 (Figure 4.1B) similar with WT control, revealing that *in vitro* differentiation induced germ cells production and progressed further into the maturation state. We then further

investigated whether rHD germ cells developed to SSCs in spermatogenesis. SALL4 is a pluripotency marker, which is presented during initiation of male spermatogenesis and shows expression-regulation in undifferentiated Spg together with a PLZF specific Spgs marker (Gassei and Orwig, 2013; Lovelace et al., 2016; Chan et al., 2017). Here, rHD, as well as rWT SSCs, exhibited SALL4 positive cells by immunofluorescence staining at ESCs state and SSCs day 10 (Figure 4.2, 4.3), similarly as with PLZF positive cells observed in day10, suggesting that rHD progressed forward to early SSCs as both Spgs marker detection. Furthermore, quantitative SALL4 mRNA expression was observed at the same level with rWT SSCs. rESCs exhibited highest level of SALL4 mRNA and then significantly decreased the expression from day 5 throughout 10 days differentiation (Figure 4.1C). The results revealed the transition of PSCs into undifferentiating Spgs around day5 and then persistently reduced the expression as the transformed population of immature germ cells into further mature state in later differentiation time at day 10.

4.4.2 rHD *in vitro* spermatogenesis interrupted Spgs progression, while reducing a meiotic or post meiotic SSCs marker

Spermatogonia stem cells (Spgs) or undifferentiating Spg are the basic foundation to maintain spermatogenesis. Similar with others stem cells, Spgs progress forward to differentiating spermatogonia and further mature germ cell of male gametogenesis. Taken together, Spgs have a self-renewing ability to maintain themselves to support the cells in the next round of spermatogenesis. Therefore, Spgs play a crucial role as a regenerative that is preserved to supply male gametogenesis. To define the presence of Spgs in our experiment, we examined the expression of PLZF (promyelocytic leukemia zinc-finger) a transcriptional repressor, which is restricted to undifferentiated Spgs and play a role in maintenance of undifferentiated state (Buaas et al., 2004; Phillips et al., 2010). The expression of PLZF was detected by ICC in both rWT and rHD SSCs day10 (Figure 4.2, 4.3). However, in order to quantify where PLZF population arise in our *in vitro* differentiation, we next carried out

PLZF gene expression by qRT-PCR at the time points during rSSC differentiation. Interestingly, we observed significantly different of PLZF expression between rWT and rHD. PLZF mRNA was first elevated the expression level at day5 after differentiation in both rWT and rHD, which that showed the arising point of highest undifferentiating Spgs population during *in vitro* spermatogenesis. rWT SSCs showed normal progression of Spgs to further maturing state as PLZF expression significantly declined until day10. On the other hand, rHD SSCs persistently expressed high PLZF population from day 5 to day 10, which were significantly different from rWT SSCs (Figure 4.4A, B), demonstrating that Spgs was arrested during *in vitro* spermatogenesis. This was supported by the results of Piwil1 expression, a later SSCs marker. Piwil1 is a murine piwi gene (miwi) that encodes a cytoplasmic protein specifically expressed in meiotic to post meiotic cells of diplotene spermatocyte through spermatid (Deng and Lin, 2002; S Tushir et al., 2009; Thomson and Lin, 2009; Bak et al., 2011). We observed that rWT SSCs increased Piwil1 expression throughout 10 days differentiation (Figure 4.4C). Taken together with PLZF, rWT SSCs progressed from early SSCs to more mature of meiotic or post-meiotic cells (Figure 4.4D), whereas rHD SSCs showed less progression of PLZF in to mature Piwil1 cells throughout the differentiation (Figure 4.4D). These results demonstrated that transgenic rHD interrupted the developmental process of mitotically inactive Spgs progressing forward to mature cells during *in vitro* spermatogenesis.

4.4.3 *In vitro* spermatogenesis of rHD PSCs elevated mHTT mRNA expression

Mutation of *HTT* (m*HTT*) causes pathogenic misfolding HTT proteins which are related to the pathogenic HD progression and disease severity. HTT mRNA expression are unclarified but seen widely from early development and persists in adulthood (Saudou and Humbert, 2016). Despite from the brain region, HTT mRNA is highly expressed in adult testicular cells (Van Raamsdonk et al., 2007). In order to determine the effect of transgenic mutant *HTT* on HD SSCs development, we next evaluated the level of mutant *HTT*

expression during *in vitro* spermatogenesis by qRT-PCR. Our results demonstrated the expression of HTT mRNA was detected in undifferentiated ESCs with increased expression as SSCs differentiation in day 5 in both rWT and rHD cells (Figure 4.5). The expression level of mutant HTT was highly expressed in rHD RPg7 SSCs day 10 when compared with rHD RPg5 and other WT cells, but they were not upregulated to the expression level compared to day 5 differentiated cells itself. Wheareas, rWT SSCs HTT mRNA expression declined in days 10. These results revealed rHD *in vitro* spermatogenesis elevated mHTT expression after 10 days differentiation. Notably, mHTT mRNA expression was observed as similar progression of PLZF Spgs that we see the same high HTT expression in day 5, and the persistently expression in day 10 related to the in transgenic rHD SSCs. However, it is not clear whether the expression of HTT mRNA in rHD SSCs is impacted by pathogenic mutation in Exon1 of *HTT* or in other cellular mechanism during *in vitro* spermatogenesis.

4.4.4 Pathogenic effects of CAG repeats mutation on *in vitro* spermatogenesis

Mammalian germ cell development and spermatogenesis requires precise homeostasis control as around 75% of all producing male germ cells are discarded through the apoptosis pathway (Shaha et al., 2010). In HD, aggregation of misfolding HTT protein carries expanded poly Q tract (>36 glutamines) resulting in aberrant neuronal apoptotic cells in various animals (Yang et al., 2008; Heng et al., 2009; Yang et al., 2010), thus HD is represented as a neurodegenerative disorder. In addition, HTT protein showed high expression level in HD patient's SSCs. Thus, in order to identify pathogenic effects of CAG repeat mutation on HD *in vitro* spermatogenesis, we first carried out an EM48 ICC staining protein aggregate in rSSCs. Initially, we could not detect the signal from our SSC samples. We then performed apoptosis assay to investigate whether transgenic *HTT* mutation can cause apoptotic progression in rHD SSCs. FACS based Muse annexin V apoptosis assay generated apoptotic profiles based on anexin V expression in the cells (Figure 4.6A, B). *In vitro* rSSCs differentiation discarded some cells population by significantly reducing about

40% of live cell populations in both rWT and rHD cells compared to rESCs before differentiation (Figure 4.7A). In particular, rWT rSSCs showing regular apoptosis pattern slightly increased from early to late apoptotic cells (10 to 20%) and yielded small 2.8% of dead cells population (Figure 4.7B). In contrast, rHD SSC at day 10 showed a higher early of apoptotic cells (25%) then significantly reduced late apoptotic cells (10%) which subsequently reduced the number of dead cells (1%) compared rWT (Figure 4.7B). The pathogenic cellular apoptotic in brain can cause aggressive neuronal cell death. In contrast to typical apoptotic pattern that were observed in HD brain (Portera-Cailliau et al., 1995; Hickey and Chesselet, 2003; Yang et al., 2010; Bano et al., 2011), our results reveal that rHD SSCs generated large portion of early apoptotic cells while they are not progression onward to dead cells. However, we did not confirm whether apoptotic in rSSCs impacted by only *HTT* CAG repeat mutation or maybe other impacts from *in vitro* spermatogenesis as we did not observe a significant difference of apoptotic cells between two transgenic HD cells.

4.4.5 Trinucleotide repeat expansion in rHD SSC derived from *in vitro* spermatogenesis

CAG mutation in Exon1 of HD mice testicular tissue is limited in post-meiotic of haploid germ cells (Kovtun and T McMurray, 2001). In contrast to adult HD patients' testicular tissue, CAG repeats expansion is presented from spermatogenesis throughout spermiogenesis (Yoon et al., 2003). The mutation evidence in adult HD testicular tissues is limited in finding the point where the mutation arises in earlier germ cells development. Here, we studied the instability of CAG mutant allele in SSCs derived from rHD PSCs *in vitro* differentiation which revealed earlier male germ cells progress throughout spermatogenesis based on SSCs characterization. TNR analysis in two rHD and two rWT ESCs before differentiation comparing with rSSC day 10 determined CAG repeat change in Exon1 of *HTT* gene in transgenic rHD spermatogenesis. GeneScan sizing of transgenic rHD demonstrated heterozygous disease allele in both RPg7 and RPg5 and showed 6-10 CAG

repeats of wild type endogenous allele. Range of the large HD disease allele were 81 to 131 repeats in ESCs and 81 to 132 repeats in SSCs of RPg7 (Figure 4.9B), 118 to 148 repeats in ESCs and 119 to 154 repeats in SSC of RPg5 (Figure 4.9C) respectively, while WT control cells showed 6-9 CAG repeats of endogenous HD allele (data not show). Transgenic rHD RPg7 showed major allele peak intensity at 447 with 116 CAG repeats in both ESCs and SSCs. The largest fragments are presented at 491 with 131 repeats in ESCs and 494 with 132 CAG repeats in SSCs respectively (Figure 4.9B), indicating CAG repeat expansion after a 10 days SSCs induction. Expanded HD allele increased one expansion size from 15 to 16 fragment alleles in ESC and SSC respectively (Table 4.1). Taken together, increasing of CAG instability from ESCs to SSCs are observed as shown by instability index at 0.654 to 1.1 respective, which that presented the changing repeat size from main allele ESCs before differentiation (Figure 4.8B). Notably, HD RPg5 revealed the large CAG repeats change after *in vitro* induction. The highest peak intensity is represented at allele 467 with 123 CAG repeats in ESCs and 479 with 124 repeats in SSCs. The largest fragment allele of ESCs and SSCs ends at 544 with 148 CAG repeats and 560 with 154 CAG repeats respectively, indicating large expansion of 6 CAG repeats size after *in vitro* spermatogenesis (Table 4.1). Instability of CAG repeats length indicated the large CAG instability in RPg5 HD SSCs as shown by increased 3.38 to 6.81 of instability index changing repeat size from ESCs before differentiation to SSC respectively (Figure 4.8B). Our study finds that male germ cells production *in vitro* demonstrated pathogenic CAG instability in HD during spermatogenesis, as previous studies of CAG repeat expansion agree in both human and mice spermatogenesis (Kovtun and T McMurray, 2001; Yoon et al., 2003).

Our generating rHD SSCs *in vitro* have demonstrated a high level of undifferentiated Spgs population present in PLZF gene expression in day 10 (Figure 4.4A). In these experiments, we observed CAG repeat expansion at day 10 corresponded to high PLZF level in both rHD SSCs, suggesting that HD mutation is present earlier germ cells

development of Spgs in rhesus monkey spermatogenesis. Notably, rHD RPg5, the largest expansion size, revealed low levels of Piwil1 as a maker of more mature SSCs compared to smaller CAG expanded cells in rHD RPg7, which that showed higher levels of Piwil1 (Figure 4.4 C). These results demonstrating that pathogenic expanded HD allele may impacted SSCs differentiation and maturation, which that caused Spgs arrest during *in vitro* spermatogenesis.

4.4.6 *In vitro* rHD SSCs demonstrated pathogenic male germline transmissible of HD expanded allele

Mutation of CAG expansion is a germline transmission and is unstable in either sex (Leeflang et al., 1999; Kovtun et al., 2000; Pearson, 2003). Alterations of CAG repeats is dependent on gender as in males represented repeats expansion, whereas female predominantly contract the repeats (Kovtun et al., 2000; Wheeler et al., 2007). We followed heritable changes of CAG length in male rESCs derived from transgenic rHD1 sperm, that indicated pathogenic cellular phenotype of HD in male gametes to embryonic cells state (Putkhao et al., 2013). Here, we planned to evaluate whether transgenic rPSCs can further inherit mutant *HTT* transgene after *in vitro* differentiation. We demonstrated CAG expansion in RPg7 and RPg5 HD SSCs derived ESCs, which were established from blastocyst of rHD1 sperm-fertilized oocytes given that rSSCs differentiation revealed pathogenic cellular phenotype of HD. To determine whether *in vitro* rSSCs represent paternal germline transmission of HD expanded allele, we differentiated RPg12 HD iPSCs which were derived from reprogramed rHD1 dental pulp stem cells as a paternal SSCs lineage of HD RPg7 and RP5. GeneScan sizing analysis demonstrated that rHD1 sperm carries four insertions of *HTT* transgenes and showing CAG range in large HD disease allele at 82 to 148 CAG repeats. HD RPg12, a reprogramming of somatic rHD1 cells demonstrated four insertions of mutant *HTT* transgene as shown in rHD1 sperm (Figure 4.10). Larger fragment of HD disease allele was presented at between 81 to 100 CAG repeats in RPg12 iPSCs (Figure 4.9A), suggesting

somatic instability of GAC repeats length specifically in each tissue of rHD1. Although, we couldn't observe increasing in mean number of repeats in rHD RPg12 SSCs (82-107 CAG repeats) from iPSCs (82-107 CAG repeats) (Figure 4.9A), rSSCs revealed higher CAG instability as shown in the 0.60 instability index than iPSCs before differentiation (Figure 4.8B), suggesting that *in vitro* differentiation results to the stability of CAG length in *HTT* transgene. We have demonstrated CAG expansion of rHD SSCs in both progenies cells of rHD1 sperm (HD RPg7 and RPg5). Here, we observed particular expansion repeats in paternal rSSCs, revealed the instability of CAG repeat length in each generation including small expansion size in HD RPg12 (< 1 CAG repeat), medium expansion (1 CAG repeats) in HD RPg7 and the large expansion repeat size (6 CAG repeats) in RPg5 SSCs respectively. These results demonstrated a paternal germline transmissible of HD disease allele during *in vitro* spermatogenesis, as high CAG instability occurred through *in vitro* spermatogenesis. Supporting testicular germ cells pathogenesis study in adult HD male patient.

4.5 Discussions

Huntingtin has presented as a spermatogenic gene (Van Raamsdonk et al., 2007; Yan et al., 2016). Disruption of normal function of *HTT* resulted in testicular pathology including testicular degeneration, abnormal germ cell progression and infertility. The imprinting arises from CAG instability in sperm (MacDonald et al., 1993; Telenius et al., 1994; Giovannone et al., 1997) as well as SSCs during spermatogenesis, revealed *HTT* mutation is predominantly a male germline transmission (Telenius et al., 1994; Kremer et al., 1995; Kovtun et al., 2000; Wheeler et al., 2007; Putkhao et al., 2013). Progression of pre-mutation repeats allele (29-35 CAGs) to disease range (>35 CAGs) with the large expansion size (>7 CAGs) occurs exclusively in paternal intergenerational transmission (McMurray, 2010). CAG expansion during male germ cells progression is not stable. The first study of R6/1 transgenic mouse model represented the expansion is most obvious in haploid cells after meiosis is completed (Kovtun and T McMurray, 2001; McMurray and Kortun, 2003). Unlike

HD patients, germ line expansion of CAG repeat occurs from premeiotic through the post meiotic cells of spermatogenesis. Therefore, majority of HD disease allele mutation have been found from differentiating spermatogonia, spermatocytes and haploid spermatid or sperm cells. However, larger expansion repeats size was found in haploid post meiotic cells population (Yoon et al., 2003). Thus, pathogenic cellular phenotype in testicular cells of mouse model as well as HD patient demonstrated multiple time points of CAG expansion during adult male germ cells progression. However, it is not clear whether gender specific mutation arises during paternal germline expansion only present in adulthood spermatogenesis or an earlier state such as prenatal male germline development. Previously, Kovtun et al. have reported that CAG expansion in mice was influenced by gender of embryo as represented by larger expansion size in male progeny, while a female presents the contraction from the same father (Kovtun et al., 2000), suggesting that male factor influenced mutation exists from embryo state before the start of developing germ cells. Study of earlier germ cells in HD patient remains a challenge since they are a limited source of immature SSCs and difficult to isolate primary germ cells along length of seminiferous tubule (Russell et al., 1993; Yoon et al., 2003; Sharma et al., 2017). Even though the study was carried out in mice model, the inter-species difference of cellular germ cells mechanism between mice and human revealed the challenge for using mouse model to recapitulates CAG mutation in human (Pearson, 2003). Thus, a nonhuman primate is the potential model to molecular genetic of human disease (Emborg, 2007; Chan, 2013; Cann, 2015; Havel et al., 2017), especially HD. As the transgenic HD monkey (Yang et al., 2008) shares cellular genetic as well as clinical feature with HD patient, therefore study of nonhuman primate male germ cells could reveal underlying mechanism of pathogenic HD on germline development.

We report the model study of male germ cells from embryonic pluripotent stem cells. Our *in vitro* differentiation corresponded to the series of *in vivo* germ cells development (Yoshida et al., 2006, Stukenborg et al., 2014; Saitou and Miyauchi, 2016). Here, pathogenic

rHD that carry mutant *HTT* transgenes are not impacted in early germ cells development as the Oct4, VASA and SALL4 expressed the same pattern with WT control. Our *in vitro* spermatogenesis represents pre and post-natal male germ cells progression as rSSCs gene expression profiles, suggesting that we have produced PGCs, Gonocytes, Spgs, differentiation Spgs and spermatocytes to spermatid like cells population and reveals that ultimately HD patient-specific spermatogenic cells can be derived *in vitro* with only PSCs. In contrast to previous reports in higher primate *in vitro* SSCs production (Yamauchi et al., 2009; Easley IV et al., 2012; Zhou et al., 2016; Sosa et al., 2018; Zhao et al., 2018), our pathogenic HD cells induction without genetic manipulation presented the quantitative data of particular germ cells as well as specific SSC hallmarks throughout differentiation time, which are important to define the SSCs state in each time point during *in vitro* spermatogenesis. Thus, this study represents a valuable tool for pathogenic SSCs production and study of disease mechanism in HD pateint, which that pathogenic *HTT* mutation was influenced to spermatogenesis and fertility.

Our finding that *in vitro* spermatogenesis revealing HD pathogenic cellular phenotype at least two points. First, rHD SSCs *in vitro* differentiation represents CAG expansion repeat in HD disease allele and demonstrates the effects of gender on CAG repeat expansion in rSSCs supporting that male germ cells contain an expanded disease allele similar to previous reports (Norremolle et al., 1995; Kovtun et al., 2000; Wheeler et al., 2007). The expression profiles of PLZF and Piwil1 SSCs markers in rHD SSC Day10, suggesting that mutation of CAG repeats arise earlier in mitotically inactive of Spgs of rhesus *in vitro* spermatogenesis. Cellular genetic mechanism of CAG mutation during mitotic or pre-mitotic diploid cells is unclear. Previous reports hypothesize that small expansion is mediated by DNA replication slippage while lager expansion is mediated by replication or genomic repair (Pearson, 2003; Yoon et al., 2003; McMurray, 2010). Our *in vitro* study recapitulates that large CAG expansion (6 CAG repeats) in HD germ cells may even present in prenatal state of male germ

cell progression. Notably, low level of Piwil1 mature germ cells in rHD SSCs supported that if the expansion arises from mutation in a large number of mitotic division of Spgs (PLZF), that would affect the renewing of SSCs throughout adult life (Leeflang et al., 1999; Wheeler et al., 2007). Thus, HD showed pathogenic effects on SSCs or testicular cells by extensive loss of Spgs progression.

Secondly, rHD SSCs inherited transgene from germ cells lineage mimics the pattern in parent-origin germ line transmission of expanded HD allele as in HD patients (Kremer et al., 1995; Leeflang et al., 1999; Yoon et al., 2003; Wheeler et al., 2007). rHD1 the first transgenic HD monkey (Yang et al., 2008) carries two mutant *HTT* transgenes and a GFP promoter which were represented by somatic instability of CAG repeats as shown in different repeats sizes in rHD1 sperm and rHD12 iPSCs. Our results showed that rRPg12 iPSCs (rHD1) carries the four insertions of transgenes similar with rHD1 sperm and increasing CAG instability of expanded allele in rSSCs after *in vitro* differentiation. RPg7 and RPg5 SSCs, the progenies cells (First generation), that were derived from blastocyst of rHD1 sperm injection (data not show) reduced transgene cluster with three insertions in RPg7 and only presented a large disease allele of mutant *HTT* transgene in RPg5. The reduction of a heritable transgene in progenies cells (RPg5 and RPg7) of rHD1 sperm is not clear as to whether they came from a typical gene delivery system as random integration of transgenes into chromosomes (Tolmachov et al., 2013) or the silencing of lentiviral transgene expression from extensive DNA methylation of specific promotor (Herbst et al., 2012; Qin et al., 2015). Notably, a heritable mutant *HTT* transgene subsequently increased the CAG repeats length from small to intermediate (in RPg7) and larger (in RPg5) repeats size after *in vitro* spermatogenesis, suggesting the paternal germ line transmission and instability of pathogenic CAG repeat occurred *in vitro* gametogenesis of HD monkey. The instability of CAG repeat found in male germ cells *in vitro* is an underlying mechanism of a trinucleotide repeat causing disease, which not only is present in HD, but also others diseases, including

spinocerebellar ataxia type 1 (SCA1) (Koefoed et al., 1998) SMBA (Xiao et al., 2012) and dentaturbral-pallidoluysian (DRPLA) (Sato et al., 1999), thus this study may guide to cellular pathogenic in others disease using *in vitro* SSCs differentiation.

An increase of mutant HTT mRNA was observed in our producing *in vitro* SSCs. Although, mutation of CAG repeat size is not correlated with level of mHTT mRNA, it is similar with data in previous studies (Lee et al., 2013; Shin et al., 2017). Together with mHTT mRNA level, we observed increasing of apoptotic cells after *in vitro* spermatogenesis compared to WT. However, abnormal apoptotic pattern is observed and that different from apoptotic and cell dead in the brain (Portera-Cailliau et al., 1995; Hickey and Chesselet, 2003; Yang et al., 2010 Bano et al., 2011). Underlying molecular pathway on pathogenic HD apoptosis in SSCs has not been reported. Based on our results, the concept that HD mutation may disrupt the regulation of male germ cells apoptosis, which is a demanding process of normal male germ cells progression (Shaha et al., 2010; Bejarano et al., 2018) therefore induced Spgs arrested during germ cells progression. In HD brain, m*HTT* dysregulates Bcl-2 family protein (Sassone et al., 2013) that results in neuronal mitochondrial disfunction and cell death. Taken together with previous reports that overexpressing of apoptosis protecting protein Bcl-2, and that eliminating the typical apoptosis in first wave of spermatogenesis results in accumulation of spermatogonia and infertility (Shaha et al., 2010). Thus, a mutant *HTT* gene may affect the function of apoptotic related genes resulting in pathogenesis in testicular cells and tissue in HD. Further study is needed to investigate as to how the molecular pathway is involved in the apoptosis pattern on spermatogenesis

Overall, *in vitro* production of HD spermatogenic cells in our nonhuman primate model anticipates genetic mutation during HD spermatogenesis. *In vitro* study of HD confirms genetic features of disease on male gamete which can capture the mutation point were limited to study in adulthood spermatogenesis. This reduction of a time-consuming model will lead to patient specific germ cells to *in vitro* studies and predicting disease

progression. Our present work provides valuable tools for clinical scale research since disease progresses on SSCs of HD patients and it supports unlimited male germ cells study by patient specific cells production. Additional study of cellular DNA metabolism on TNR expansion during *in vitro* germ cells development and HD pathogenic association between germ cells and neuronal cells will help in understanding the serious mutation progressed in HD, which will be a guide for developing new therapeutic strategies for HD and other diseases.

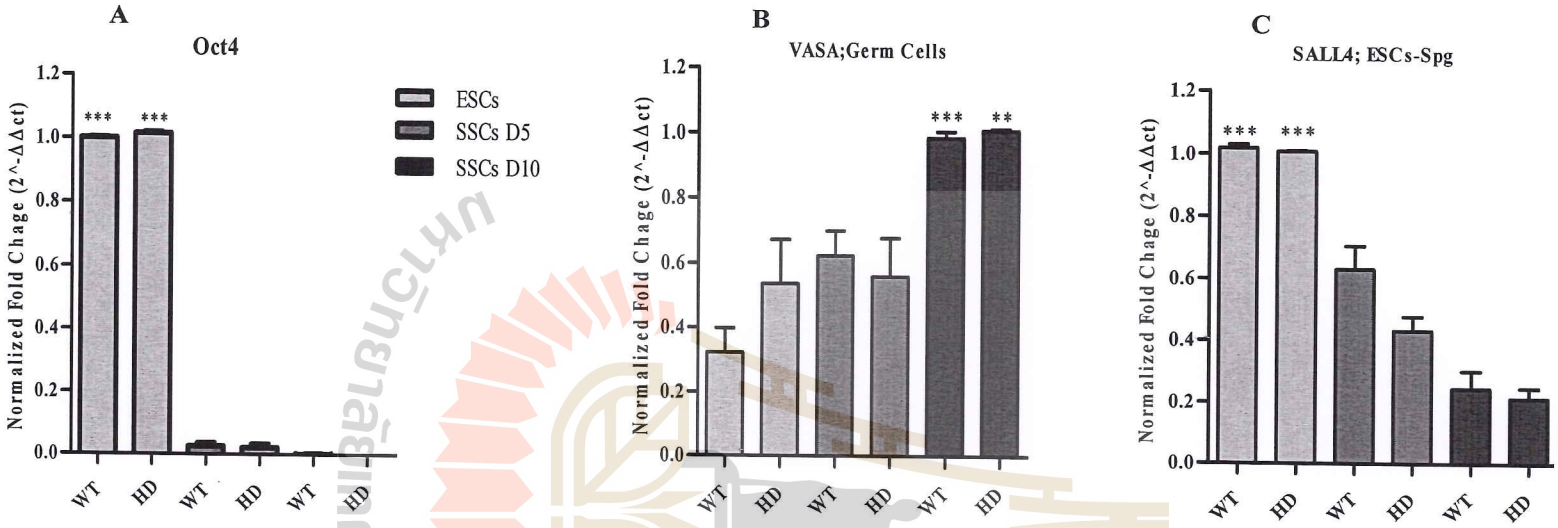

Figure 4.1 qRT- PCR analysis of early SSCs marker in rSSCs derived from *in vitro* spermatogenesis between transgenic rHD and WT control.

(A) Induction of rHD SSCs *in vitro* reduced Oct4 pluripotent marker from ESCs state throughout 10 days of differentiation.

(B) rHD SSCs elevated VASA a germ line specific marker expression throughout 10 days of differentiation similar with WT.

(C) SALL4 expression from ESCs, PGCs, GCs through Spgs state presented similarly in both rWT and rHD SSCs along 10 days of differentiation.

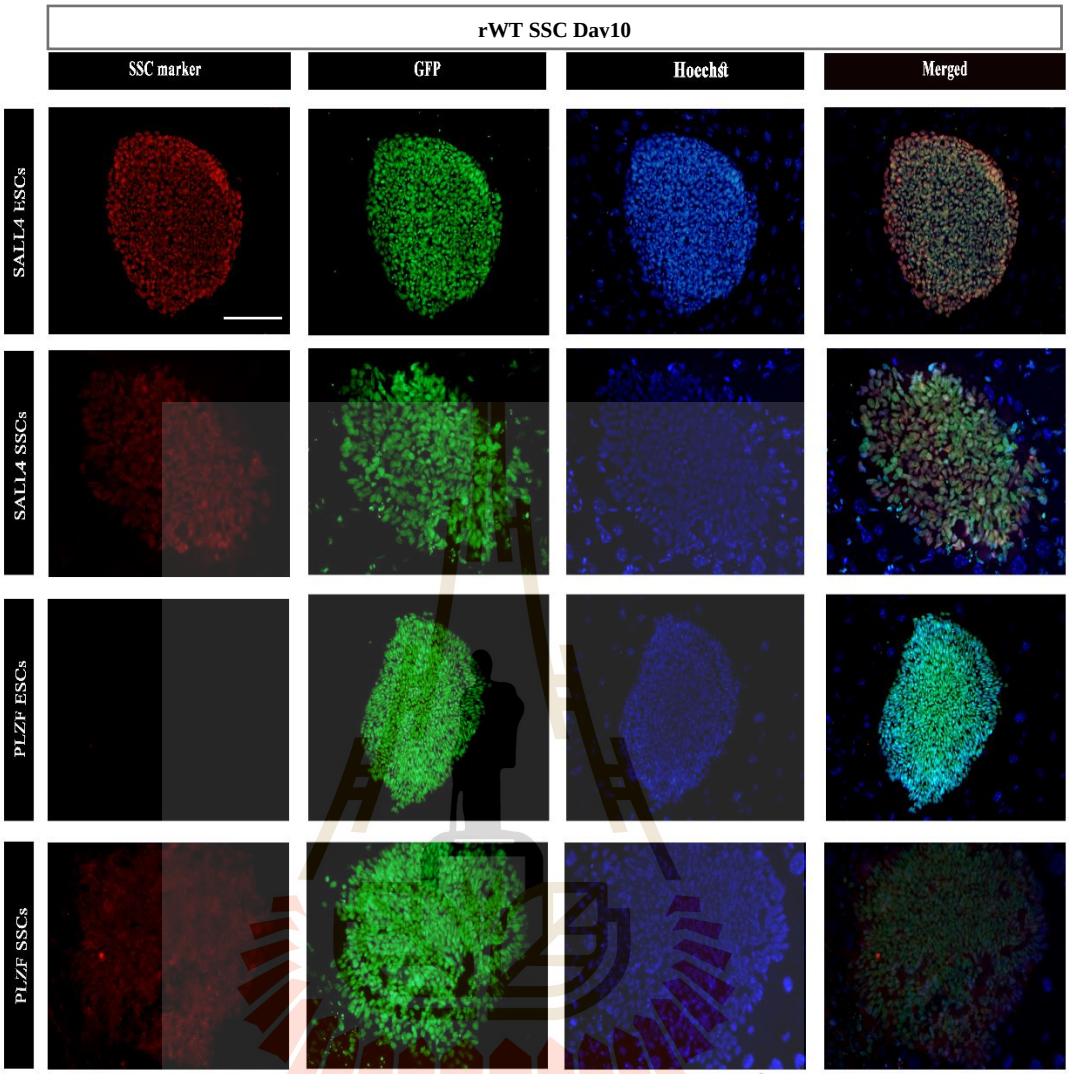

Figure 4.2 Immunofluorescence staining of rWT SSC Day 10 of *in vitro* spermatogenesis. rWT SSCs day 10 expressed SALL4+ (Red) in pluripotent ESCs and rSSCs day 10, while PLZF+, Spgs marker specifically expressed only in rSSCs differentiated cells. Endogenous green fluorescence protein (GFP; Green), Hoechst DNA staining (Blue) and scale bar = 200 µm.

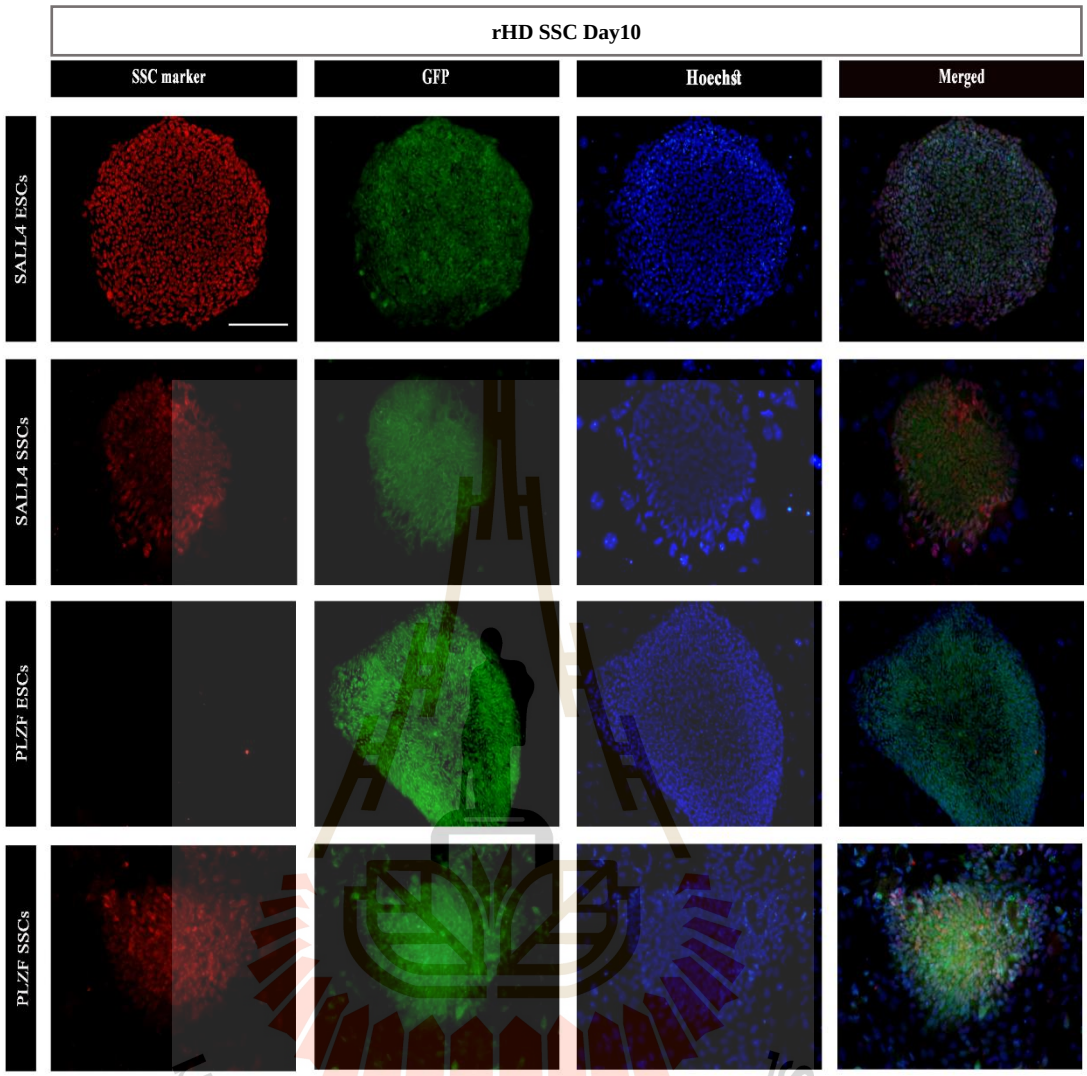

Figure 4.3 Immunofluorescence staining of rHD SSC Day 10 of *in vitro* spermatogenesis. *In vitro* spermatogenesis demonstrated male germ cells development from transgenic rHD ESCs into rHD SSCs as the same progressed in WT control.; SALL4+ (Red), PLZF+ (Red). Endogenous green fluorescence protein (GFP; Green), Hoechst DNA staining (Blue) and scale bar = 200 µm.

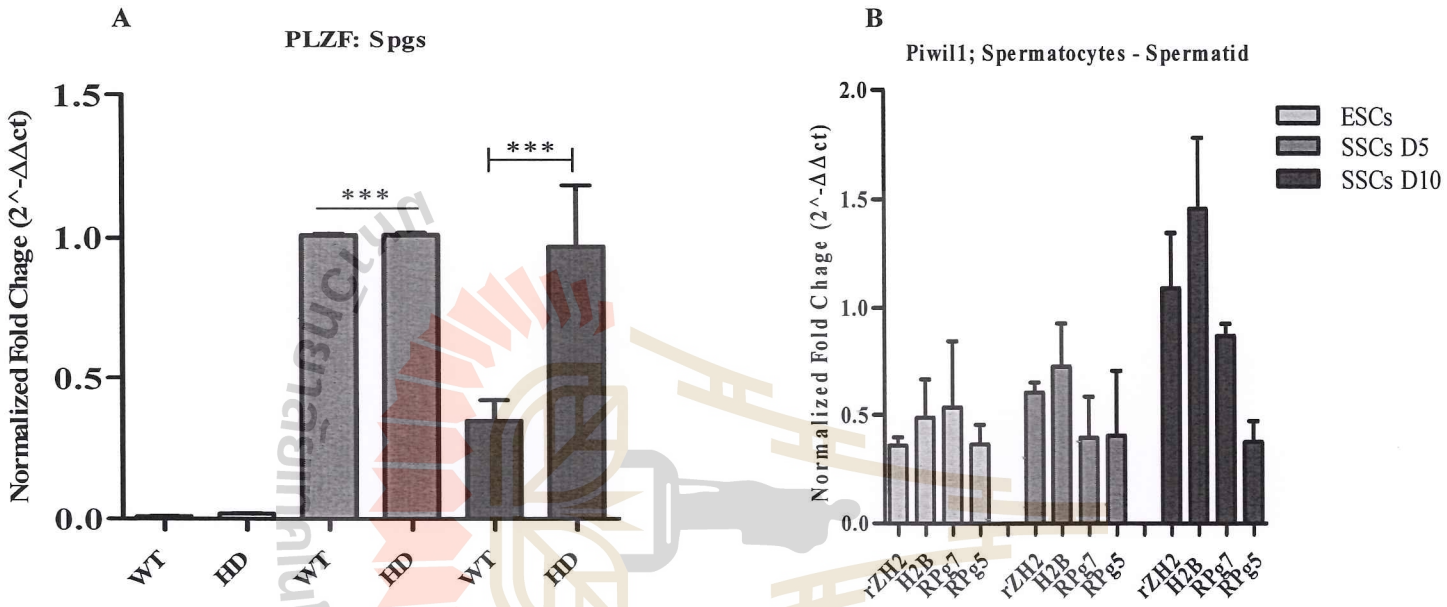

Figure 4.4 qRT-PCR analysis of rSSC markers during *in vitro* spermatogenesis.

(A) rHD elevated PLZF expression after induced for 5 days. Later days of differentiation rHD SSCs revealed significant greater of PLZF population compared WT. Results are the mean of triplicate repeats in each batch. Each data set are combined 6 biological replicates from two cell lines in each representative group. (B) Representative of PLZF, a Spgs specific marker in transgenic HD rSSCs and WT control individually. rSSCs differentiation elevated PLZF level in both WT and HD at day5. Longer induction time of rHD SSCs maintained PLZF level from day 5 throughout day 10. Data analyses from 3 different biological replicates.

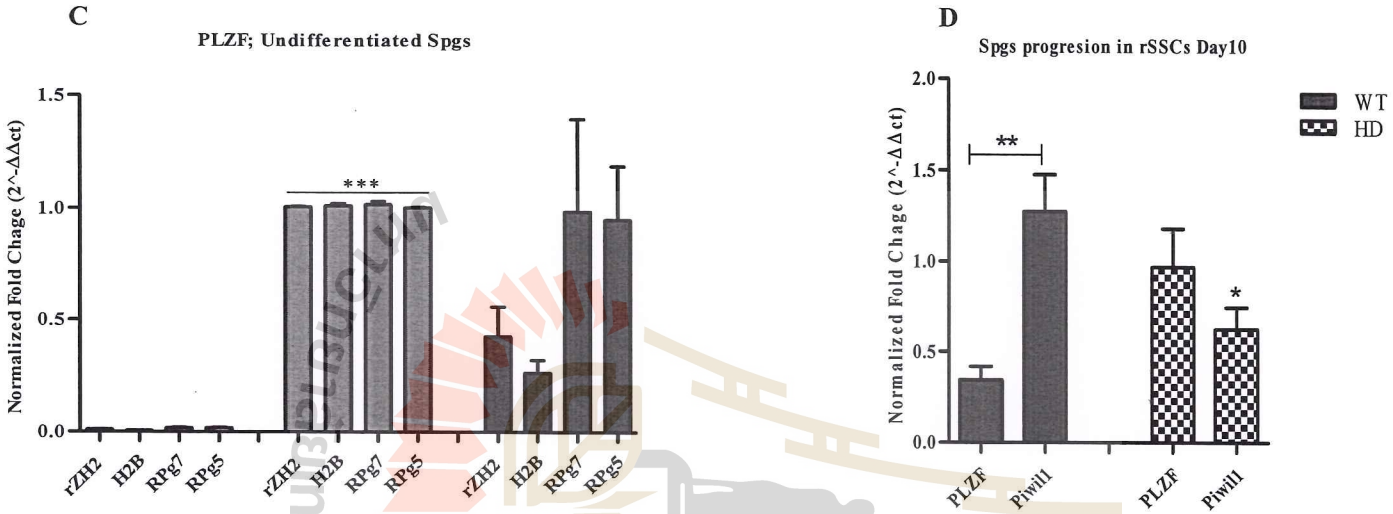

Figure 4.4 qRT-PCR analysis of rSSC markers during *in vitro* spermatogenesis.

(C) Piwil1 expression in rSSC throughout 10 days differentiation, rWT SSCs elevated Piwil1 expression over, while rHD SSCs revealed low level of Piwil1 expression especially in RPg5. Data analyses from three different biological replicates.

(D) Expression of PLZF corelated with Piwil1 level revealed differentiation potential of Spgs progressed forward mature SSCs *in vitro*. rWT SSC day10 significantly upregulated Piwil1 level compared PLZF expression. Whereas, rHD showed greater level of PLZF expression and lower level of Piwil1 expression compared rWT SSCs.

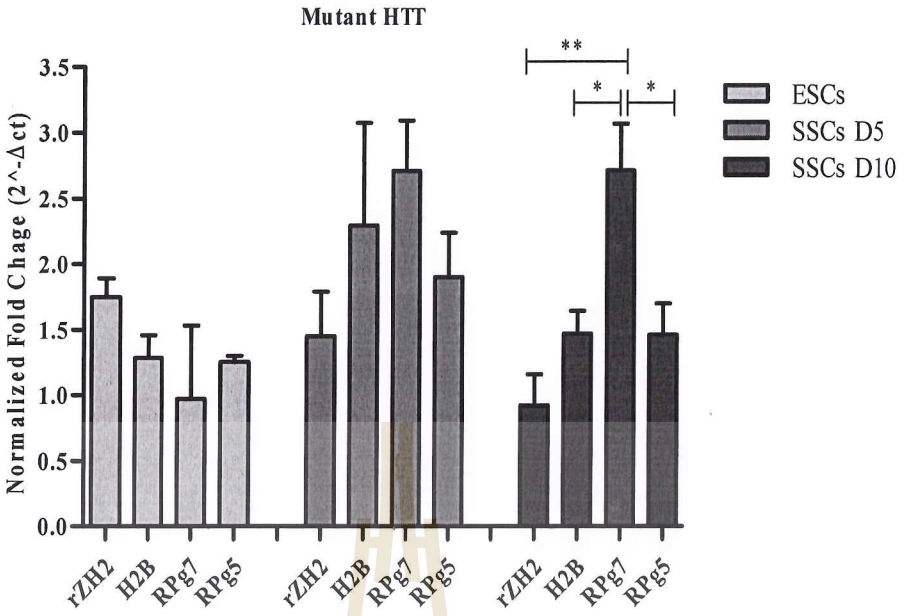

Figure 4.5 qRT PCR analysis of transgenic mutant HTT mRNA expression before and after differentiation. Demonstrative of mHTT mRNA expression in ESCs, rSSC D5 and rSSC D10. rHD SSCs differentiation elevated mHTT mRNA expression in Day5. Longer induction time of rSSCs day 10 reduced WT and RPg5 HD mHTT mRNA level, while RPg7 are maintained mHTT level from day5 through 10 days *in vitro* differentiation.

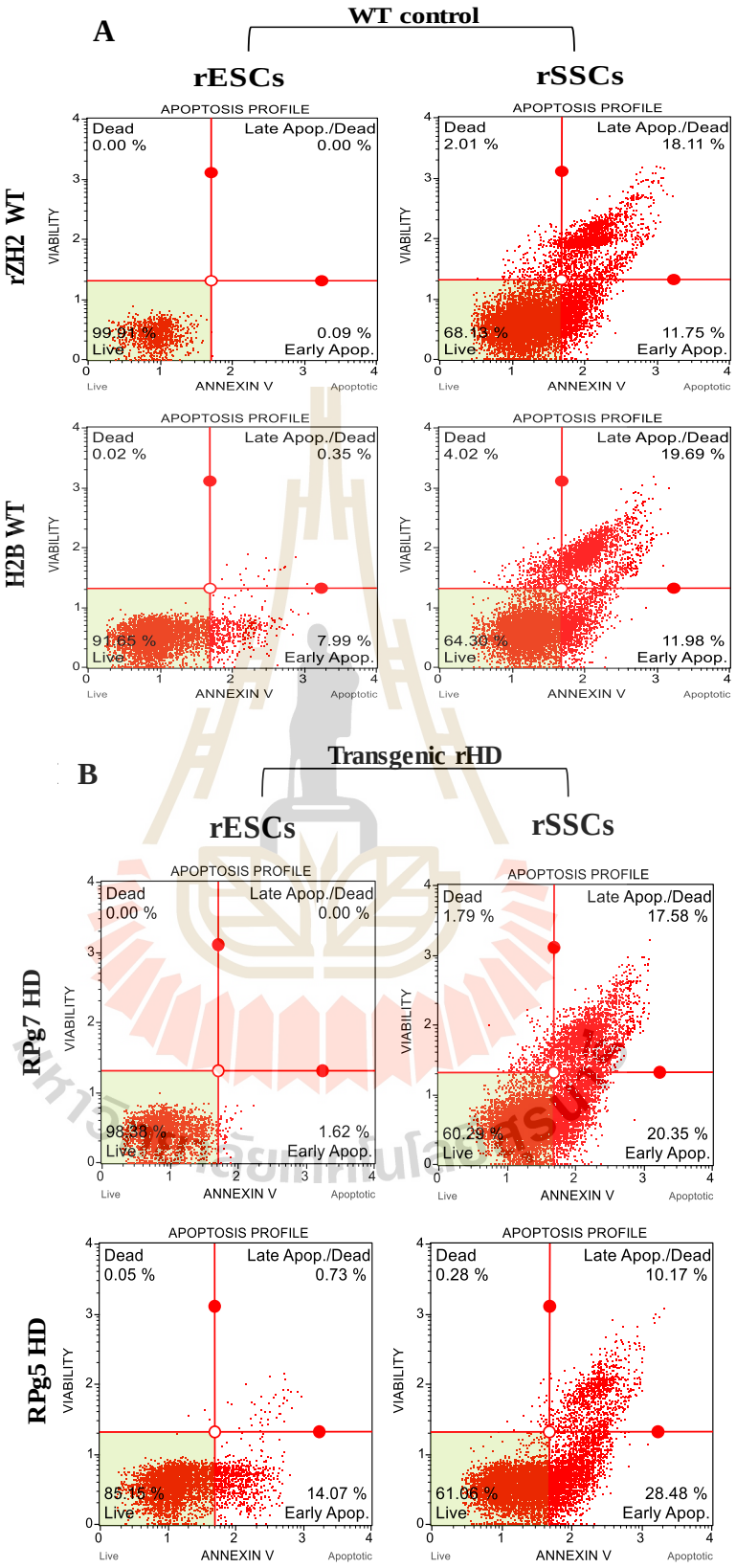

Figure 4.6 Annexin V apoptosis assay by FACS-MUSE analyzer, (A) Apoptotic profile in rWT control and (B) Apoptotic profiles in transgenic rHD cells between ESCs and differentiated cells at day 10.

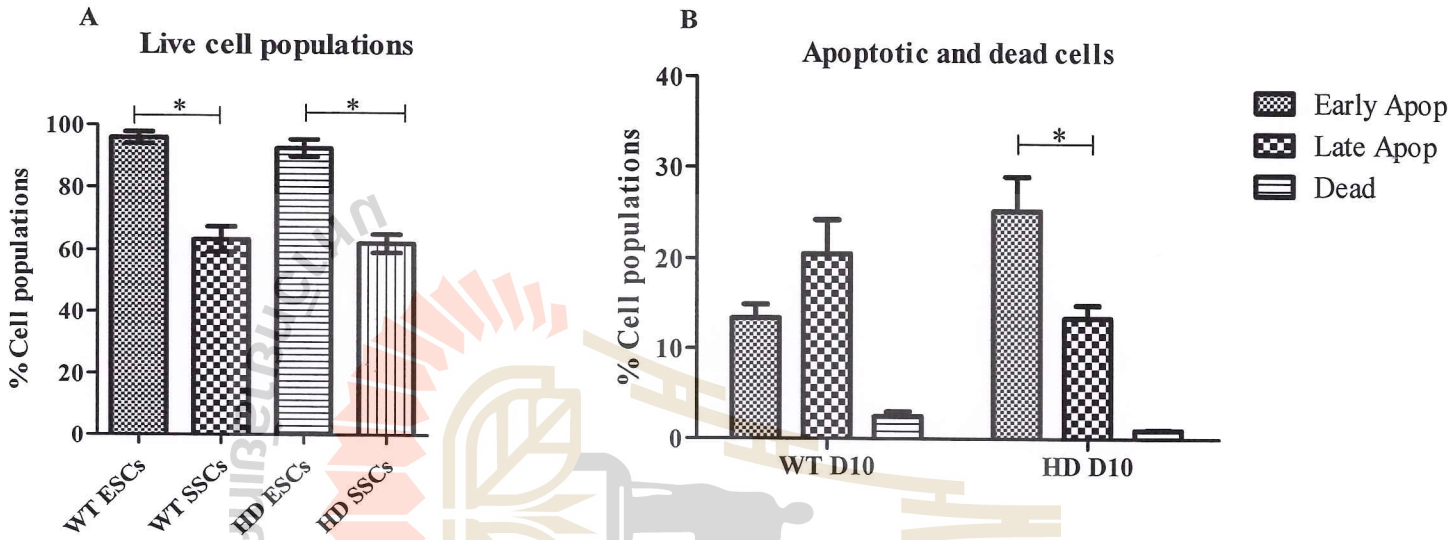

Figure 4.7 Graph representative the percentages of apoptotic cells in rHD and rWT control cells. (A) rSSCs *in vitro* differentiation discarded live cells population in both rHD and WT control. (B) rWT SSCs showing regular apoptosis pattern which are slightly increase of Early to Late apoptotic cells before progression forward to dead cells. Whereas, rHD SSC altered typical HD pathogenic cell death, as showing highly apoptotic cells in early state then significantly reduced in late apoptotic and dead cells. Results are the mean of triplicate repeats in each batch. Each data set are combined six biological replicates from two cell lines in each representative group.

Table 4.1 GeneScan trace in exon1 of *HTT* revealed allele fragments in rHD and rWT control between PSCs and SSCs Day10 after differentiation.

Samples	rPSCs					rSSCs				
	Normal allele (<26 CAG)	Intermediated allele (27-39 CAG)	Disease allele		Total allele	Normal allele (< 26 CAG)	Intermediated allele (27-39 CAG)	Disease allele		Total allele
			(>40 CAG)	Expanded allele (from main allele)				(>40 CAG)	Expanded allele (from main allele)	
rHD Pg12	25	12	32	4	73	25	12	32	5	74
rHD Pg7	28	16	24	6	75	30	15	32	7	76
rHD Pg5	27	14	22	10	73	26	14	28	16	84
rZH2 WT	8				8	11				11
YRES5 WT	6				6	5				5

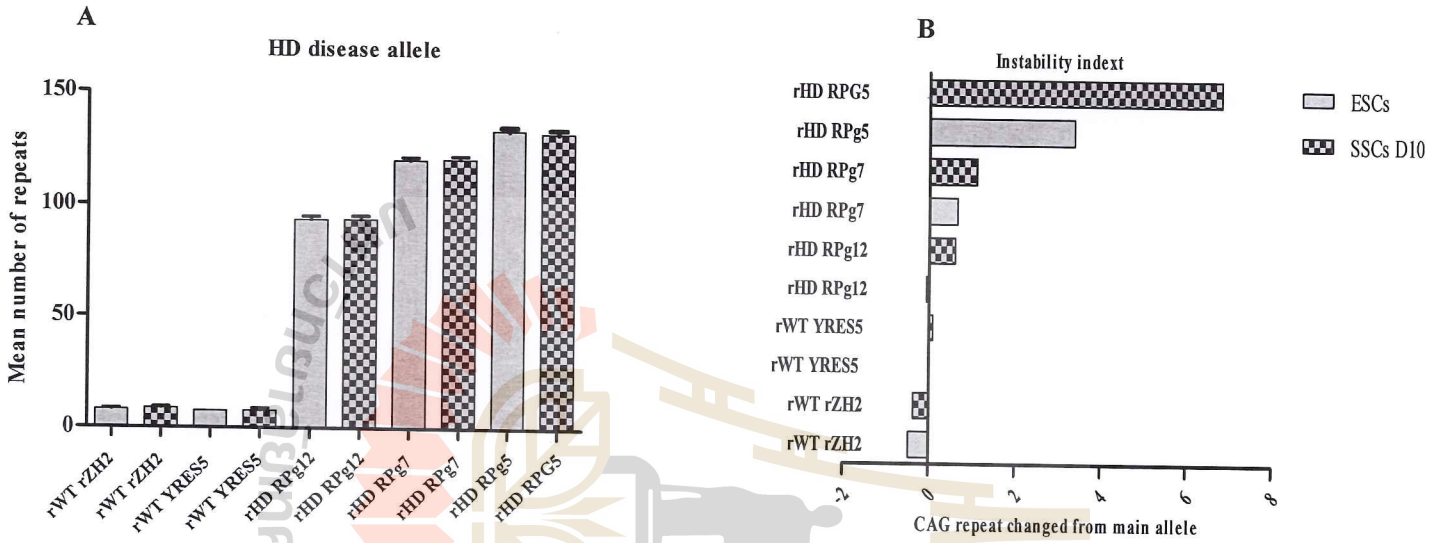

Figure 4.8 CAG repeat length and instability quantification. (A) Mean number of CAG repeat length in rHD before and after differentiation in rHD and rWT control. (B) Instability index of CAG repeats change from rESCs main allele before differentiation in rESCs compared rSSCs day10. All of rHD SSCs derived from *in vitro* spermatogenesis showed CAG instability with small (RPg12), medium (RPg7) and large (RPg5) CAG expansion repeat changed from rESCs before differentiation. While rWT control cells are observed contraction repeats in rESCs and after *in vitro* spermatogenesis.

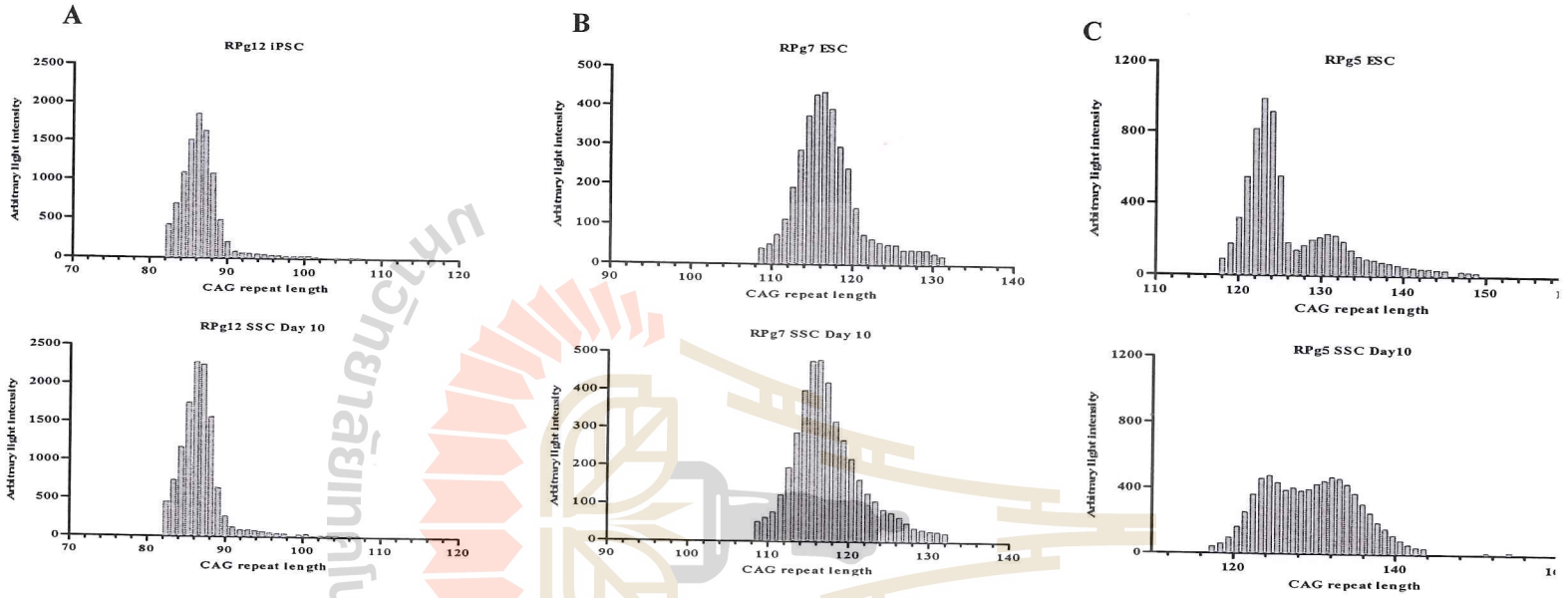

Figure 4.9 GeneScan sizing represented distribution of HD allele size (largest insertion size) of transgenic rHD SSCs Day 10 compared rPSCs before differentiation. (A) RPg12 revealed allele distribution from 40-100 CAG repeats in both iPSCs before and SSCs day10 after differentiation. (B) RPg7 revealed allele distribution from 40-87 and 40-89 CAG repeats in rESCs and rSSCs day10 respective. (C) RPg5 revealed largest distribution of HD disease allele with CAG length 40-115 and 40-122 repeat in ESCs and SSCs respective.

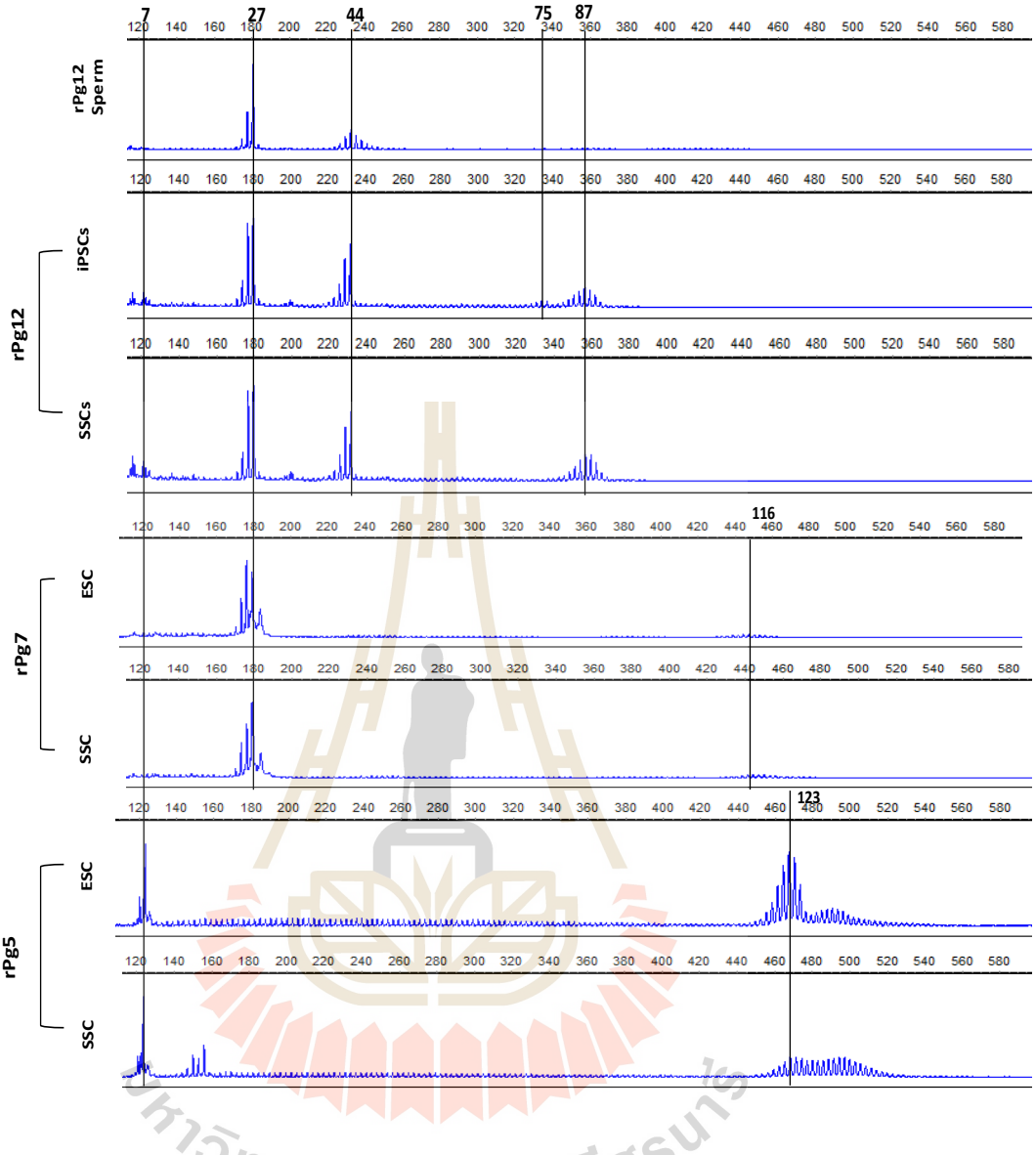

Figure 4.10 GeneScan sizing represented four insertions of *HTT* transgene inherited from rHD1 sperm to rPg12 iPSCs, rPg5 and rPg7 with increasing the expansion repeat size in large allele of rSSCs derived from in vitro spermatogenesis compared rESC in each group.

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CHAPTER V

OVERALL CONCLUSION

In vitro spermatogenesis is now becoming a powerful tool for male germ cells production outside the body. Significant progress has been made to better understand of male germ cells biology at the cellular and molecular level that is important to biomedical and male fertility preservation. The present study shows that male germ cells lineage including early germ cells (PGCs and GCs) and SSCs (Spgs, spermatocyte, spermatid) could be produced from *in vitro* differentiation of rhesus monkey PSCs. Using molecular characterizations, our derived germ cells from PSCs represented male germ cells with characteristics mimicking *in vivo* spermatogenesis, revealing that our rapid differentiation model has potential to induce pluripotent plasticity of PSCs into advanced male germ cells. The significance of deriving advanced male germ cells from higher primate, as in rhesus monkey, provides the greatest promising model for a longitudinal study of human germ cells biology. Artificially generated rSSCs *in vitro* may someday warrant the production of germ cells from PSCs as relevant to achieve an alternative source for infertility treatment in men.

Because no genetically modified rSSCs are derived from rPSCs differentiation, our method is suitable for the study of molecular genetic disorders of human disease. Our current work reveals that transgenic HD PSCs could be induced into male germ cells by expressing similar SSCs markers with wild type PSCs. Pathogenesis of *HTT* mutation in HD has an impact on *in vitro* spermatogenesis by maintaining PLZF spgs progression and reducing mature germ cells population throughout the differentiation time. Moreover, rHD SSCs derived through *in vitro* spermatogenesis increased CAG repeats expansion, which was observed from paternal rSSCs through their progeny's rSSC populations, revealing that

in vitro spermatogenesis could elucidate paternal germline transmissions of *HTT* transgene throughout the generations and represent the paternal bias of CAG repeat expansion which predominantly observed in HD patient. Production of rSSCs from transgenic rHD cells provides a valuable model for study of cellular pathogenic of HD on male germ cells biology, which will be an important step forward in biomedical and therapeutic strategy of HD research.

BIOGRAPHY

Sujittra Khampang was born in Surin, Thailand on February 2, 1990. She finished her high school at Phanomdongrakwitthaya School in Surin. In 2013, she received a Bachelor's Degree (B.Sc.) of Science in the Medical Science Program, Burapha University of Thailand. Then, she started her Ph.D. in Embryonic Stem Cells Technology at the School of Biotechnology, Institute of Agricultural Technology, with her Ph.D. program supported by SUT-PhD scholarship from Suranaree University of Technology. Her Ph.D. thesis title includes both *In vitro* spermatogenesis of rhesus monkey pluripotent stem cells and Polyglutamine instability in *in vitro* derived male germ cells from HD monkey PSCs. Part of this work was presented as an oral presentation at Human Genetic Seminar Retreat 2018 on September 7-8, 2018, at Chateau Elan Hotel and Resort, Braselton, Georgia, USA, and at a poster presentation at "The 7th Annual Meeting of the Society for Stem Cells Research" on February 6, 2019, Siriraj Hospital, Bangkok, Thailand.