การตรวจสอบคุณสมบัติและการเลี้ยงเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้
ในแมวก่อนและหลังวัยเจริญพันธุ์

นายณรงค์ ทิพธนวัฒนะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

CHARACTERIZATION AND CULTURE OF SPERMATOGONIAL STEM CELLS IN PRE- AND POST-PUBERTAL CATS

Mr. Narong Tiptanavattana

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	CHARACTERIZATION AND CULTURE OF SPERMATOGONIAL STEM CELLS IN PRE- AND POST-PUBERTAL CATS
By	Mr. Narong Tiptanavattana
Field of Study	Theriogenology
Thesis Advisor	Assistant Professor Theerawat Tharasanit, D.V.M., Ph.D.
Thesis Co-Advisor	Professor Mongkol Techakumphu, D.V.M., Doctorate de 3e cycle

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Veterinary Science
(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Sudson Sirivaidyapong, D.V.M., Ph.D)

.....Thesis Advisor
(Assistant Professor Theerawat Tharasanit, D.V.M., Ph.D.)

.....Thesis Co-Advisor
(Professor Mongkol Techakumphu, D.V.M., Doctorate de 3e cycle)

.....Examiner
(Nawapen Phutikanit, D.V.M., Ph.D.)

.....Examiner
(Professor Surasak Taneepanichskul, M.D., M.P.H., L.L.B., F.R.T.C.O.G.)

.....External Examiner
(Sasitorn Rungarunlert, D.V.M., Ph.D.)

ณรงค์ ทิพธนวัฒนะ : การตรวจสอบคุณสมบัติและการเลี้ยงเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ ในแมวก่อนและหลังวัยเจริญพันธุ์ (CHARACTERIZATION AND CULTURE OF SPERMATOGONIAL STEM CELLS IN PRE- AND POST-PUBERTAL CATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร.ธีรวัฒน์ ธาราศานิต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. น.สพ. ดร.มงคล เตชะกำพุ, 92 หน้า.

การศึกษาที่ 1 ศึกษาการเลี้ยงและการตรวจคุณสมบัติของเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ในแมว การทดลองที่ 1 ศึกษาอัณฑะของแมววัยเจริญพันธุ์ที่แสดงออกต่อโปรตีนจำเพาะของ GFRalpha-1 c-kit และ DDX-4 การทดลองที่ 2 และ 3 เซลล์อัณฑะที่ถูกย่อยและเลี้ยงภายนอกร่างกายด้วยระบบการเลี้ยงเฉพาะสำหรับเซลล์ต้นกำเนิดในแมว จากนั้นทำการเก็บกลุ่มเซลล์คล้ายเซลล์ต้นกำเนิดมาทำการตรวจคุณสมบัติ (การทดลองที่ 2) และการเลี้ยงภายนอกร่างกายร่วมกับเซลล์พี่เลี้ยง (การทดลองที่ 3) ผลการศึกษาพบว่า เซลล์สืบพันธุ์ในระยะต่างๆ มีการแสดงออกของโปรตีนแต่ละชนิดแตกต่างกัน โดยพบว่าเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้จะอยู่ที่บริเวณฐานของท่อสร้างอสุจิ และมีการแสดงออกของโปรตีนจำเพาะกับ GFRalpha-1 and DDX-4 เท่านั้น สำหรับการเลี้ยงภายนอกร่างกายนั้นพบว่า กลุ่มเซลล์ต้นกำเนิดจะมีลักษณะคล้ายพวงองุ่นที่ประมาณ 13-15 วันของการเลี้ยง ซึ่งกลุ่มเซลล์นี้มีการแสดงออกของยีน *GFRA1* และ *ZBTB16* แต่ไม่พบการแสดงออกของยีน *KIT* การศึกษานี้ประสบความสำเร็จในการแยกและเลี้ยงเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ในแมวได้ โดยเซลล์คล้ายเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้สามารถเลี้ยงภายนอกร่างกายได้ประมาณ 57 วัน

การศึกษาที่ 2 ศึกษาการจำแนกระยะการเปลี่ยนแปลงของเซลล์โกโนไซต์และเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ (G/SSC) เพื่อการพัฒนาการเลี้ยงเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ โดยแบ่งอัณฑะแมวออกเป็น 3 กลุ่ม (I. อายุน้อยกว่า 4 เดือน II. อายุ 4-6 เดือน III. อายุมากกว่า 6 เดือน) การทดลองที่ 1 ศึกษาการเปลี่ยนแปลงรูปร่างของเซลล์สืบพันธุ์โดยโครงสร้างทางจุลกายวิภาค โครงสร้างระดับจุลทรรศน์อิเล็กตรอนแบบส่องผ่านและเทคนิคอิมมูโนฮิสโตเคมี การทดลองที่ 2 ศึกษาการแสดงออกของโปรตีน GFRalpha-1 DDX-4 และ c-kit และหาจำนวนเซลล์ GFRalpha-1^{+} โดยวิธีโฟว์ไซโตมิเตอร์ การทดลองที่ 3 ศึกษาการแสดงออกของยีนและโครงสร้างระดับอิเล็กตรอนของกลุ่มเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ที่ได้จากอัณฑะกลุ่มที่ II และ III ผลการศึกษาพบว่าเซลล์สืบพันธุ์มีการเปลี่ยนแปลงโดยพบระยะ G/SSC โดยพิจารณาจากขนาดและรูปร่างของเซลล์โกโนไซต์และเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ อัณฑะของกลุ่มที่ II มีปริมาณเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ต่อท่อสร้างอสุจิเท่ากับ 17.66±2.20% และเซลล์ GFRalpha-1^{+} เท่ากับ 14.89±5.66% โดยพบว่าประสิทธิภาพการเกิดกลุ่มเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้จากกลุ่มที่ II นั้นสูงกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มที่ III (74.33±2.64% vs. 23.33±2.23%, p<0.001) และกลุ่มเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้มีการแสดงออกของยีน *GFRA1 ZBTB16 RET* และ *POU5F1* การศึกษานี้สรุปได้ว่าระยะ G/SSC เกิดขึ้นที่ช่วงอายุ 4-6 เดือน

การศึกษาที่ 3 ศึกษาความแตกต่างของการแยกความบริสุทธิ์ของเซลล์คล้ายเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ด้วยวิธีบนจานเพาะเลี้ยงที่เคลือบผิววัสดุของสารระหว่างเซลล์ชนิดต่างๆ และการปั่นเหวี่ยงด้วยสาร Percoll^{TM} การทดลองที่ 1 ศึกษาลักษณะทางจุลกายวิภาคและการแสดงออกของโปรตีน laminin SSEA-4 DDX-4 และ GFRalpha-1 ของอัณฑะแมววัยเจริญพันธุ์ (n=6) การทดลองที่ 2 ศึกษาจำนวนเซลล์คล้ายเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ที่ยึดเกาะกับผิววัสดุที่เวลา 15 30 และ 60 นาที โดยเปรียบเทียบจากเซลล์อัณฑะที่ถูกเลี้ยงบนจานเพาะเลี้ยงที่เคลือบผิววัสดุด้วยสารลามินินและเจลาติน การทดลองที่ 3 ศึกษาประสิทธิภาพของการใช้การปั่นเหวี่ยงด้วยสาร Percoll^{TM} ร่วมกับวิธีบนจานเพาะเลี้ยงที่เคลือบผิววัสดุเพื่อเพิ่มประสิทธิภาพของการแยกความบริสุทธิ์ ผลการศึกษาพบว่า ลักษณะทางจุลกายวิภาคแสดงถึงอัณฑะมีกระบวนการสร้างอสุจิโดยสมบูรณ์ และมีการแสดงออกของลามินินที่ท่อสร้างอสุจิ นอกจากนี้เซลล์สเปอร์มาโตโกเนียมยังแสดงออกโปรตีน SSEA-4 และ GFRalpha-1 ร่วมกับ DDX-4 อีกด้วย นอกจากนี้เซลล์คล้ายเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ที่ได้จากผิววัสดุลามินินที่ 15 นาที โดยประเมินจากเซลล์ที่แสดงออกโปรตีน SSEA-4 (59.42±2.18%) และ GFRalpha-1 (42.70±1.28%) เมื่อเปรียบเทียบกับกลุ่มเซลล์ที่ได้จากผิววัสดุเจลาติน (p<0.05) และพบว่าวิธีการปั่นเหวี่ยงด้วยสาร Percoll^{TM} ร่วมกับการคัดเลือกด้วยผิววัสดุลามินินนั้นจะให้เซลล์คล้ายเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้ที่มีอัตราการรอดชีวิตเพิ่มขึ้น (91.33±0.14%, p<0.001) และมีความบริสุทธิ์เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (83.82±2.05% ของ SSEA-4 และ 64.39±1.51% ของ GFRalpha-1, p<0.05) โดยเซลล์ที่เกาะนั้นมีลักษณะรูปร่างที่จำเพาะกับเซลล์ต้นกำเนิดเซลล์สืบพันธุ์เพศผู้และมีการแสดงออกของยีน *POU5F1 RET* และ *ZBTB16*

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5375952531 : MAJOR THERIOGENOLOGY
KEYWORDS: DEVELOPMENT / DOMESTIC CAT / EXTRACELLULAR MATRIX / GENE EXPRESSION / IN VITRO CULTURE / ISOLATION / PURITY / SPERMATOGONIAL STEM CELL / TRANSITION PHASE

NARONG TIPTANAVATTANA: CHARACTERIZATION AND CULTURE OF SPERMATOGONIAL STEM CELLS IN PRE- AND POST-PUBERTAL CATS. ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., CO-ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorate de 3e cycle, 92 pp.

Study 1 : aimed to culture and characterize feline SSCs. In Exp. 1, testes from pubertal domestic cats were immunolabeled to examine the expression of markers (GFRalpha-1, c-kit and DDX-4). In Exp. 2 and 3, testicular cells were digested and cultured *in vitro* using a modified SSC culture system. The resultant presumptive SSC colonies were then collected for SSC identification (Exp. 2), or further cultured *in vitro* on feeder cells (Exp. 3). Morphology, gene expression and immunofluorescence were used to identify the SSCs. Exp. 1 demonstrated that varying types of spermatogenic cells exist and express different germ cell/SSC makers. A rare population of SSC located at the basement membrane of the seminiferous tubules was specifically identified by co-expression of GFRalpha-1 and DDX-4. Following enzymatic digestion, grape-like colonies formed by 13-15 days of culture. These colonies expressed *GFRA1* and *ZBTB16,* but did not express *KIT*. Although we successfully isolated and cultured feline SSCs *in vitro*, the SSCs could only be maintained for 57 days. In conclusion, this study demonstrates for the first time, that SSCs from testes of pubertal domestic cats can be isolated and cultured *in vitro*. These cells exhibited SSC morphology and expressed SSC specific genes

Study 2 : aimed to identify G/SSC transition and also to improve the SSC establishment *in vitro*. The testes were divided into 3 groups according to donor age (I:<4 months, II:4-6 months and III:>6 months). Exp. 1 studied the development and morphology of testicular cells by histology, transmission electron microscopy, immunohistochemistry. Exp. 2 determined expression of GFRalpha-1, DDX-4 and c-kit. The numbers of GFRalpha-1^{+} cells were also analyzed using flow cytometry. The established SSCs isolated from group II and III were characterized by mRNA expression and TEM (Exp. 3). Chronological changes of testicular cells, in terms of morphology, proliferation and apoptosis markers at G/SSC transition were demonstrated. The size and morphology including the ultrastructure of SSCs were apparently distinguishable from the gonocytes. The results demonstrated that group II testes contained highest numbers of SSC per seminiferous cord/tubule (17.66±2.20%) and GFRalpha-1^{+} cells (14.89±5.66%) compared with other groups. The findings coincided with an increased efficiency of SSC derivation in group II when compared to group III (74.33±2.64%vs.23.33±2.23%, $p<0.001$). The resulted colonies expressed mRNA essentially importance for SSCs (*GFRA1*, *ZBTB16*, *RET* and *POU5F1*). This study concludes that the G/SSC phase occurs at 4-6 months of age.

Study 3 : aimed at purifying SSC-like cell using different types of extracellular matrixes and the discontinuous gradient density. In Exp. 1, the testes (n=6) were analyzed for histology and protein expressions of laminin, SSEA-4, DDX-4 and GFR alpha-1. Cell suspension after enzymatic digestion was plated onto either laminin or gelatin coated dish. The number of SSC like cells in relation to total attached cells were determined at 15, 30 and 60 min of culture (Exp. 2). The Exp. 3 was performed to test whether or not the additional step of Percoll™ gradient density could really improve purification of SSCs. Testicular histology represented the complete spermatogenesis with laminin expression at the extracellular matrixes surrounded SSC and basal lamina of the seminiferous tubules. SSEA-4 and GFRalpha-1 co-localized with DDX-4 essentially expressed in spermatogonia. The relative percentage of SSC-like cells, as determined by SSEA-4 (59.42±2.18%) and GFRalpha-1 (42.70±1.28%) expressing cells revealed that the highest SSC-like cell purity was obtained for the 15-min incubation on laminin coated dish compared with other incubation times and gelatin treatment ($p<0.05$). The Percoll™ treatment prior to laminin selection (15 min) significantly improved SSC-like cell recovery (91.33±0.14%, $p<0.001$) and purity (83.82±2.05% for SSEA-4 and 64.39±1.51% for GFRalpha-1, $p<0.05$). These attached cells demonstrated a typical SSC morphology and also expressed *POU5F1*, *RET* and *ZBTB16* mRNA. The double SSC selection by Percoll™ treatment and laminin plating is a simplified technique for SSC purification.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This part of thesis is free to write whatever from “thanking” mind to someone and something in my fortunate PhD life. My PhD achievement has been supported by the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej and by the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University.

My sincere gratitude goes to my excellent advisor, Assistant Professor Dr. Theerawat Tharasanit, who has always supported me all of my life. Thank you for your incessant dedication for scientific training, tons of valuable commentaries and suggestions, including your superb English teaching (be relaxed, it will be okay!). Besides, a number of fruitful instructions and comments from co-advisor, Professor Dr. Mongkol Techakumphu, have been considerably appreciated. Distinguished Scholar Dr. Chainarong Lohachit, you are my very best "teacher" in small animal reproduction; you always teach and give me several decent opportunities through my CU life. I am really grateful for senior project advisor, Associate Professor Dr. Wijit Banlunara, for giving the IHC experience. For spending my life abroad, I wish to express my respectfulness to Professor Dr. Poul Hyttel and Hanne Holm, for your fantastic discussions on the experimental results and weather forecast.

The accomplishment of this thesis will not take place if these people does not exist on my world. Dr. Chommanart Thongkittidilok, you not only provide me the kindness as my colleague, but you are also my initial PCR trainer. Dr. Pachara Pearodwong, thank you for your help in fighting with “E”-thesis. Many thanks to TT’s lab partners: Dr. Sirirak Buarpung, Dr. Nuttha Klincumhom, Dr. Praopilas Phakdeedindan, and Dr. Piyathip Setthawong. To the “very” perfect "working!!" team: Dr. Thitida Pakdeesaneha, Dr. Nicole S. Mehl and Dr. Raya Treecharoen, I never regret spending more time with both of you because of “More LINE, much closer”. Thank you for your support and friendship to my brothers and sisters: Dr. Sirichai Techarungchaikul, Dr. Atthaporn Roongsitthichai, Dr. Siriluck Luckanahasaporn, Dr. Piyanon Prasarnwong, Dr. Panyarat Chongsawat, Dr. Nothethawat Theeramoke, Dr. Cholayuth Bhanmeechao, Dr. Kajohnwat Seikulthanakij, and Dr. Nawaphat Somboonpoonpol. To my dear and best friends, Dr. Chartpat Pholpark, Dr. Nopgavee Rieosanguanwong, Dr. Kamut Tepsuthammarat, Dr. Kna Mutithanon, Dr. Khunti Saduakkarn, Dr. Araya Radtanakatikanon, Dr. Thitiporn Thongsima, Dr. Nae Tanpradit, and Dr. Kasemsri Sajjaviriyakul, you all have made me “you will never walk alone.” Furthermore, the appreciation goes for all those, whose names are not listed, who has supported me even in the shadow.

Special thanks to my father, my mother, and Nong P, as well as Mamee and Foithong, who always give me love and stand closely by my side. Eventually, I wish to thank my another half, You and Me, to walk with hand-in-hand.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

°C, C	Degree centigrade, Celsius
µg/ml	Micrograms per millilitre
µm	Micrometre
µM	Micromolar
ANOVA	Analysis of Variance
bp	Base pairs
BTB	Blood-testis barrier
BSA	Bovine serum albumin
CDH1	E-cadherin type 1
CDK2	Cyclin dependent kinase 2
cDNA	Complementary deoxyribonucleic acid
cm^2	Square centrimetre
CO_2	Carbondioxide
CSF-1	Colony stimulating factor 1
DDX-4	Dead (Asp-Glu-Ala-Asp) box polypeptide 4
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EthD-1	Ethidium homodimer-1
EpCAM	Epithelial cell adhesion molecule
ES	Ectoplasmic specialization
ESC	Embryonic stem cell
Exp	Experiment
FACs	Fluorescence-activated cell sorting
FGF2, bFGF	Fibroblast growth factor (basic)
G/SSC	Gonocyte/spermatogonial stem cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFRα-1, *GFRA1*	GDNF family receptor α-1
GIST	Gastrointestinal stromal tumor
h	Hour
HBSS	Hanks' balanced salt solution
H&E	Hematoxylin and eosin

IF	Immunofluorescence
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IU/ml	International units per millilitre
KIT, c-kit	c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LIN28	Lin-28 homolog A
M	Molar
MACs	Magnetic-activated cell sorting
min	Minute
MEF	Mouse embryonic fibroblast
mg/ml	Milligrams per millilitre
ml	Millilitre
mRNA	Messenger ribonucleic acid
ng/ml	Nanograms per millilitre
nm	Nanometre
NPH	Non-human primate
NTC	No template control
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLZF	Promyelocytic leukemia zinc finger
POU5F1	POU domain class 5 homeobox 1
TBE	Tris-Borate-EDTA
TEM	Transmission electron microscopy
Thy-1, CD90	Thymus cell antigen-1
RET	RET proto-oncogene
RNA	Ribonucleic acid
rrGDNF	Recombinant rat glial cell line-derived neurotrophic factor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCF	Stem cell factor
SD	Standard derivation
sec	Second
SEM	Standard error of mean
SSC	Spermatogonial stem cell
SSEA-4	Stage-specific embryonic antigen-4

U/ml	Units per millilitre
µg/ml	Micrograms per millilitre
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume
ZBTB16	Zinc finger and BTB domain containing 1

CHAPTER 1

INTRODUCTION

1.1 Importance and rationale

Research in stem cell has enormous impact, directly and indirectly to human/animal health and also to biological research models. Various cell types derived from stem cells have become a promising tool for treatment of incurable diseases principally via cell-based regenerative medicine. Furthermore, the stem cells can also generate *in vitro* diseased models, and thus the use of laboratory animals can be reduced. Spermatogonial stem cells (SSCs) are only the adult stem cells that preserve and pass the genetic materials to the next generation (Sato et al., 2011). The SSCs are the subpopulation of undifferentiated spermatogonia that regulate the spermatogenesis and produce more than billion sperm throughout pubertal life (Tagelenbosch and de Rooij, 1993). The SSCs have been classified as unipotent stem cells in the testis but, later on, some evidences also suggested that they also can differentiate into several cell types of three germ layers (Guan et al., 2006; Seandel et al., 2007; Ko et al., 2009; Kossack et al., 2009). During the past decade, an advancement of SSC technology, aimed specifically at developing the SSC culture system and attempting to produce sperm *in vitro*, has been made (Kanatsu-Shinohara et al., 2003a). However, such progress has been highly-developed only in some species (*i.e.* rodent and non-human primate (NPH)), while the mechanism that maintains the SSCs in culture is largely unknown in other species, including in domestic cat (Aponte et al., 2008).

SSCs have been believed to serve as potential approach for treating germ cell-depleting problems, restoring the testicular functions and also for a novel strategy in male germ cell banking. As cat genome is highly conserved and exhibits the most similarity to that of human, it is therefore preferable to use cat as a model for inherited and infectious diseases such as acquired immune deficiency syndrome (AIDS) (Vester et al., 2010; Wongsrikeao et al., 2011). In addition, domestic cats whose have similar

reproductive physiology to wild cats, have become an important model for conservation of wild cat species; most of wild cats are classified as threatening, valuable or endangered. It is therefore preferable to develop the reproductive technology, essentially in order to develop a novel strategy for producing gametes *in vitro*. Successful culture of SSCs in domestic cats would allow the researcher to study the factors that regulate the spermatogenesis, and the knowledge obtained may facilitate the development of *in vitro* production of germ cells in domestic and wild cats. However, the SSC research in domestic cat and other felids have not been reported and the knowledge related to the spermatogenesis is very scant (Kim et al., 2006). To increase our knowledge, the ultrastructure of SSCs and their niche should be examined in order to underpin the structural complexity of the cells and its surrounding structures. Furthermore, the development of germ cells inside the seminiferous tubule during pre- and post-pubertal ages also help to determine the transition period of gonocyte to undifferentiated spermatogonium.

Hence, this study aims specifically at developing the novel strategy to produce SSCs in domestic cats. The ultrastructure of germ cells and its surrounding structures, together with the examination of undifferentiated and differentiated markers of SSCs in domestic cat, will be studied. In addition, the factors that potentially maintain the SSCs *in vitro* will also be studied. These studies would allow us to in-depth understanding the fate and factors that regulate the viability and functions of SSCs.

1.2 Objectives of the thesis

1. To characterize SSC germ cell markers and to examine the efficacy of *in vitro* culture in domestic cats

2. To study on the development and changes of male germ cells in the seminiferous tubules in relation to age of domestic cats

3. To characterize the cellular changes during transition of gonocyte to spermatogonial stem cell (G/SSC)

4. To study on ultrastructure of undifferentiated spermatogonium and its surrounding structures

5. To examine the efficacy of enrichment assays of spermatogonial stem cells in domestic cats

1.3 Research delimitation

This research was undertaken from January 2012 to February 2015. All testes of this research were collected from domestic cat (*Felis catus*) following castration at the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Bangkok, Thailand and the Division of Obstetrics, Gynaecology and Reproduction, The Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. In chapter 3, testes from 5 pubertal cats were studied on the immunohistochemistry of SSC-related markers (GFRα-1, DDX-4 and c-kit) and identification and longevity culture of *in vitro* SSC. The chapter 4 was designed to define the gonocyte/ spermatogonial stem cell (G/SSC) transition during postnatal cat and analyse the phenotype expression and flow cytometry of SSC markers. In addition, the efficiency of *in vitro* SSC derivation was compared between the pre- and post-pubertal testicular cells. The final article (Chapter 5), testicular cells of pubertal cat were used to examine the effects of different extracellular matrixes and discontinuous gradient density on efficiency of SSC enrichment.

1.4 Research benefits

1. The first report on cat SSCs including the development, characterization, morphology and ultrastructure and *in vitro* culture system.

2. Providing a novel knowledge on the development of germ cells in the seminiferous tubules in relation to age of domestic cats and also determination the transition period of gonocyte to spermatogonial stem cell.

3. Demonstration the effective technique for enrichment the SSC populations from digested testicular cells of pubertal cats.

CHAPTER 2

LITERATURE REVIEW

Spermatogonial stem cells (SSCs) are subpopulation of spermatogenic precursor cells in men and male animals, also called undifferentiated spermatogonia. They have been believed to be used for regenerative medicine and male gamete sperm banking (Simon et al., 2010; Silva et al., 2012). Interestingly, these cells have also been demonstrated to express POU5F1 and fully differentiated into three germ layers and formed teratoma similar to that of embryonic stem cells (Guan et al., 2006; Seandel et al., 2007; Dann et al., 2008; Ko et al., 2009; Kossack et al., 2009). Thus, these SSCs can be used as alternative pluripotent model. Until recently, *in vitro* culture of SSCs has successfully been established in several species, but not in felids.

2.1 Development of embryonic male germ cell lineage

During development of mouse embryos (Fig. 1), primordial germ cells (PGCs) are derived from the proximal epiblast or embryonal ectoderm. During day 7.25 to day 10.5 after fertilization, the PGCs migrate from the base of allantois and pass the hindgut to genital ridges (Hayashi et al., 2007). The PGCs within XY-determinated embryos then proliferate and develop in the testicular cords or seminiferous cords (Stevens, 1967). Subsequently, these cells are transformed to primitive male germ cells so-called “gonocytes”. They migrate from the center to basement membrane of seminiferous cords and then are covered by primitive Sertoli cells (de Rooij, 1998). At this stage, the gonocytes have lost their pluripotency, thereafter referred to as unipotent cells (de Rooij, 1998; Durcova-Hills et al., 2008). By contrast to *in vivo* situation, these cells can be converted to pluripotent embryonic germ cells (EGCs) (Resnick et al., 1992).

The number of gonocytes is increasing for a while and remains at G0/G1 phase of mitosis until birth (Vergouwen et al., 1991). The gonocytes will change their

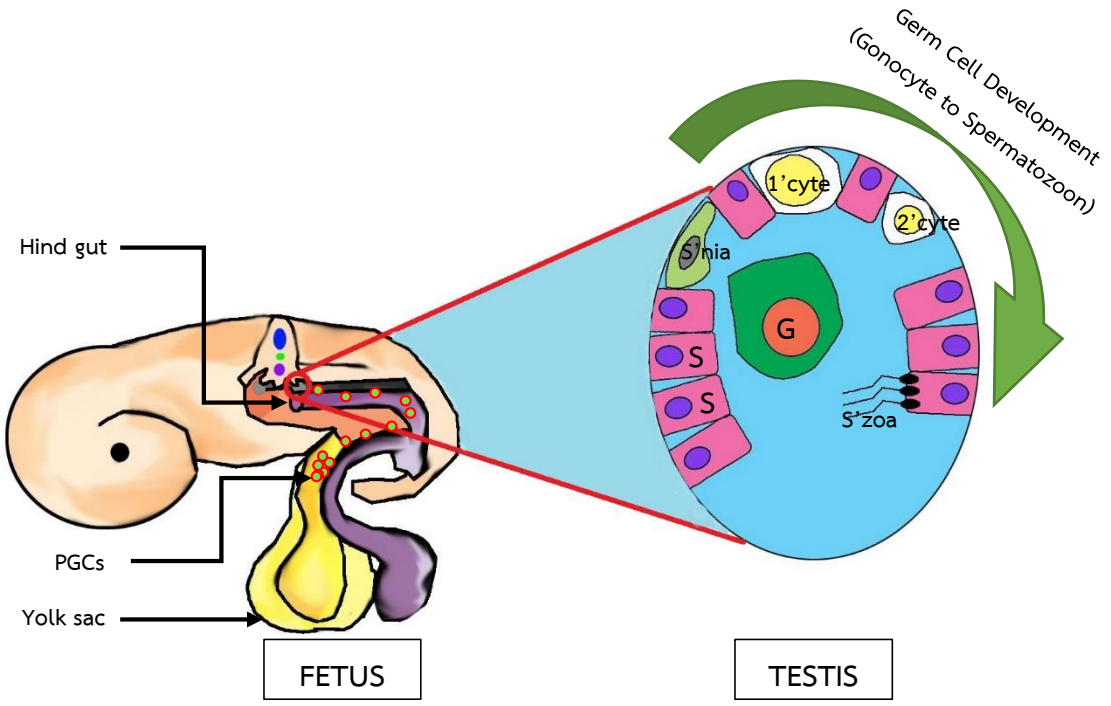

Figure 1 Development of male germ cell lineage.

The primordial germ cells (PGCs) migrate to the genital ridges (embryonal testes) pass along the hindgut. The primordial germ cell transform to gonocyte (G) and then move down to the basement membrane. The spermatogonium (S'nia) derived from gonocyte and entrapped by Sertoli cells (S) proliferates and enters to the meiosis for primary (1' cyte) and secondary (2' cyte) spermatocytes. Finally, spermatozoa (S'zoa) or sperms are produces by complete spermatogenesis after male animal entering to the puberty.

morphological and molecular characteristics and ultimately develop to undifferentiated spermatogonia. This process occurs after birth. The complete spermatogenesis and maturation of sperm occur when man and male animal acquire their pubertal age.

2.2 Spermatogenesis in mammals

Spermatogenesis takes place within the seminiferous tubules. The spermatogenesis can be divided into three phases.

1. Proliferation phase

Diploid cells (or spermatogonia), attached on basement membrane of the seminiferous tubule, generally divided into A (undifferentiated spermatogonia, A_{undiff} and differentiated spermatogonia, A_{diff}), intermediate (In) and B types. B type spermatogonia transform into spermatocytes (de Rooij and Grootegoed, 1998).

2. Meiotic phase

During the second phase, the number of chromosomes in the spermatogonia is reduced to be haploid cells by meiosis. The primary spermatocytes immediately enter to the prophase I including preleptotene, leptotene, zygotene, pachytene and diplotene. The preleptotene phase completes DNA replication, forming tetrads without separation, and then crossing over of DNA is taken place. At this stage, blood-testis barrier (BTB) composing of basal ectoplasmic specialization (ES), tight junctions, desmosome and gap junctions is broken down, allowing the preleptotene spermatocytes to move across the BTB. The preleptotene spermatocytes become leptotene spermatocytes in the intermediate compartment of BTB (Cheng and Mruk, 2002; Mruk and Cheng, 2004; Cheng and Mruk, 2011). After that, the leptotene spermatocytes further develop to round spermatids.

3. Spermiogenesis

Spermiogenesis involves the morphological change of haploid germ cell (round spermatid). The spermiogenesis consists of the golgi phase, cap phase, acrosomal phase and maturation phase. The acrosomal proteins, such as the acrosin and proteolytic enzymes, are synthesized at the pachytene spermatocyte stage throughout the round and elongating spermatid (Anakwe and Gerton, 1990; Escalier et al., 1991). During the golgi phase, the acrosomal vesicles attached to the nuclear envelope will be fused with golgi-derived vesicles (Burgos and Fawcett, 1955). At later stages (cap and acrosomal phases), the acrosomal vesicle is flattened over the nucleus, covering up to two-third of sperm head. Acrosomal proteins are condensed and packed in the acrosomal granules, which eventually form as the acrosomal matrix in mature sperm (Anakwe and Gerton, 1990; Escalier et al., 1991). At acrosomal and maturation phases, the golgi apparatus moves down and the acrosome is formed. The golgi apparatus remained in the cytoplasmic lobe will be discarded into cytoplasmic droplet together with other organelles (Burgos and Fawcett, 1955).

The mature sperm will release from Sertoli cells into lumen of the seminiferous tubules, so-called spermiation. At this stage, the sperm are mature but they are immotile. The non-motile sperm will be transported to epididymis by peristaltic contraction (Cheng and Mruk, 2011).

In domestic cat, complete spermatogenesis begins when the cats enter to 8-12 months of age (Siemieniuch and Woclawek-Potocka, 2007). The total duration of spermatogenesis in domestic cat is 46.8 days (4.5 cycles) (Franca and Godinho, 2003). However, the knowledge involving the spermatogenesis in domestic cat has been markedly limited, in particular the seminiferous epithelial cycle and sperm production.

2.3 Expansion of spermatogonial derivatives

Until recently, models of male germ cell expansion have been committed and referenced in 2 models, non-primate and primate mammal models. For non-primate mammal model (Huckins, 1971; Oakberg, 1971), the spermatogonia are generally divided into A (undifferentiated spermatogonia, A_{undiff} and differentiated spermatogonia, A_{diff}), intermediate (In) and B types. A_{undiff} spermatogonia can be subdivided by topographic arrangement on basement membrane into 3 subtypes (A_{single}, A_{paired} and $A_{aligned}$; Fig. 2). A_{single} (A_s) spermatogonium is believed to be SSC, and only about 35,000 cells have been estimated in the mouse testis (Tegelenbosch and de Rooij, 1993). A_s spermatogonium can be mitotically divided into 2-spermatogonium (A_{paired}, A_{pr}) that are connected each other with an intercellular bridge. This cellular bridge functions to allow migration of the organelles and genetic substances of cloned cells, resulting of the synchronization of the A_{pr} spermatogonia (Lee et al., 1995). A_{pr} spermatogonia randomly divide into chains of four, eight, sixteen and sometimes thirty-two $A_{aligned}$ (A_{al}) spermatogonia. Therefore, the number of precursor cells of spermatogenesis is continuously increased about 2^n (n is the number of mitosis).

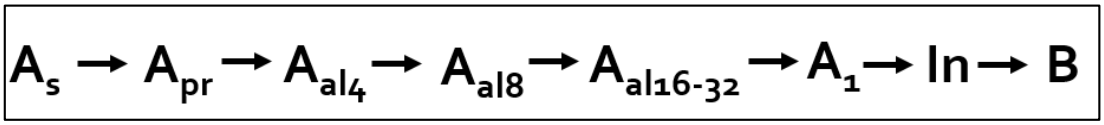

Figure 2 Scheme of spermatogonium derivation in non-primate model.

A_{single} (A_s) is promised to be the SSC which provide the SSC pool and undifferentiated spermatogonia (A_{undiff}) populations. The A_{paired} (A_{pr}) is mitotically divided of A_s connected with intercellular bridge. The division of A_{pr} produce the $A_{aligned}$ (A_{al}) from 4 to 16 (or 32) cells. Afterthat, $A_{aligned}$ differentiate into A_1, Intermediate (In) and B type spermatogonia, respectively.

By contrast, A_{undiff} spermatogonia regulate themselves by apoptosis phenomenon in order to control the suitable physiological numbers using density-dependent regulation (Huckins, 1971; Hamer et al., 2003). The A_{al} spermatogonia are transformed to A1 spermatogonia that are subsequently divided to B type spermatogonia. At this point, B type spermatogonia give rise to primary spermatocytes prior to meiosis (de Rooij and Grootegoed, 1998). The A, In and B type spermatogonia can be distinguished by morphological characteristics of nuclear chromatin (De Rooij and Russell, 2000). Nuclear morphology of A type spermatogonia demonstrates euchromatin with a few heterochromatins. The transition of the chromatin structure occurs during the development of In type spermatogonia, and thus abundant of heterochromatin is usually found in the B type spermatogonia. However, the heterochromatin amount and intercellular distance to classify the types of spermatogonia are unique and may be different among species.

For primate mammal model, type A spermatogonia are characterized into 2 subtypes, A_{pale} and A_{dark}. The differences of the two types are defined by nuclear intensity following hematoxylin staining (Clermont and Leblond, 1959). A_{pale} spermatogonia are active SSCs and often found to locate on the basement membrane. They play a central role to balance and reserve the undifferentiated spermatogonia throughout adult life (Hermann et al., 2007; Hermann et al., 2009). A_{dark} spermatogonia are also designated as SSC reserve but these cells are normally quiescent. However,

they will be triggered to divide when the spermatogenesis is severely damaged by gonadotoxic substances (van Alphen and de Rooij, 1986).

2.4 Spermatogonial stem cell niche and its regulation

Histological structure of testis is classified only 2 types of testicular cells in seminiferous tubules, including germ cells and Sertoli cells. Sertoli cells and spermatogonia settled on basement membrane of the tubules are surrounded with peritubular myoid cells and connected with interstitial tissues, composing of the Leydig cells, vasculature structure (arterioles and venules) and other components. Inside the tubules, germ cells develop from basement membrane directly to innermost lumen together with the Sertoli cells (Fig. 3). This process is accompanied by both local and systemic regulations. Sertoli cells play roles as neighboring cells to support the development of germ cells and also form blood-testis barrier (BTB) as a biological barrier (Mruk and Cheng, 2004). It has been demonstrated in mouse that a spermatogonium is supported by about 100 Sertoli cells (Tegelenbosch and de Rooij, 1993). However, almost SSCs in the tubules are preferred to locate nearby the vascular structure (Chiarini-Garcia et al., 2001; Shetty and Meistrich, 2007).

The SSC niche regulated the SSC property by several factors. Glial cell line-derived neurotrophic factor (GDNF) is a Sertoli cells secreted-growth factor that functions to promote SSC activity. GDNF is a member of transforming growth factor beta (TGFβ) superfamily that binds specifically to its co-receptors, RET (Rearranged during transfection) tyrosine kinase and GFRα-1 (GDNF family receptor-1) receptors (Airaksinen and Saarma, 2002; He et al., 2008). The signal activates both Src-PI3K/Akt and Ras/ERK1/2 signaling pathways via kinases and followed by the stimulation of N-myc expression to maintain the SSC activity and c-Fos expression to increase cyclin A and cyclin dependent kinase 2 (CDK2) activity in S phase of cell cycle (He et al., 2008; Hofmann, 2008). Recently, most established SSC culture system in a number of species studied usually add GDNF into the medium (Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004d; Aponte et al., 2008; Kanatsu-Shinohara et al., 2008a; Sadri-Ardekani et al., 2009; Sato et al., 2011).

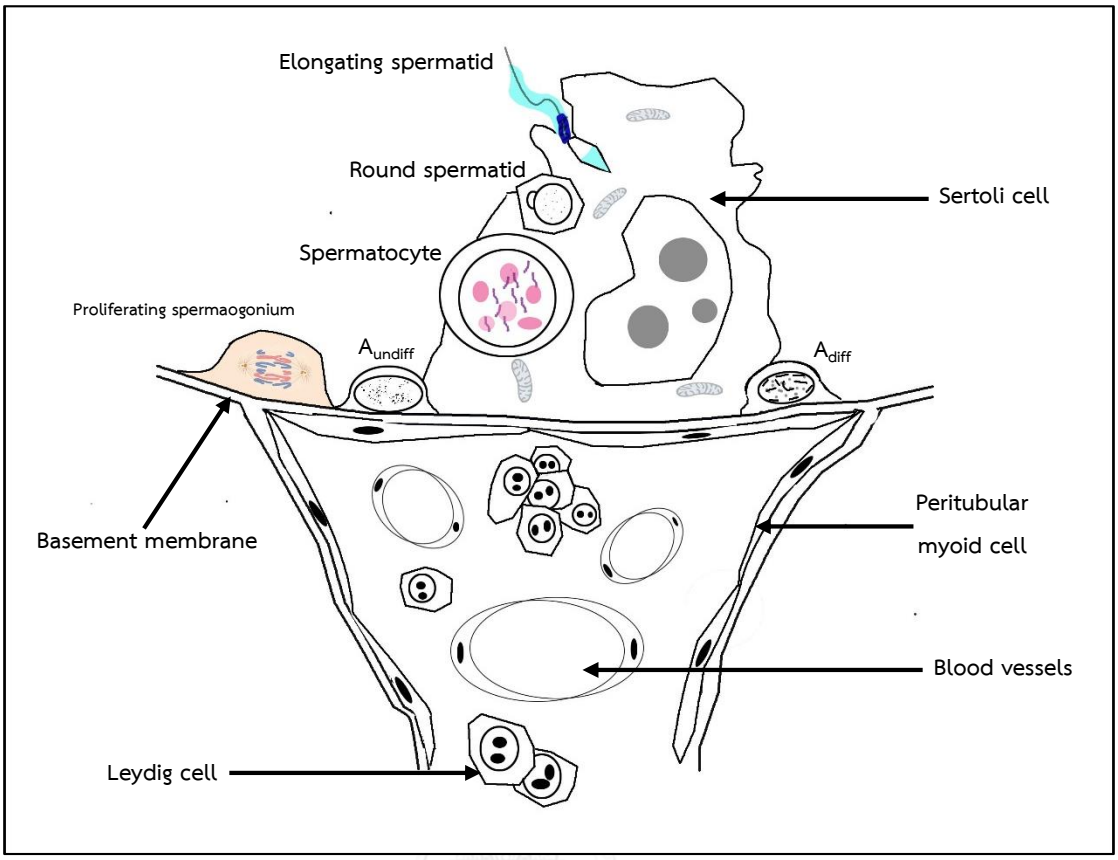

Figure 3 Spermatogonial stem cell niche.

Histology of testis consists of 2 compartments (tubular compartment and interstitial compartment). Tubular compartment which surrounded by basement membrane of connective tissues and peritubular myoid cells has only 2 types of testicular cells i.e. germ cells (such as spermatogonium, spermatocyte and spermatid) and somatic cell (Sertoli cell). Interstitial compartment resides at the outer part of seminiferous tubule.

As Sertoli cells also secrete fibroblast growth factor 2 (FGF2; also called basic fibroblast growth factor; bFGF), this FGF2 has therefore been tested and demonstrated to increase GDNF/GFRα-1 mechanisms (Ryu et al., 2005). In addition, colony stimulating factor 1 (CSF-1) and insulin-like growth factor 1 (IGF-1) have also been to maintain SSC activity. These factors are produced and secreted by Leydig cells and peritubular myoid cells (Huang et al., 2009; Oatley et al., 2009).

On the other hand, the differentiation of SSCs is regulated by microenvironment of stem cell niche via stem cell factor (SCF). SCF is secreted by Sertoli cells and acts on plasma membrane receptor of differentiating spermatogonia called c-kit. This signal pushes the transformation of A_{al} to A1 spermatogonia.

2.5 Characterization of spermatogonial stem cells

It is well accepted that the number of SSCs in the seminiferous tubules is very low when compared with the total number of testicular cells. Therefore, the characterization of the SSCs including phenotypic and molecular examinations is essential to distinguish between the SSC population and other cells. Although several markers have been demonstrated to be expressed in the SSCs, the universal SSC markers for particular species remain to be elusive. Formally, testis side-population (T-SP) sorted cells using a flow cytometer expressed putative SSC markers, including Sca-1, Ep-CAM, Stra8 and α_6-integrin (Falciatori et al., 2004; Lassalle et al., 2004). However, Lassalle et al. (2004) demonstrated that only T-SP expressing α_6-integrin colonized into recipient's seminiferous tubules after 10 weeks of transplantation. Later studies therefore used laminin-coated dish to enrich the SSCs for transplantation (Shinohara et al., 1999; Kent Hamra et al., 2004). Nevertheless, it is important to note that, although α_6-integrin ligands bind specifically to laminin and can be used for SSC selection (Ooba et al., 2008), laminin γ_3 is also found to express at apical ectoplasmic specialization (ES) between Sertoli cells and developing spermatids (Yan and Cheng, 2006). In addition, CD90 (THY1) positive testicular cells were also used to select the SSCs in rodent, non-human primate and bull (Kubota et al., 2003; Ryu et al., 2004; Hermann et al., 2009; Maki et al., 2009; Reding et al., 2010). However, this THY1 marker would need other markers to increase the efficacy of SSC sorting (Kubota et al., 2003). Furthermore, several previous studies also showed that other markers can also successfully enrich the SSC population. These markers include CD9, CD24 and POU5F1 (Kubota et al., 2003; Kanatsu-Shinohara et al., 2004b; Ohmura et al., 2004).

More importantly, GFRα-1, RET and Foxo1 have been studied and demonstrated to present in undifferentiated spermatogonia (Morimoto et al., 2009;

Goertz et al., 2011). Sorted testicular cells with GFRα-1 could generate the colonies into recipient seminiferous tubules of mice (Morimoto et al., 2009). In addition, only A_s and A_{pr} spermatogonia expressing promyelocytic leukemia zinc finger (PLZF or ZBTB16) also expressed GFRα-1. However, 10% of A_s spermatogonia did not show the GFRα-1 expression and 5% of paired spermatogonia had asymmetrically GFRα-1 expression (Grisanti et al., 2009; Nakagawa et al., 2010; Goertz et al., 2011). For non-human primates, the GFRα-1 have been used to identify the localization and characteristics of SSCs and co-expressed with DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX-4), a germ cell marker (Hermann et al., 2007; Maki et al., 2009). Moreover, undifferentiated spermatogonia including SSCs expressed NEUROG3 (NGN3) and E-cadherin (E-CAD or CDH1). In contrast, Nakagawa et al. (2010) mentioned that NGN3 particularly expressed in A_{al} spermatogonia and transition phase of A1 spermatogonia. These findings therefore indicated that aforementioned markers are not "true" SSC markers but they are more likely to help during the enrichment of the SSC population for further culture and/or transplantation.

2.6 Enrichment of spermatogonial stem cells

At the basement membrane of seminiferous tubule, the SSCs are subpopulation of spermatogonia type A. The estimated numbers of SSCs have been documented to be 0.02-0.03% of total testicular cells in adult rat (Tagelenbosch and de Rooij, 1993; van Pelt et al., 1996). The purification of the SSCs in pubertal age has therefore become an important step of isolation because the purification can eliminate the negative effects derived from somatic testicular cells (van Pelt et al., 1996; Oatley and Brinster, 2006; Kubota and Brinster, 2008). Recently, the efficacy of enrichment techniques (differential plating, discontinuous Percoll™ gradient density and Flow cytometry) has been demonstrated among the species studies (mouse, rat and cattle) (Shinohara et al., 1999; Izadyar et al., 2002; Kent Hamra et al., 2004; Kubota et al., 2004d; Herrid et al., 2009). The SSCs contacted the basement membrane of seminiferous tubules by various types of extracellular matrixes (ECMs) (Shinohara et al., 2000; Oatley et al., 2011). The ECMs have been used to enrich the SSC population

such as laminin, fibronectin, collagen type I and IV and gelatin (Kanatsu-Shinohara et al., 2008n; Kim et al., 2010; Lim et al., 2014). The gelatin as a denaturing collagen plays as a connective tissue, and is also interacted with laminin and fibronectin. The laminin-coated culture dish could be enriched the number of SSC by more than 3 folds compared before treatment in mouse, rat and bull SSCs (Shinohara et al., 2000; Orwig et al., 2002a; Herrid et al., 2009). The SSC selection by laminin provided the high efficiency related with its receptors (Shinohara et al., 1999). α_6-integrin is the specific surface marker on SSCs in mouse that binds with laminin (Hynes, 1992; Shinohara et al., 1999). In addition to differential plating, Percoll^{TM} treatment has been used to recover the SSC population by different gradient density. This technique provided the high recovery and viability rate of SSCs up to 96% in rat, buffalo and pig (van Pelt et al., 1996; Rafeeqi and Kaul, 2013; Han et al., 2014)

2.7 Factors affecting the establishment of *in vitro* culture spermatogonial stem cells

Culture system of SSC was firstly reported by Nagano et al. (1998). They reported that long-term *in vitro* culture of mouse SSCs could be performed using a basic medium supplemented with only 10% (v/v) fetal bovine serum. However, subsequent studies indicated that co-culture of mouse SSCs with feeder cells (conventional culture system) using low serum concentration and defined medium with growth factors were also efficient (Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004d); the GDNF has been convinced as an essential growth factor for *in vivo/ in vitro* SSC self-renewal property (Meng et al., 2000). More recently, feeder-free system using extracellular matrix substrates have also been reported to support SSC survival (Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara et al., 2011). It is worth to note that well-defined culture medium is preferable because serum and feeder cells can either promote or inhibit the SSC growth (Nagano, 2011). However, SSC culture in an absence of serum and feeder cells reduced the SSC colonies after transplantation in mouse when compared with conventional culture system, though these cultured cells could still produce the fertile offspring (Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara et

al., 2011). In some species for example hamster, the SSCs preferred to growth under feeder-free condition rather than culture with feeder cells (mouse embryonic fibroblasts; MEFs) (Kanatsu-Shinohara et al., 2003a; Guan et al., 2007; Kanatsu-Shinohara et al., 2008n; Huang et al., 2009; Aeckerle et al., 2012). In bull and human, Sertoli cells can be used to support SSC proliferation (Aponte et al., 2006; Liu et al., 2011). At present, it seems likely that there is no particular culture system that suit to all species and thus the adaptation and modification of culture system would be needed. These aspects have become a major obstacle in the development of SSC technology. For example, different protein sources (such as serum, bovine serum albumin (BSA) and fetuin) differently supported the SSC proliferation (Aponte et al., 2006; Kanatsu-Shinohara et al., 2008a; Kanatsu-Shinohara et al., 2011).

In addition to the effect of culture medium, the age of SSC donor is also critical for successful SSC establishment. SSC culture generally prefers to use neonatal or pre-pubertal donors because their testes contain with highly relative percentage of SSC numbers. Lacking of differentiated germ cells also increases the chance to obtain SSCs since the differentiated germ cells may negatively affect to SSC derivation (Kanatsu-Shinohara et al., 2003a; Aponte et al., 2006; Aponte et al., 2008; de Rooij et al., 2008; Kanatsu-Shinohara et al., 2008a; Guan et al., 2009; Liu et al., 2011; Li et al., 2012)

2.8 The clinical and research implications of spermatogonial stem cells

Until now, the SSCs prospects for future implications have become increasingly important means of regenerative therapy, laboratory animal model development and animal conservation because the SSCs have been proven as pluripotent stem cells. By its definition, the SSCs could be used as a source of many cell types, also possibly germ cells (Guan et al., 2006; Durcova-Hills et al., 2008; Geijsen and Hochedlinger, 2009; Golestaneh et al., 2009; Guan et al., 2009; Huang et al., 2009; Ko et al., 2009; Kossack et al., 2009; Guan et al., 2012; Im et al., 2012). This type of stem cells may also have less ethical concern when compared to embryonic stem cell (ESC) researches. Therefore, the SSCs have now been proposed as “alternative ESC” (Kee et al., 2010). More strikingly, one of the properties of SSCs is the capability to colonize and restore

spermatogenic functions following transplantation (Brinster and Avarbock, 1994; Ogawa et al., 2000; Mikkola et al., 2006; Kim et al., 2008). This ability of SSCs on restoring spermatogenesis can be applied in patients treated with gonadotoxic therapy (Hermann and Orwig, 2011). These SSCs would also be isolated and cultured for germ cell banking prior to gonadotoxic treatment. Although semen collection is clinically performed before gonadotoxic chemotherapy. However, this technique may not be feasible in pre-pubertal patients or in the case of obstructive azoospermia.

In animal, the SSC technology has become a promising tool for generating transgene animals. These animals can be used for study the mechanism of many diseases and developmental biology (Kim et al., 2006; Kim et al., 2007; Wongsrikeao et al., 2011). However, knowledge of SSCs in large animals is fairly restricted, and limitation of intra- and inter-species transplantation is recently problematic (Ogawa et al., 1999). There would also be a possibility to generate the SSC culture for valuable animals such as wild species that would unexpectedly die. The cultured cells can be cryopreserved as germ cell banking. It is very changing in the recent year that the development of SSC culture system will contribute to unlimitedly spermatogenesis *in vitro* (Kim et al., 2006; Kim et al., 2007).

CHAPTER 3

CHARACTERIZATION AND *IN VITRO* CULTURE OF PUTATIVE SPERMATOGONIAL STEM CELLS DERIVED FROM FELINE TESTICULAR TISSUE

Narong Tiptanavattana, Chommanart Thongkittidilok, Mongkol Techakumphu, Theerawat Tharasanit

Journal of Reproduction and Development
Vol. 59, No 2, 189-195, 2013 (Published)

3.1 Abstract

Spermatogonial stem cells (SSCs) function to regulate the balance of self-renewal and differentiation of male gametes. SSCs have been successfully isolated and cultured *in vitro* in a number of species, but not in feline. Therefore, in this study, we aimed to culture and characterize feline SSCs. In experiment 1, testes from pubertal domestic cats were cryosectioned and fluorescently immunolabeled to examine the expression of markers which represent SSC (GFRα-1), differentiated spermatogonium (c-kit) and germ cell (DDX-4) markers. In experiments 2 and 3, testicular cells were digested and subsequently cultured *in vitro* using a modified SSC culture system. The resultant presumptive SSC colonies were then collected for SSC identification (experiment 2), or further cultured *in vitro* on feeder cells (experiment 3). Morphology, gene expression and immunofluorescence were used to identify the SSCs. Experiment 1 demonstrates that varying types of spermatogenic cells exist and express different germ cell/SSC makers. A rare population of SSC located at the basement membrane of the seminiferous tubules was specifically identified by co-expression of GFRα-1 and DDX-4. Following enzymatic digestion, grape-like colonies formed by 13-15 days of culture. These colonies expressed *GFRA1* and *ZBTB16*, but did not express *KIT*.

Although we successfully isolated and cultured feline SSCs *in vitro*, the SSCs could only be maintained for 57 days. In conclusion, this study demonstrates for the first time, that SSCs from testes of pubertal domestic cats can be isolated and cultured *in vitro*. These cells exhibited SSC morphology and expressed SSC specific genes. However, long-term culture of these SSCs was compromised.

Keywords: Domestic cat, Gene expression, *In vitro* culture, Isolation, Spermatogonial stem cells

3.2 Introduction

Spermatogonial stem cells (SSCs) play a central role in perpetuating the genetic information via spermatogenesis throughout adulthood, as long as functional SSCs are present within the seminiferous tubules of the testis. These cells share some molecular features and have capability to differentiate into three germ layer lineages (Guan et al., 2006; Seandel et al., 2007; Ko et al., 2009; Kossack et al., 2009). Therefore, they hold great promise, not only for treating male-related infertility, by *in vitro* spermatogenesis (Sato et al., 2011), but also for cellular differentiation, which could be useful for patient-specific cell therapy (Guan et al., 2006; Guan et al., 2009). It is also believed that SSCs may be useful for gamete banking for males with a valuable genetic background, which could be used for future propagation, differentiation and cell transplantation.

Within the testis, the SSCs are located at the basement membrane of the seminiferous tubules, and are entrapped by the stem cell niche, comprising the contacting domain of Sertoli cells, vascular structure, interstitial cells and noncellular portions (Shetty and Meistrich, 2007). This SSC niche communicates with internal and external testicular factors, which are important in maintaining SSC properties. Factors necessary for the propagation of SSCs *in vitro* are largely unknown and may differ between species. Identification of these factors is important for development of successful culture conditions for SSCs. Furthermore, the numbers of SSCs within the testis are extremely low (e.g., approximately 0.02-0.03% of mouse testicular cells) (Tagelenbosch and de Rooij, 1993). These shortcomings could be addressed by

identification of SSC markers and also by examining the factors that regulate the fate of SSCs during *in vitro* culture. Although putative SSC markers, such as GDNF family receptor α-1 (GFRα-1), $\alpha_6\beta_1$-integrins, epithelial cell adhesion molecule (EpCAM), promyelocytic leukemia zinc finger (PLZF; ZBTB16), thymus cell antigen-1 (Thy-1, CD90), LIN28, E-cadherin type 1 (CDH1), POU domain class 5 homeobox 1 (POU5F1) and Nanos 2 and 3 are promising candidates for purification and characterization of SSCs in a number of species (Shinohara et al., 1999; Kanatsu-Shinohara et al., 2003q; Kubota et al., 2003; Hermann et al., 2007; Tokuda et al., 2007; Grisanti et al., 2009; Morimoto et al., 2009; Suzuki et al., 2009; Zheng et al., 2009; Reding et al., 2010; Li et al., 2012), the definite characterization of "true" SSCs is confirmed if these cells can colonize and produce sperm following transplantation into the recipient's seminiferous tubules (Sato et al., 2011). To date, GFRα-1 receptor is mostly used as a consensus marker for SSC identification in several species, including rodents (Sariola and Immonen, 2008). GFRα-1/RET is a co-receptor of GDNF, an SSC factor that plays a central role in regulating *in vivo* and *in vitro* SSC activity (Sariola and Saarma, 2003). GDNF is often added to SSC culture medium, although successful culture of SSCs with this factor varies considerably between species (Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004d; Hamra et al., 2005; Ryu et al., 2005; Aponte et al., 2008; Kanatsu-Shinohara et al., 2008a; Kossack et al., 2009). Several factors have been shown to improve the success of SSC culture, such as the culture medium, age of donor and the culture system used (Kanatsu-Shinohara et al., 2008a). In the domestic cat, information regarding the factors regulating SSCs *in vivo*, and the techniques for identification, isolation and *in vitro* culture of SSC is currently lacking. The objectives of this study were therefore to characterize SSC germ cell markers and to examine the efficacy of *in vitro* culture in domestic cats.

3.3 Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise indicated.

3.3.1 Experimental designs

3.3.1.1 Experiment 1: immunolabeling of germ cell, SSC and differentiating spermatogonium markers

A total of 5 pubertal cat testes were cryosectioned and then fluorescently labeled with 1) an SSC marker (GFRα-1, GDNF family receptor α-1), 2) a germ cell marker (DDX-4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4), and 3) a differentiated spermatogonium marker (c-kit, CD-117). Secondary antibody staining without primary antibody was used as a negative control. The immunofluorescently labeled samples were then examined using fluorescent microscopy. The characteristics and localization of each marker were explained by descriptive analysis.

3.3.1.2 Experiment 2: identification of feline SSCs cultured *in vitro*

This study was performed to observe the characteristics and proliferative activity of feline SSCs. Dissociated testicular cells were cultured in a SSC culture medium. The free-floating presumptive SSC colonies (as shown in Fig. 6a, replicate I = 37 colonies; II = 25 colonies) were manually collected with a fine-ended glass pipette and tested for the expression of spermatogonial marker genes (*GFRA1*, GDNF family receptor α-1; *ZBTB16*, Zinc finger and BTB domain containing 16; and *KIT*, c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) using RT-PCR (reverse transcriptase-polymerase chain reaction). Gene expression of both dissociated testicular cells and presumptive SSC colonies were analyzed. To verify the designed primers, the PCR products of testicular cells (positive control tissue) were sequenced and blasted in GenBank to determine the nucleotide homology. Occasionally, some SSC colonies were also immunolabeled with GFRα-1 to detect protein expression.

3.3.1.3 Experiment 3: culture of feline SSCs

The feline SSCs were isolated and cultured as described in experiment 2; however, the SSC colonies were further cultured on feeder layers (CF-1 MEFs/Sertoli cells). The success of *in vitro* culture was assessed daily for colony morphology and growth characteristics using a phase-contrast microscope (CKX41, Olympus, Shinjuku, Japan).

3.3.2 Sample collection and immunolabeling of germ cell, SSC and differentiating spermatogonium markers

The testes (weighing between 0.3-0.5 grams) were obtained from pubertal domestic cats (of unknown age) following routine castration at the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Bangkok, Thailand. They were transported in 0.9% (w/v) normal saline solution at room temperature (approximately 30 C) to the laboratory. The epididymides were dissected and cut into 2-3 pieces. The presence of motile sperm observed after smearing the epididymides onto a glass slide indicated the complete spermatogenesis of pubertal cat's testes. After extraneous tissues were dissected from the testes, they were then fixed in 4% (w/v) paraformaldehyde for 24 hours. The testes were maintained in 20% (w/v) sucrose in phosphate buffered saline solution (PBS) until being processed. Testicular tissues to be used for cryosectioning were first frozen in OCT compound (Jung, Wetzlar, Germany). Cryosections were sectioned at 7 µm using a Cryostat-microtome (Leica Microsystems, Wetzlar, Germany). To perform immunolabeling, the sections were first incubated in PBS supplemented with 2% (w/v) bovine serum albumin (BSA) and 5% (v/v) normal goat serum in order to block nonspecific antigens. The sections were incubated with mouse monoclonal GFRα-1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal c-kit (1:300, Dako, Carpinteria, CA, USA) antibodies at 4 C overnight or in rabbit polyclonal DDX-4 (1:100, Abcam, Cambridge, MA, USA) antibody at 37 C for 1 hour. After washing twice with PBS, the sections were labeled with the secondary antibodies at 37 C for 1 hour using goat anti-mouse IgG TRIT-C at a dilution of 1:250 (for GFRα-1) and goat anti-rabbit IgG FIT-C at a dilution of 1:100 (for c-kit and DDX-4). 4',6-Diamidino-2-phenylindole (DAPI) was used to label DNA. The fluorescently labeled samples were then examined under an epifluorescent microscope (BX5, Olympus, Shinjuku, Japan). Photomicrographs of individual fluorescent channels were recorded using the DP2-BSW program (Olympus, Shinjuku, Japan) and merged using Adobe Photoshop CS5 Version 12.0 (Adobe Systems, San Jose, CA, USA).

Presumptive SSC colonies obtained from culture were fluorescently labeled with GFRα-1 (specific SSC marker). The colonies were first treated with 2% (w/v) BSA (bovine serum albumin) and 5% (v/v) normal goat serum in PBS. They were then incubated with mouse monoclonal GFRα-1 and the secondary antibody (goat anti-mouse IgG TRITC) as described above.

3.3.3 Isolation of testicular cells

Testes were obtained from pubertal cats after castration as previously described. Upon arrival, they were weighed and decapsulated from the tunica albuginea in Hanks' balanced salt solution (HBSS) containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin. Only testes weighing between 0.3-0.5 grams were used in this study. Feline testicular cells were digested as previously described by Ogawa et al. (1997) and Anway et al. (2003) with some modifications. A two-step enzymatic digestion was used in this study. In brief, the tunica albuginea was first removed from the testicular parenchyma, and the seminiferous tubules were then separated from the interstitial compartments by treating the testicular parenchyma for 15 min with 0.5 mg/ml type IV collagenase and 0.016 mg/ml DNase I (Roche, Indianapolis, IN, USA) in HBSS and placed in a shaking water bath (37 C, 135 strokes/min). The second enzymatic digestion was performed by incubating the separated seminiferous tubules at 37 C for 30 min with 0.04 mg/ml type IV collagenase, 0.03% (v/v) trypsin-EDTA (Gibco, Grand Island, NY, USA) and 0.001 mg/ml DNase I in HBSS. The digested contents were filtered through a nylon mesh (100 µm and 40 µm, respectively, BD Falcon^{TM}, Bedford, MA, USA). Finally, the cell suspension was washed with HBSS and centrifuged at 201 x g for 5 min at 4 C.

3.3.4 Assessment of testicular cell viability

Viability of the testicular cells was evaluated by labeling the cells with fluorescent probes and then visualized using an epifluorescence microscope (BX51, Olympus, Japan). Plasma membrane integrity was assessed using the non-membrane permeant DNA stain ethidium homodimer-1 (EthD-1; Molecular Probes, Eugene, OR,

USA), while intracellular esterase enzyme activity was examined using calcein AM (Molecular Probes, Eugene, OR, USA). A total of 200 cells were evaluated per testicular digestion. The examined cells were classified into 2 categories as either viable (intact plasma membrane: positive for calcein AM and negative for EthD-1) or dead cells (damaged plasma membrane, EthD-1 positive). Only testicular cell suspensions demonstrating more than 80% viable cells were used in this study.

3.3.5 Preparation of feeder cells

This study was designed to analyze 2 types of feeder cells that have been previously reported to support SSCs *in vitro*, i.e., CF-1 MEFs (mouse embryonic fibroblasts, CRL-1040, ATCC) and feline Sertoli feeder cells.

For isolation of feline Sertoli cells, the testicular cells were digested using the same procedure described above. Sertoli cells were isolated using a hypoosmotic shock technique as previously described by Anway et al. (2003) with minor modifications. The Sertoli cells were then examined for cell purity by means of cell morphology using a phase contrast microscope (Olympus, Shinjuku, Japan) and immunolabeling with vimentin (1:200). Sertoli cell morphology was typically recognized within a mixed population of testicular cells by their large cell size, extended cytoplasm and varied size of cytoplasmic vacuoles (Griswold et al., 1988). The purity of Sertoli feeder cells is shown in Fig. 5b. The CF-1 MEFs and Sertoli cells were cultured in knockout DMEM/F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum ((Gibco, Grand Island, NY, USA), 2 mM GlutaMAX^{TM} (Gibco, Grand Island, NY, USA) and 0.25% (w/v) Penicillin-Amphotericin B (Gibco, Grand Island, NY, USA). The CF-1 MEFs (passage 3-5) and Sertoli cell (passage 1) feeder cells were treated with mitomycin-C for 2.5 and 3.0 h, respectively.

3.3.6 Culture of spermatogonial stem cells

The SSC culture medium (modified from Kanatsu-Shinohara et al. (2003a)) used in this study was StemPro-34 SFM (Invitrogen, Carlsbad, CA, USA) supplemented with StemPro Supplement (Invitrogen), 25 µg/ml insulin, 100 µg/ml transferrin, 60 nM

sodium selenite, 60 µM putrescine dihydrochloride, 6 mg/ml D-(+)-glucose, 30 mg/ml MEM sodium pyruvate, 1 µM DL-lactic acid, 5 mg/ml BSA, 2 mM GlutaMAX™ (Gibco, Grand Island, NY, USA), 0.5 µM 2-mercaptoethanol (Gibco, Grand Island, NY, USA), 1x MEM amino acids solution, 1x MEM nonessential amino acids solution, 10^{-4} M L-ascorbic acid, 10 µg/ml D-biotin, 0.25% (w/v) penicillin-amphotericin B (Gibco, NY, USA) and 1% (v/v) fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). This culture medium was further supplemented with 20 ng/ml mouse epidermal growth factor (EGF), 10 ng/ml human basic fibroblast growth factor (FGF2, BioVision, Milpitas, CA, USA), 10^3 U/ml recombinant human leukemia inhibitory factor (rhLIF, Millipore, Temecula, CA, USA) and 50 ng/ml recombinant rat glial cell line-derived neurotrophic factor (rrGDNF, R&D Systems, Minneapolis, MN, USA).

Cell culture was performed at 37 C in a humidified environment of 5% CO_2 in air. Partial purification of the SSC population was performed using negative selection by plating onto 0.1% (w/v) gelatin coated-dishes 2-3 times. This procedure allows fibroblasts and other testicular somatic cells to attach to a Petri dish. Putative SSCs were then counted and cultured at a final concentration of $2x10^5$ cells/ml on mitomycin-treated CF-1 MEFs. The SSC colonies were passaged manually every 2-3 weeks depending on the proliferation rate of the SSCs. For long-term culture of SSCs, the SSC colonies were co-cultured on Sertoli cells. The SSC colonies were observed daily for morphology and proliferative characteristics using a phase-contrast microscope (CKX41, Olympus, Shinjuku, Japan)

3.3.7 RNA extraction, RT-PCR analysis and gene expression

Total cellular RNA was extracted from dissociated testicular cells and SSCs using an Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, CA, USA). The extracted RNA was kept at –80 C until use. For reverse transcription (RT), first-strand cDNA was synthesized from total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was stored at -20 C. These procedures were performed according to the manufacturer's instructions.

The Primer Express® Software v.3.0 (Applied Biosystems, Carlsbad, CA, USA) was used to design specific primers (*GFRA1*, *ZBTB16* and *KIT*). Conserved regions of the bovine *GFRA1* mRNA sequences (accession number: NM_001105411) and the dog *ZBTB16* mRNA sequences (accession number: XM_845250.3) were used to design primers. A domestic cat *KIT* mRNA sequence (accession number: NM_001009837.3) was used to design *KIT*. In all cases, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, 5'-GGAGAAAGCTGCCAAATATG-3' and 5'-CAGGAAATGAGCTTGACAAAGTGG-3') designed from previous study (Sano et al., 2005) was used as the internal control.

PCR was performed on template cDNA containing either reverse transcriptase (RT+) or without reverse transcriptase (RT-) as a control. Briefly, the PCR reaction was performed as follows: 2 min at 95 C for initial denaturation, followed by 30 cycles of 30 sec at 95 C, 30 sec at annealing temperature for each primer (*GFRA1* [product length: 250 bp; accession number JX984462], 60 C, 5'-CAACTGCCAGCCAGAGTCAA-3' and 5'-AGCCATTGCCAAAGGCTTGA-3'; *ZBTB16* [product length: 119 bp], 63 C, 5'-GCAAGAAGTTCAGCCTCAAGC-3' and 5'-GCTTGATCATGGCCGAGTAGTC-3'; *KIT* [product length: 533 bp; accession number JX984463], 60 C, 5'-TCCTGCTCCGCGTCCAGACA-3' and 5'-CTTGCCCTTCCGGTCCGCAG-3') and 30 sec at 72 C. Incubation for 2 min at 72 C was used for the final extension. The PCR products were electrophoresed in 2% (w/v) agarose gel (Bio-Rad, Hercules, CA, USA) in TBE buffer containing 0.4 mg/ml ethidium bromide (Promega, Madison, WI, USA). The amplified products were examined under UV light using a Gel Documentation system (Syngene, Cambridge, CB, UK).

The PCR products (from testicular cells) were extracted using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, CA, USA) and sequenced. The extracted products were confirmed for purity by electrophoresis using 1-2% agarose gel. The sequences derived from both strands were aligned using BioEdit Version 7.0.8.0 (T.A. Hall Software, Raleigh, NC, USA). These sequences were blasted in GenBank in order to determine the nucleotide identity compared with other species.

3.3.8 Statistical analysis

The expression of germ cell, SSC and differentiating spermatogonium markers (experiment 1 and 2) and SSC morphology (experiment 3) were descriptively analyzed. Viability of testicular cells after dissociation is expressed as a mean±SD.

3.4 Results

3.4.1 Experiment 1: immunolabeling of germ cell, SSC and differentiating spermatogonium markers

Feline testicular cryosections were immunofluorescently labeled in order to verify the specificity of SSC, germ cell and differentiating spermatogonium markers in domestic cats. In general, testicular cryosections contained the various stages of spermatogenesis, and each type of spermatogenic cells differently expressed germ cell/SSC/differentiating spermatogonia makers. DDX-4 (germ cell marker) was expressed in spermatogonia, primary/secondary spermatocytes and some round spermatids (Fig. 4a). GFRα-1 (SSC marker) staining was predominantly found at the plasma membrane of a single cell located at the basement membrane of the seminiferous tubule (Fig. 4b). However, faint GFRα-1 expression was also occasionally observed in the cytoplasm. Expression of c-kit (differentiating spermatogonia marker) was found at the basement membrane of the seminiferous tubule, similar to GFRα-1. However, the numbers of c-kit-positive cells were found to be greater than those of GFRα-1. Furthermore, c-kit-positive cells were present, either as single cells or as pairs (Fig. 4c). A rare population of SSCs situated at the basement membrane of the seminiferous tubules was specifically identified by co-localization of GFRα-1 and DDX-4 (Fig. 4d).

3.4.2 Experiment 2: identification of feline SSCs cultured *in vitro*

After enzymatic isolation, the viability of dissociated testicular cells was 92.8±1.9% (mean from 4 replicates) (Fig. 5a). They were subsequently cultured under modified SSC conditions. Approximately 7 days after cell seeding, 2-cell presumptive

SSCs with incomplete cytokinesis were first observed. These paired cells continued to proliferate and form a tightly packed 'grape-like' structure containing 3-6 cells per SSC colony by day 13–15 of *in vitro* culture (Fig. 6a). In order to identify the SSCs, these SSC colonies were collected and examined for mRNA expression using RT-PCR. The results revealed that these colonies expressed *GFRA1* (SSC marker) and *ZBTB16* (early spermatogonium marker) but did not express *KIT* (differentiated spermatogonium marker) (Fig. 6b). This result was in an agreement with the finding that these colonies also strongly expressed GFRα-1 (Fig. 6c). In addition, the partial feline 3 mRNA nucleotide sequences (*GFRA1*, *ZBTB16*, *KIT*) obtained from RT-PCR were subsequently blasted to verify the nucleotide identity with the sequences previously reported in other species. The nucleotide identity of *GFRA1* mRNA sequences between feline and other species ranged from 86-94%. Moreover, the mRNA sequences of *ZBTB16* and *KIT* showed high nucleotide identity (92-99% and 99 %, respectively) (Table 1).

3.4.3 Experiment 3: culture of feline SSCs

For long-term culture, both CF-1 MEFs and Sertoli cells were used as feeder cells. A total of 30 SSC colonies (each colony contained 3-6 cells) were first cocultured with CF-1 MEFs. Of these colonies, we found only 6 colonies loosely attached onto the CF-1 MEFs, and only 2 colonies (mean diameter: 46.5 μm) were observed following passage of the SSC colonies to new CF-1 feeders. Unfortunately, the proliferative activity appeared to decline by day 30 of *in vitro* culture (maximal diameter approximately 30 μm). We subsequently layered the colonies onto feline Sertoli cells as homologous derived-feeder cells (since Sertoli cell feeders have been reported to support the proliferation ability of SSCs) (Aponte et al., 2006; Liu et al., 2011). These two colonies attached onto the Sertoli cell feeders. However, only one colony continued to proliferate. Its diameter increased to 45.12 μm by day 47 of *in vitro* culture (Fig. 6d), and the colony underwent degeneration at approximately 57 days of *in vitro* culture.

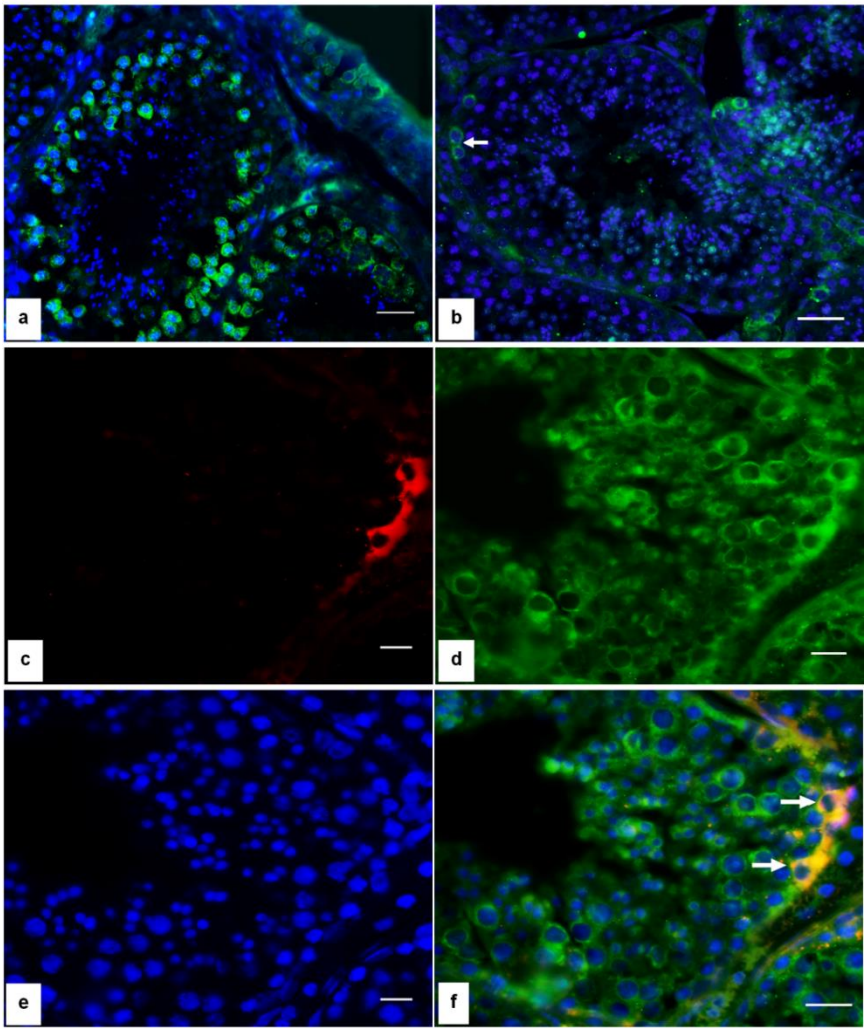

Figure 4 Expression of germ cell-specific (DDX-4), differentiated spermatogonium (c-kit) and, spermatogonial stem cell markers (GFRα-1) and co-localization between DDX-4 and GFRα-1 in cryosectioned feline testes.

(a) The expression of DDX-4 (green) was found in all stages of germ cells except some spermatids and Sertoli cells. DDX-4 expression was found within fine granules in the cytoplasm. (b) c-kit was labeled at the plasma membrane and cytoplasm of differentiating spermatogonial cells (green). c-kit was expressed on 2-pairing cells (arrow). Multicolor photomicrographs illustrate the expressions of putative spermatogonial stem cells that highly expressed GFRα-1 (red, c), DDX-4 (green, d) and DAPI (blue, e). Co-expression of GFRα-1 and DDX-4 is shown by arrows (f). a and b: scale bars = 30 µm. c-f: scale bars = 10 µm.

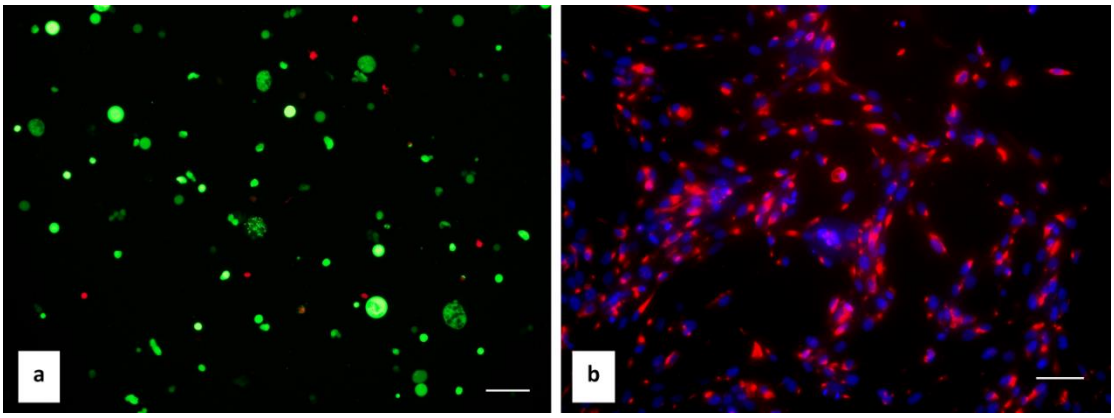

Figure 5 The viability of digested testicular cells and the vimentin expressing Sertoli cells.

(a) The viability of testicular cells after enzymatic digestion. Calcein AM-positive (green) cells indicate the esterase enzyme activity (viable cells), while EthD-1 (red) binds specifically to the nucleus of membrane-disrupted testicular cells (dead cells). (b) High proportions of cells positive for vimentin (red) were obtained after hypoosmotic shock treatment and cultured *in vitro*. This vimentin binds specifically to intermediate filaments of Sertoli cells. Scale bars = 50 μm.

3.5 Discussion

In the current study, we reveal that spermatogenic cells within the seminiferous tubules of the feline testis differently expressed germ cell and SSC markers. Three markers (GFRα-1, c-kit and DDX-4) were used to define SSC, differentiated spermatogonium, and germ cell markers, respectively. Furthermore, we demonstrated for the first time that feline SSCs could be successfully isolated and cultured *in vitro*, although long-term culture of these SSCs was compromised.

In experiment 1, we examined the expression pattern of several markers used for identifying SSCs and differentiating spermatogonial cells, since the specific markers for SSCs remain elusive in domestic cats. From our results, it is clear that the GFRα-1 is a consensual SSC marker in domestic cats, as the expression pattern

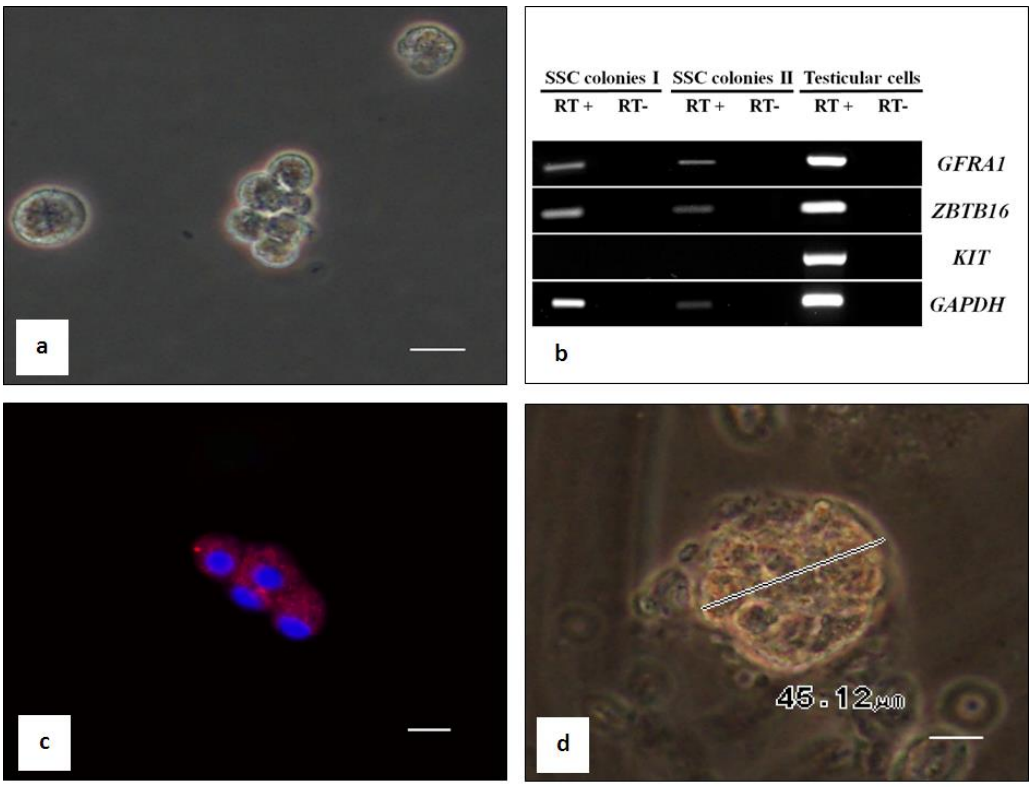

Figure 6 SSC colonies were isolated and cultured in vitro.

(a) Floating "grape-like" SSC colonies were found between days 13-15 of *in vitro* culture. (b) RT-PCR analysis of presumptive SSC colonies and testicular cells was performed. PCR product bands indicate that presumptive SSC colonies expressed *GFRA1* and *ZBTB16* but no *KIT* PCR product was observed. (c) Immunolabeling of an SSC floating colony revealed the expression of GFRα-1 at the cell membrane and within the cytoplasm. (d) An SSC colony (day 47 of *in vitro* culture) was cultured on mitomycin-treated Sertoli cells. Scale bars = 10 μm.

was similar to those of other species such as the rodent (expressed in A_{single} and possibly in A_{paired} spermatogonia) and monkey (expressed in A_{dark} and A_{pale} spermatogonia) (Sariola and Immonen, 2008; Hermann et al., 2009). This marker has successfully been used to enrich (more than 90%) undifferentiated spermatogonial stem cells in the mouse (Hofmann et al., 2005; Braydich-Stolle et al., 2007). In addition, while only a rare population of GFRα-1-positive cells was found at the basement membrane of the seminiferous tubule (approximately 2-4 cells per

Table 1 Sequence alignments of *GFRA1*, *ZBTB16* and *KIT* amplicon products with mRNA sequences previously reported in GenBank.

Species	GenBank accession number	Nucleotide identity (%)
GFRA1 (product length: 250 bp)	JX984462	
Mouse (*Mus musculus*)	NM_010279.2	86
Rat (*Rattus norvegicus*)	NM_012959.1	88
Human (*Homo sapiens*)	NM_145793.3	93
Bovine (*Bos taurus*)	NM_001105411.1	92
Dog (*Canis Familiaris*)	XM_846994.2	94
ZBTB16 (product length: 119 bp)	HF678120*	
Mouse (Mus musculus)	NM_001033324.2	92
Rat (*Rattus norvegicus*)	NM_001013181.1	96
Human (*Homo sapiens*)	NM_006006.4	97
Bovine (*Bos taurus*)	NM_001037476.1	99
Dog (*Canis Familiaris*)	XM_845250.3	95
KIT (product length: 533 bp)	JX984463	
Cat (*Felis catus*)	NM_001009837.3	99

*ENA accession number

cryosection), all of these cells also co-expressed DDX-4 (a specific germ cell marker) (Fig. 4d). However, since all stages of spermatogenic cells (except elongated spermatids and spermatozoa) were positive for DDX-4, the results confirm that this marker can only be used as a general germ cell marker (Raz, 2000; Toyooka et al., 2000). Indeed, co-localization of GFRα-1 and other SSC or undifferentiated spermatogonium markers such as $\alpha_6\beta_1$-integrins, POU5F1 and LIN28 would be required to conclusively identify the "true" SSCs in the domestic cat (Airaksinen and Saarma, 2002; Tokuda et al., 2007; de Rooij et al., 2008; Kanatsu-Shinohara et al., 2008n; Suzuki et al., 2009; Zheng et al., 2009; Wu et al., 2010; Gillis et al., 2011).

GFRα-1 and its co-receptor, RET tyrosine kinase located on the cell plasma membrane, are the specific binding sites of glial cell line-derived neurotrophic factor (GDNF) (Meng et al., 2000). This ligand receptor binding signals via the Ras/ERK1/2 pathway to stimulate DNA synthesis and cell proliferation, which in turn maintain function and survival of the SSCs both *in vivo* and *in vitro* (Sorrentino et al., 1991; Sariola and Saarma, 2003; Kubota et al., 2004d; He et al., 2008). By contrast to GFRα-1, DDX-4 and c-kit were expressed in a more advanced stage of spermatogonia, similar to the pattern previously reported in rodents and juvenile rhesus macaques (Sariola and Immonen, 2008; Hermann et al., 2009; Maki et al., 2009). Moreover, expression of c-kit in feline testes was occasionally observed at the basal compartment of seminiferous tubules, as reported in adult rhesus macaques (Schrans-Stassen et al., 1999). Although the expression of c-kit in undifferentiated spermatogonia (A_{paired} spermatogonia) is still controversial, we found in domestic cats, that c-kit was expressed in paired cells at the basement membrane of the seminiferous tubules, similar to the finding that c-kit was expressed in A_{paired} spermatogonia in other species (Fig. 4c) (Hofmann et al., 2005; Kanatsu-Shinohara et al., 2006).

Following enzymatic digestion of the feline testes, only small numbers of SSC-like colonies (3-6 cells) formed within approximately 2 weeks in the modified culture system used in this study. This finding is in an agreement with experiment 1, where the numbers of GFRα-1-positive cells were low compared with other germ cells (DDX-4-positive cells). We confirmed for the first time that these colonies were SSCs by immunolabeling with a GFRα-1 fluorescent probe and also by mRNA expression of SSC-specific genes (*GFRA1* and *ZBTB16* mRNA). Furthermore, these cells did not express the differentiated spermatogonium marker (*KIT*). We also additionally demonstrated that the nucleotide sequences of these genes were similar to other species (Table 1), suggesting that these genes are relatively conserved between species. Nevertheless, although these genes have been shown to be potential markers for identification of rodent SSC subpopulations (Kim et al., 2006; Dann et al., 2008; Grisanti et al., 2009), there is a further requirement to determine other genes that may also be expressed in SSCs within the domestic cat. In fact, information regarding gene expression and cell

signaling in feline SSCs has yet to be fully established. Furthermore, transplantation of the positive GFRα-1 colonies obtained in this study into the seminiferous tubules is still required in order to examine the biological assay of the SSCs in terms of colonization and *in vivo* spermatogenesis in the recipient testis. However, this SSC transplant technique has yet to be established, since cat mixed germ cells xenotransplanted into mouse testes colonized within the seminiferous tubules but failed to reinitiate sperm production (Silva et al., 2012). Furthermore, long-term establishment of a germ cell-depleted model in tom cats remain unsuccessful (Oatley et al., 2004; Silva et al., 2012).

In the current study, we isolated and identified SSCs from domestic cats as a molecular assay. The SSC colonies were maintained *in vitro* for only approximately 57 days. This indicated that GDNF supplementation is not an exclusive factor for maintaining the self-renewal and function of SSCs *in vitro*, despite the five-fold increase (50 ng/ml) in GDNF concentration in our study compared with an original mouse SSC protocol (Kanatsu-Shinohara et al., 2003a). GDNF is well recognized to increase SSC proliferation *in vitro* in a dose-dependent manner (He et al., 2008). This proliferative activity of GDNF has been reported in a number of species including the mouse, rat, hamster and bull (Anway et al., 2003; Kubota et al., 2004d; Ryu et al., 2005; Braydich-Stolle et al., 2007; Aponte et al., 2008; Hofmann, 2008; Kanatsu-Shinohara et al., 2008a). Moreover, Kanatsu-Shinohara et al. (2008a) revealed in the hamster, that addition of FGF2 was necessary to promote GDNF activation, while EGF supplementation adversely affected hamster SSCs. It is therefore essential to determine the interaction of these growth factors on derivation of feline SSCs, since the culture system may be species-specific. In addition to growth factor supplementation, feeder cell layers are also one of the critical factors determining the success of SSC derivation. For example, mouse embryonic fibroblasts (MEFs) and testicular somatic cells have been successfully used to support the culture of SSCs in rodents, bovine and humans (Anway et al., 2003; Kanatsu-Shinohara et al., 2003a; Guan et al., 2006; Aponte et al., 2008; Liu et al., 2011). In the current study, we found that MEFs were not suitable for SSC culture, as only 2 colonies (of 30 colonies) were

maintained, while the proliferative activity appeared to decrease over time. We therefore decided to transfer the two colonies to Sertoli cell feeders because this feeder type has been demonstrated to support SSCs *in vitro* in several species (Aponte et al., 2006; Liu et al., 2011). We found that feline Sertoli cells could reactivate their proliferative activity and further support SSC growth for 57 days. Sertoli cells and SSCs interact *in vivo* by forming an SSC niche and by secreting GDNF and other growth factors to activate SSC proliferation (Hofmann, 2008; Wu et al., 2010). However, it is worth noting that the use of feeder cells to support SSC activity remains largely controversial because feeder-free culture systems have been demonstrated to be preferable for hamster SSCs rather than culture systems containing feeder cells (Dann et al., 2008; Kanatsu-Shinohara et al., 2008a).

In the current study, we demonstrated for the first time that spermatogonial stem cells can be isolated from testes of pubertal domestic cats. These SSCs expressed SSC-specific genes and could be successfully cultured *in vitro*. However, long-term culture of these SSCs was compromised. Further studies investigating other factors that regulate the proliferation and senescence of SSCs in the domestic cat are required.

CHAPTER 4

DETERMINATION PHASE AT TRANSITION OF GONOCYTES TO SPERMATOGONIAL STEM CELLS IMPROVES ESTABLISHMENT EFFICIENCY OF SPERMATOGONIAL STEM CELLS IN DOMESTIC CATS

Narong Tiptanavattana, Araya Radtanakatikanon, Poul Hyttel, Hanne M. Holm, Supranee Buranapraditkun, Piyathip Setthawong, Mongkol Techakumphu, Theerawat Tharasanit

(Submitted)

4.1 Abstract

The development of germ cells has not entirely been documented in cat especially the transition phase of gonocyte to spermatogonial stem cell (G/SSC). The aims of study were to examine testicular development and also to identify G/SSC transition in order to isolate and culture of the SSC *in vitro*. The testes were divided into 3 groups according to donor age (I:<4, II:4-6 and III:>6 months).

Exp.1 studied testicular development by histology, transmission electron microscopy and immunohistochemistry. Exp.2 determined expression of GFRα-1, DDX-4 and c-kit and flow cytometry. The SSCs isolated from group II and III were characterized by RT-PCR and TEM (Exp.3). Chronological changes at G/SSC transition were demonstrated. The size, morphology and ultrastructure of SSCs were distinguishable from gonocytes. The results demonstrated that group II contained highest numbers of SSC per seminiferous cord/tubule (17.66±2.20%) and GFRα-1^{+} cells (14.89±5.66%) compared with other groups. The findings coincided with an increased efficiency of SSC derivation in group II compared with group III (74.33±2.64% vs. 23.33±2.23%, p<0.001). The colonies expressed mRNA for *GFRA1*, *ZBTB16*, *RET* and

POU5F1. Our study concludes that the G/SSC transition occurs at 4-6 months of age. This period is useful for isolation and improves efficiency of cat SSC *in vitro*.

Keywords: Development, Transition phase, Spermatogonial stem cell, *In vitro* culture

4.2 Introduction

In male mammal, primordial germ cells derived from extra-embryonic ectoderm essentially develop to gonocytes, as an origin of male germline lineage, in seminiferous cord before birth (Lawson and Pedersen, 1992). The gonocytes subsequently reproliferate and relocalize to the privileged testicular niche formed by primitive Sertoli cells by day 12.5-14 of mouse embryo development (de Rooij, 1998; Nagano et al., 2000). In rodent, the transformation of gonocyte to spermatogonial stem cell (SSC) or transition phase of gonocyte/spermatogonial stem cell (G/SSC) takes place shortly after birth in the seminiferous cord. This transition phase is, in part, mediated by Notch signaling and anti-MÜllerian hormone from active Sertoli cells of developing and mature testis (Garcia et al., 2013; Su et al., 2014). However, heterogeneous population of gonocytes in terms of their maturation and apoptosis results in variably committed programs of G/SSC transition after quiescent period of the testicular cell development (Orwig et al., 2002a; Zogbi et al., 2012). The mature gonocytes migrate down to the periphery of the seminiferous cord by KITL/KIT signaling and then, some *POU5F1* expressing gonocytes are committed to be the SSCs with self-renewal ability (Yoshida et al., 2006). The SSCs permanently forms and settles at the basement membrane of the seminiferous cord (Orth et al., 1997). The SSCs, thereafter, play a key role to regulate the balance of stemness and differentiation of SSC populations after puberty in mammals.

The G/SSC transition is short and rapidly occurs during days 3-6 and days 5-8 after birth in mouse and rat, respectively. By contrast to the findings in rodents, human G/SSC phase is generally presented between 3-12 months after birth (Bellve et al., 1977; Zogbi et al., 2012; Su et al., 2014). Thus, the duration of the G/SSC transition is largely variable among species predominantly because of the differences in the

quiescent period of the gonocytes. The quiescent period of the gonocytes is important as the checkpoint of gonocytes that are capable of undergoing the transformation to SSCs. Although the SSCs resume the mitosis to increase their stemness activity, the numbers of SSCs within the testis is extremely low (approximately 0.03% of total testicular cells) (Tagelenbosch and de Rooij, 1993). Until recently, informative data on testicular development particularly the G/SSC transition in relation to derivation of SSCs in domestic cats is very limited.

The gonocytes have a unique and uniform in morphology. Generally, the gonocyte is large size with prominent nucleoli and low complexity of cytoplasmic organelles (Orwig et al., 2002a). The gonocytes migrate to the basal lamina of seminiferous cord by the cytoplasmic projection (Orth et al., 1997; Orth et al., 2000). The spermatogonia define by their small flattened shape with round to oval nucleus. There are 2 main populations of chromatin distribution, homogenous chromatins and condensation of granular chromatins (Schulze, 1988). Until recently, testicular development and the puberty age (complete spermatogenesis) takes place around 8-12 months of age with the spermatogenic cycle of 46.8 days (4.5 cycles) (Franca and Godinho, 2003). However, information on pre-pubertal and post-pubertal testicular development in cat has been restricted (Siemieniuch and Woclawek-Potocka, 2007). Given that only specific stage of testicular development can enrich the SSC population, study of G/SSC transition is essential for isolation and culture of SSCs.

In vitro culture of SSCs is only well understood in mouse and rat in terms of mechanisms that control SSC signaling and self-renewal ability (van Pelt et al., 2002; Kanatsu-Shinohara et al., 2003a; Hamra et al., 2005; Ryu et al., 2005; Sato et al., 2013). Of several factors contributing to successful isolation and propagation of SSCs, age of donor at SSC isolation, specific growth factors and types of culture system (such as feeder vs. feeder-free or serum vs. serum-free system) centrally control fate of SSCs *in vitro* (Kanatsu-Shinohara et al., 2008a). However, effects of these factors on *in vitro* culture of SSCs in other domestic species remain elusive. The G/SSC phase occurs in a time- and species-specific manners. This phase of testicular development improves efficiency of SSC isolation and culture by enrichment of SSC population with minimal contamination of other differentiated germ cells during culture (de Rooij and

Grootegoed, 1998; Ryu et al., 2004; Ryu et al., 2005; Drumond et al., 2011; Kanatsu-Shinohara and Shinohara, 2013). Our previous study demonstrated that the SSCs could be isolated from pubertal cats, and the resultant SSC-like colonies had the SSC activity (Tiptanavattana et al., 2013). However, the efficiency of establishment of SSC *in vitro* is still limited probably due to lacking information of the G/SSC transition. This study therefore aimed to examine chronological changes of testicular cell development in order to define the specific period of the G/SSC transition that would be useful for increased efficiency of isolation and culture of SSCs in domestic cats.

4.3 Materials and methods

4.3.1 Experimental designs

4.3.1.1 Experiment 1: The transition of testicular germ cells during different phases of post-natally testicular development

The testes obtained from domestic cat (*Felis catus*) were collected and categorized into 3 groups depending on donor ages: less than 4 months (group I, neonatal (n=2); 2.5 months (n=1); 3 months (n=3)), 4 to 6 months (group II, 4 months (n=3); 5 months (n=3)) and more than 6 months (group III, 7 months (n=2); 8 months (n=1); 9 months (n=1); 12 months (n=1); 24 months (n=1)). The data on testicular weight and the presence of epididymal sperm were also recorded. The cat testes were descriptively analyzed for conventional histology (H&E staining), immunohistochemistry for apoptosis and proliferation using TUNEL assay and Ki-67 and ultrastructure by transmission electron microscopy (TEM). The average percentage of gonocyte and spermatogonium numbers per tubule was calculated from 30 counted cross-sectioned seminiferous cords and tubules.

4.3.1.2 Experiment 2: Phenotypic analysis of testicular cells with SSC markers and flow cytometry analysis of SSC population

Exp. 2.1 To identify the SSC marker, GFRα-1 (GDNF family receptor α-1), DDX-4, (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) and c-kit were used for immunohistochemistry.

Exp. 2.2 Testes of group II. (n=5) and group III. (n=5) were used in this experiment. The testes were dissociated by modified two-step enzymatic digestion. The dissociated testicular cells were fixed and stained with SSC markers. The immunolabelled cells were examined by flow cytometry.

4.3.1.3 Experiment 3: *In vitro* culture and identification of derived SSC colonies

Independent testes from group II (n=17) and group III (n=18) were dissociated and cultured as previously described (Tiptanavattana, et al., 2013). This experiment was designed to observe the SSC morphology and activity. The SSC colonies (5 colonies/group) were manually collected with a fine-ended glass pipette and detected the mRNA expression of *GFRA1*; GDNF family receptor α-1, *ZBTB16*; Zinc finger and BTB domain containing 16, *RET*; RET proto-oncogene and *POU5F1*; POU class 5 homeobox 1) using RT-PCR. The ultrastructure of SSC colonies were also analyzed by TEM.

4.3.2 Animals

Cat testes were obtained from domestic cats (*Felis catus*) following routinely castration at the Small Animal Teaching Hospital of Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. This study were ethically performed and approved by the Chulalongkorn University of Animal Care and Committee.

They were transported in a 0.9% (w/v) sodium chloride solution (Thai Otsuka pharmaceutical, Bangkok, Thailand) supplemented with 1% (v/v) penicillin-streptomycin (Gibco, NY, USA) at room temperature. After the surrounded tissues were separated and discarded from the testes, the testes were individually weighed and used for further analysis. For extraction of epididymal sperm, the caudal epididymides were separated from surrounding tissue and then cut into small pieces. The presence of epididymal sperm was observed under a light microscope at x400 magnification.

4.3.3 Testicular Histology

Testes were fixed in 4% (w/v) paraformaldehyde (VWR BDH Prolabo, Poole, UK) in phosphate-buffered saline (PBS) for 24 h, rinsed with 70% ethanol, embedded in paraffin and sectioned at 4 μm thickness and layered on a silane coated glass slide.

Routinely histological study was performed on each tissue section by hematoxylin and eosin (H&E) staining. For immunohistochemistry (IHC), the expressions of SSC-related markers (Ki-67, DDX-4, GFRα-1, and c-kit) were detected by chain polymer-conjugated with peroxidase system. In short, the slides were heated at 121 ℃ for 15 min in citric acid buffer (VWR BDH Prolabo, Poole, UK; pH=6.0) supplemented with either 0.02% (v/v) Tween-20 (for Ki-67 and DDX-4) or 0.03% (v/v) Triton X-100 (for GFRα-1 and c-kit). Hydrogen peroxide (1%, v/v) in methanol (VWR BDH Prolabo, Poole, UK) was used to block endogenous peroxidase. The sections were incubated with 2% (w/v) bovine serum albumin (BSA) (Sigma-aldrich, CA, USA) in PBS and subsequently with primary antibodies diluted in sterile PBS at 4 ℃ for 24 h. Dako REAL™ EnVision™ Detection System (Dako, Glostrup, Denmark) at room temperature for 45 min. The sections were immersed in DAB and counterstained with Mayer's hematoxylin. The primary antibodies were mouse monoclonal Ki-67 (1:100, Dako, Glostrup, Denmark), rabbit polyclonal DDX-4 (1:100, Abcam, MA, USA), rabbit polyclonal GFRα-1 (1:50, Abcam, MA, USA) and rabbit polyclonal c-kit (1:300, Dako, Glostrup, Denmark). The primary antibodies were replaced with sterile PBS as for negative controls. For the TUNEL assay (ApopTaq® Peroxidase In Situ, Millipore, CA, USA), the slides were heated and quenched as previously described. The procedures were performed following manufacturer's recommendation.

The morphology of different stages of testicular germ cells and the expression of protein markers were observed using a BX5 light microscope (Olympus, Shinjuku, Japan). The percentage of testicular cells per tubule (gonocyte, spermatogonium and Sertoli cell) was calculated from a total of 30 centered cross-sectioned seminiferous cords/tubules. The photomicrographs were recorded using the cellSens program (Olympus, Shinjuku, Japan) and Adobe Photoshop CS6 Version 13.0.1 (Adobe systems, CA, USA).

4.3.4 Transmission electron microscope (TEM)

Testes, cut into small blocks, and SSC colonies were fixed in 3% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer (pH=7.2) for 24 h, then after washing in

fresh buffer they proceeded to fixation in 1% (w/v) osmium tetroxide for 1 h. The tissues were further dehydrated with gradient concentrations of ethanol and embedded in Epon (TAAB laboratories equipment, Aldermaston, UK). The Epon embedded blocks were cut as semithin and ultrathin sections on an ultramicrotome (Reichert Ultracut S, Leica, Microsystems). The semithin sections (1 μm thickness), which were stained with 1% (v/v) basic toluidine blue, were used for identifying an area of interest of an approximate size of 200 x 200 μm. Ultrathin sections (60 nm thickness) were prepared from the area of interest and contrasted with uranyl acetate (30 min) and lead citrate (10 min) and then observed using a transmission electron microscope (Philips CM 100 TEM, Darmstadt, The Netherlands)

4.3.5 Flow cytometry

The dissociated testicular cells were fixed in 4% (w/v) paraformaldehyde (VWR BDH Prolabo, Poole, UK) in sterile PBS for 24 h at 4 °C and washed/maintained in sterile PBS for further process. Testicular cell suspensions were incubated with rabbit polyclonal GFRα-1 (1:50, Abcam, MA, USA) for 24 h at 4 °C and washed with sterile PBS for 2 times. The secondary antibody was Alexa Fluor® 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes, CA, USA). The suspensions maintained in sterile PBS were analyzed using BD FACSCalibur™ (Becton Dickinson, NJ, USA). For negative control, GFRα-1 primary antibody was replaced with sterile PBS to determine the baseline level of expression.

4.3.6. Culture of spermatogonial stem cells

The SSC culture medium modified from Kanatsu-Shinohara et al. (2003a) were used in this study. Dissociated testicular cells were counted and cultured at a final concentration of $2x10^5$ cells/ml at 37 °C in a moisture incubator with 5% CO_2 in air. The SSC colonies were cultured in feeder-free system and observed daily for morphology and proliferative characteristics using a phase-contrast microscope (CKX41, Olympus, Shinjuku, Japan) for 4 weeks as previously described (Tiptanavattana et al., 2013).

4.3.7 mRNA expression of SSCs cultured *in vitro*

A total of 5 SSC colonies were collected and extracted for total RNA using an Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, CA, USA). The extracted RNA was reverse transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) for cDNA systhesis (RT+). Negative control (RT-) were prepared by removal of Superscript™ III reverse transcriptase. The synthesized cDNA was used as the template for PCR.

PCR was performed with RT+ and RT- using GoTag® Green Master Mix (Promega, WI, USA). Briefly, the PCR reaction consisted of initial denaturation (2 min at 95 °C), 30 cycles of PCR cycling (30 sec at 95 °C, 30 sec at annealing temperature for each primer and 30 sec at 72 °C; Table 2) and final extension (2 min at 72 °C). The PCR products were electrophoresed in 1% (w/v) agarose gel (Bio-Rad, CA, USA) and 5% (v/v) RedSafe™ nucleic acid stain (iNtRON Biotechnology, Gyeonggi-do, Korea) in TBE buffer. The amplified products were examined under UV light using a Gel Documentation system (Syngene, CB, UK). The primers used in this study are described in Table 2.

4.3.8 Statistical analysis

The data are presented as mean±SEM. Data were analyzed with SPSS version 20.0.0 (IBM corporation, Armonk, NY, USA). The statistical differences between groups were tested using the Analysis of Variance and Bonferroni post hoc test (Exp. 1) and

Table 2 Sense and antisense of primers description of target genes for SSC characterization

Gene	Sequence (5'-3' orientation)		Amplicon size (bp)	Annealing temperature (°C)	GenBank accession number or Reference
GFRA1	Sense:	CAACTGCCAGCCAGAGTCAA	250	60	(Tiptanavattana et al., 2013)
	Antisense:	AGCCATTGCCAAAGGCTTGA			
ZBTB16	Sense:	GCAAGAAGTTCAGCCTCAAGC	119	63	(Tiptanavattana et al., 2013)
	Antisense:	GCTTGATCATGGCCGAGTAGTC			
*RET**	Sense:	TGTGCATGACTACAGGCTGG	193	63	XM_003994195.2
	Antisense:	CCTGCTCACAGTGAAGGTGT			
POU5F1	Sense:	TGAGAGGCAACCTGGAGAAC	112	55	(Filliers et al., 2012)
	Antisense:	AACCACACTCGGACCACATC			
GAPDH	Sense:	GGAGAAAGCTGCCAAATATG	191	55	(Sano et al., 2005)
	Antisense:	CAGGAAATGAGCTTGACAAAGTGG			

*newly designed primer.

independent samples Student's t-test (Exp. 2 and 3). A *p*-value ($p<0.05$) was set to determine statistical significance.

4.4 Results

4.4.1 The transition of testicular germ cells during different phases of post-natally testicular development

The histologically cell types in cat testes observed in different age groups (group I, II and III; Fig 7) were gradually presented within seminiferous cords/tubules of the testes as advanced development of testicular germ cells proceeded. These cells included the development of testicular germ cells from gonocytes and undifferentiated spermatogonia (SSCs) to spermatocytes, round spermatids, elongated spermatids and spermatozoa, respectively. At early age of testicular cell development (group I), gonocytes were predominantly found in the seminiferous cords. Pleomorphic shaped gonocytes had average size of 17.17±2.82 µm (ranged between 10.9-25.77µm). Prominent nucleolus and pseudopod were also observed. Their ultrastructure represented homogenously euchromatin nucleus and less of cellular organelles (Fig 8a). However, a small numbers of cells already had

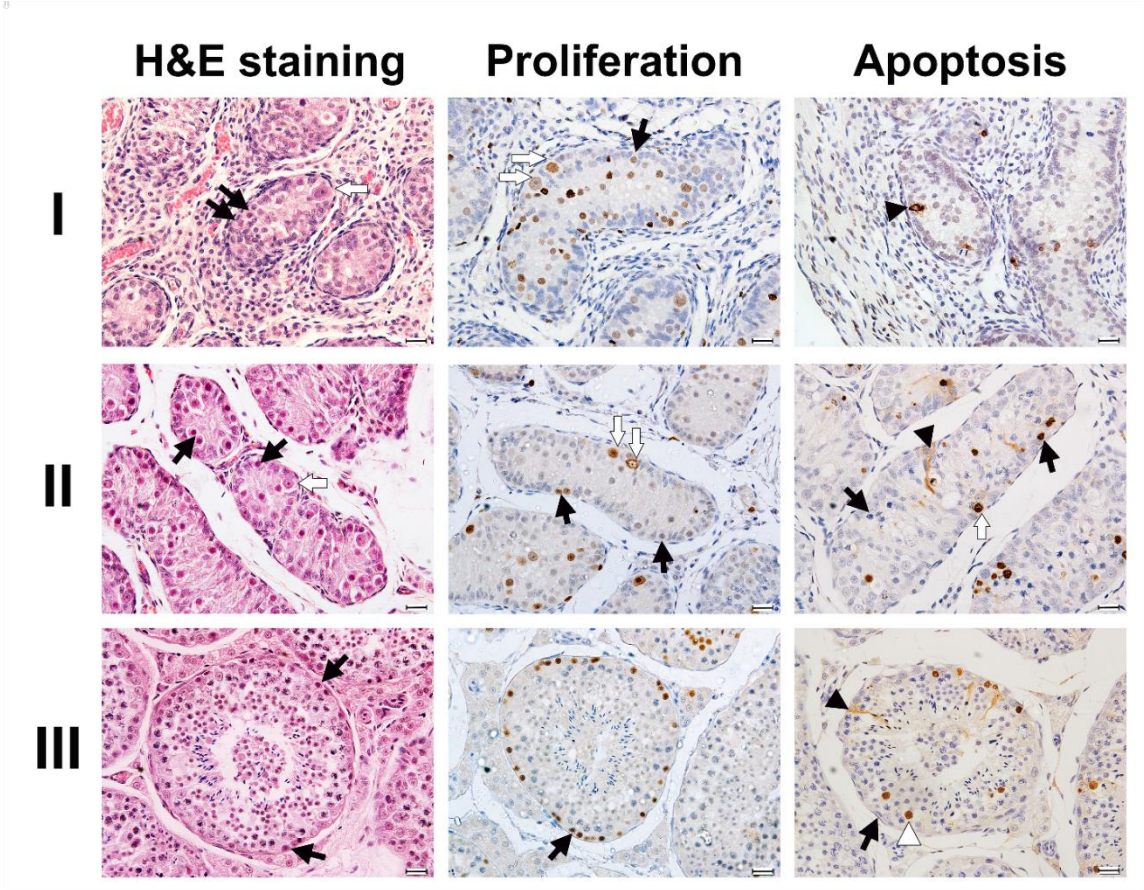

Figure 7 Conventional histology, proliferative activity and apoptotic activity of cat testicular sections.

Conventional histology (left panel), proliferative activity (Ki-67 expression, middle panel) and apoptotic activity (TUNEL assay, Right panel) of cat testicular sections in group I, II and III. Testicular section of group I (<4 months) demonstrated a large proportion of gonocytes presented in the seminiferous cords. These cells had high proliferative activity, while apoptotic gonocytes were occasionally phagocytized by Sertoli cells. Group II (4-6 months) presented the mix populations of gonocytes and spermatogonia in the seminiferous cords. The Ki-67 activity was absent in some gonocytes and spermatogonia (black arrow). However, rest of them were also expressed the proliferative and apoptosis activity. The complete spermatogenesis was only observed in Group III testes. Spermatogonia and primary spermatocytes obviously expressed proliferative and apoptosis activities, respectively. Gonocyte (white arrow); Spermatogonium (black arrow); phagocytizing Sertoli cell (black arrow head); primary spermatocyte (white arrow head). Scale bar = 20 μm

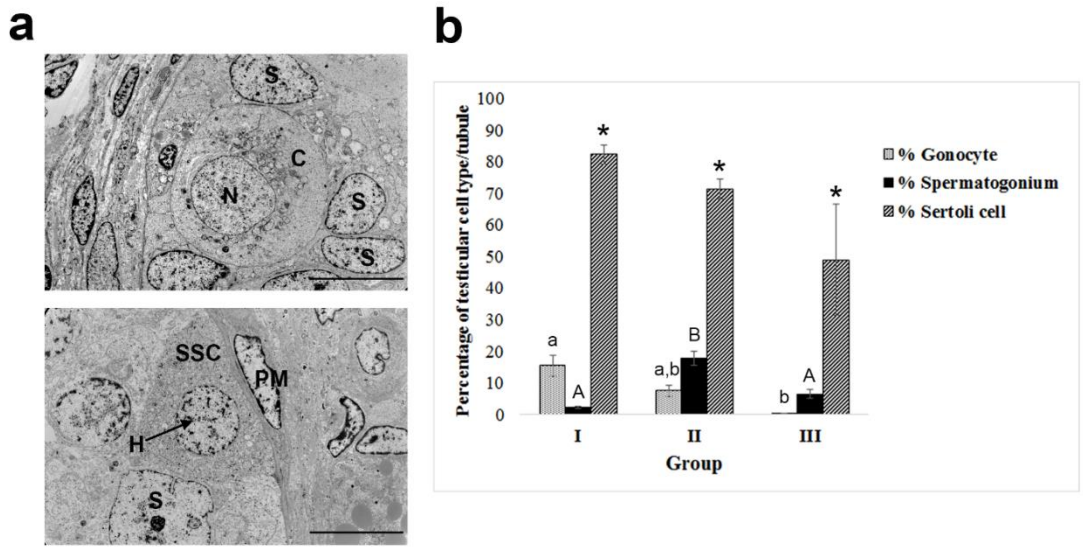

Figure 8 Morphology and numbers of testicular germ cell types within seminiferous cords/tubules.

a) The ultrastructure of gonocyte (upper panel) and spermatogonium (lower panel). The gonocyte presents abundant euchromatin chromatin in nucleus (N) and sparse organelles in the cytoplasm (C). The spermatogonium (SSC) presents small clumps of heterochromatin (H) and numerous of mitochondria. b) Mean percentage of gonocytes and spermatogonia per seminiferous cord/tubule. Different letters indicate values that are significantly different ($p<0.05$). Sertoli cell (S), peritubular myoid cell (PM). Different characters (a, b, A, B and *) within type of cells indicate values that are significantly difference ($p<0.05$). Scale bar = 10 μm

heterochromatin clumps within the nucleus. The mitotic activity was presented along with proliferative gonocytes and Sertoli cells as determine by the expression of Ki-67 proliferative marker (Fig 7). This stage of testicular development had higher percentage of gonocytes per seminiferous cord (15.49±3.34) compared to those observed in testes of group II (7.52±1.79%) and group III (0.11±0.10%) (Fig 8b). In addition, apoptotic marker (TUNEL positive cells) was only expressed in phagocytic Sertoli cells and occasionally in the gonocytes (Fig 7).

Predominant changes of germ cell types were evidently demonstrated in testis of group II, especially at the age of 4 months. At this time point, major populations of testicular cells in the seminiferous cord were gonocytes, spermatogonia and Sertoli

cells. The gonocyte dramatically transformed to undifferentiated type of spermatogonium (referred to SSC) as determined by an increasing of nucleus to cytoplasm ratio (reduced cell size) with darkening cytoplasm and chromatin condensation. This cell transformation occurred spontaneously with the migration of spermatogonia from innermost structure to periphery of the seminiferous cords. The putative SSCs presented as round to oval shaped cells then localized onto the basement membrane of the seminiferous cord. The mean size of these putative SSCs was 10.31±0.89 μm (ranged between 8.11-13.55 μm). At this stage of post-natally testicular development, the mean percentage of SSC numbers per seminiferous cord/tubule (17.66±20%) was significantly higher than those found in testes of group I (2.18±0.36%) and III (6.45±1.56%) (Fig 8b, $p<0.001$). For ultrastructure, nucleus of the SSCs was distinctly euchromatin with small clumps of heterochromatin. The TEM examination also revealed a marked increase in mitochondria in the cytosol compared with that observed in earlier stage of testicular cell development (i.e. group I testes). This finding coincided with intensely stained cytoplasm of the SSCs observed in H&E examination (Fig 8a). For group II, the histology demonstrated no expression of Ki-67 in some gonocytes and spermatogonia (Fig 7). Apoptosis was predominantly observed in gonocytes which was occasionally engulfed by Sertoli cells (Fig 7).

By 6 month of age (group III), initiation of meiosis of spermatogenesis took place as indicated by the presence of prophase I spermatocytes within the seminiferous cords. With an advanced age of the cats (group III), the development of seminiferous tubules was formed along with the presence of advanced stages of testicular germ cells. While the gonocytes were rarely present from all testicular samples, spermatogonia and spermatocytes were distinctly the major population of testicular cells population in the seminiferous tubules. However, complete spermatogenesis was firstly observed in 9-month old testes. The Ki-67 was found to be strictly expressed in spermatogonia. Apoptosis was episodically found in a small population of spermatogonia and premeiotic spermatocytes (Fig 7).

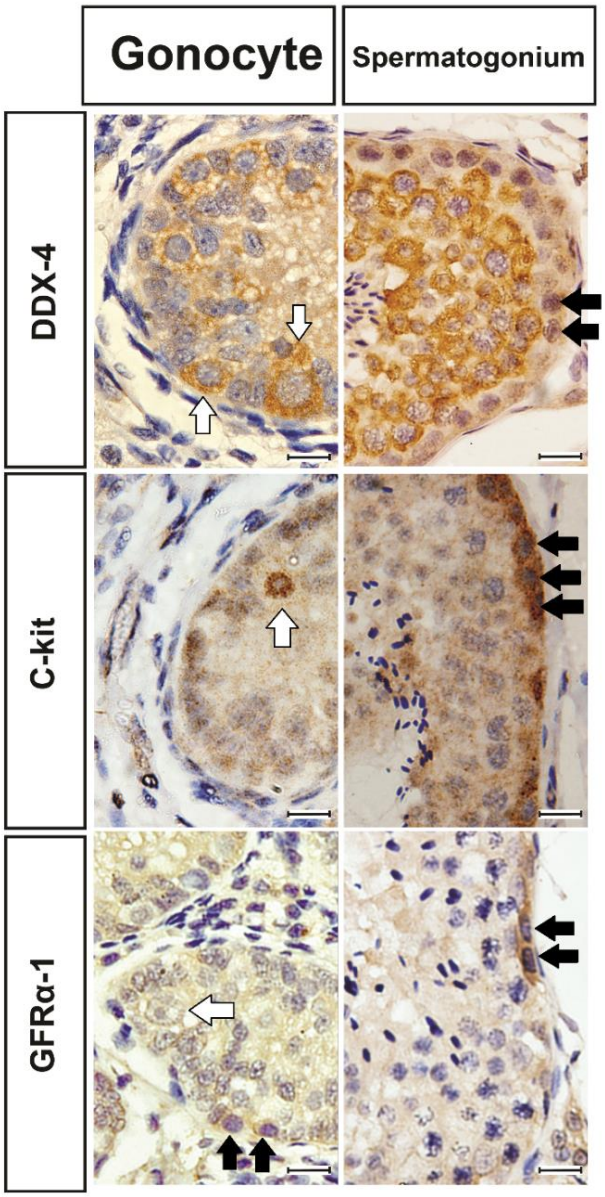

Figure 9 The protein expression of DDX-4, c-kit and GFRα-1 in the cat testes.

The DDX-4 expressed in cytoplasm of all types of testicular germ cells. Spermatogonia and primary spermatocytes expressed DDX-4 at lower levels than secondary spermatocytes and round spermatids. The c-kit was localized at peri-nuclear area (golgi pattern) of gonocyte, plasma membrane and cytoplasm of most spermatogonia. Single and paired spermatogonia expressed GFRα-1 at plasma membrane and in the cytoplasm. Gonocyte (white arrow); Spermatogonium (black arrow). (gonocytes; left panel and spermatogonia; right panel). Scale bar = 10 μm

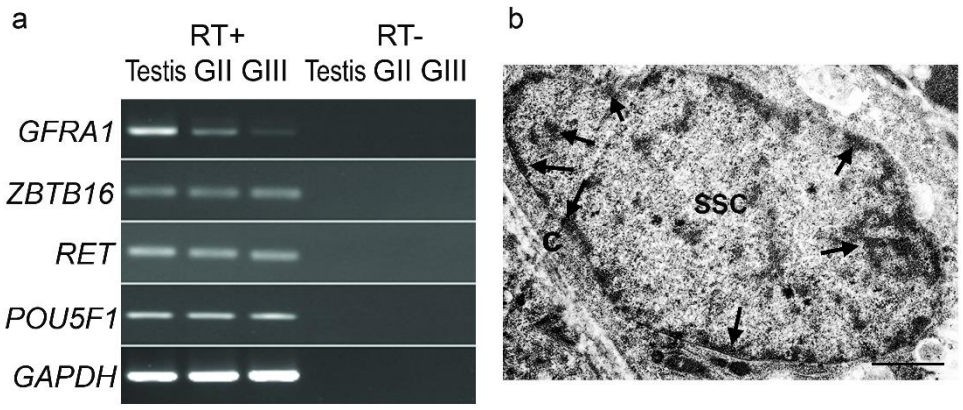

Figure 10 The mRNA expression and ultrastructure of SSC colonies.

a) the RT-PCR products from group II (GII), III (GIII) testes. RT+ and RT- were performed to detect mRNA expression of SSC genes (*GFRA1*, *ZBTB16*, *RET* and *POU5F1*). b) Nuclear ultrastructure of *in vitro* cultured SSCs demonstrated a typical nuclear ultrastructure containing euchromatin and small clumps of heterochromatin (arrow). Cytoplasm (C); Scale bar = 1 μm

4.4.2 Phenotypic analysis of testicular cells with SSC and testicular markers

Immunohistochemistry was used to study the expression of GFRα-1, DDX-4 and c-kit as SSC, germ cell and differentiated spermatogonium markers, respectively. In the seminiferous cords/tubules, the DDX-4 protein were localized at the cytoplasm of gonocytes, spermatogonia, primary and secondary spermatocytes and some of round spermatids (Fig 9). The DDX-4 expression was descriptively lower expressed in both of spermatogonia and primary spermatocytes when compared to other cell types. The c-kit expression was strictly found in both gonocytes and spermatogonia. The patterns for c-kit expression were different between gonocytes and spermatogonia in that the peri-nuclear membrane (golgi pattern) was predominantly found in gonocytes while the c-kit protein was translocalized to express in the cytoplasm and plasma membrane of the spermatogonium (Fig 9). The GFRα-1 essentially expressed in plasma membrane and cytoplasm of the SSCs or spermatogonia (Fig 9) but not in the gonocytes.

4.4.3 Flow cytometry analysis of SSC population

To determine the numbers of SSCs (GFRα-1 positive cells) within the testes, testes from group II and III were only selected as group I testes did not contain SSCs. Of the total cells isolated from testicular digestion, the percentage of GFRα-1^+ cells obtained from group II testes (14.89±5.66%) were significantly higher than the group III (1.46±1.64%) (p<0.001).

4.4.4 *In vitro* culture and identification of derived SSC colonies

Following testicular cell digestion and SSC culture, the SSC colonies containing 2-6 cells were observed within 2 weeks. The SSC were gradually increased the cell numbers to approximately 12 cells around 4 weeks of culture. The SSC colonies were tightly packed with small intercellular space. Of SSC culture, the SSC colonies obtained from group II testes had a higher success rate than group III testes, as determined by the number of colonies observed at 4 weeks of culture (74.33±2.64% vs. 23.33±2.23%, p<0.001).

The SSCs from both groups expressed *GFRA1*, *ZBTB16* and also *RET*, and also demonstrated a typical ultrastructure morphology (TEM) (Fig 10). The TEM revealed that the SSCs had round to oval shaped nucleus with flatten cytoplasm. The nucleus contained euchromatin with a small number of heterochromatin clumps underneath the nuclear membrane similar to the ultrastructure found in testicular tissues (Fig 10b).

4.5 Discussion

This study revealed that the development of testicular germ cells occurred asynchronously depending on cell types and was donor age dependent. Here, we reported for the first time that the G/SSC transition phase primarily initiated by 4 months of age in domestic cats. In addition, this phase of testicular development was appropriated for SSC derivation as these periods contained the highest numbers of GFRα-1^+ SSCs and significantly improved efficiency of SSC colony forming.

In this study, we described chronological development of testicular germ cells in cat testes as these cellular changes have been proposed to critically affect to successful derivation of SSC *in vitro* (Kanatsu-Shinohara et al., 2008a). It was clearly demonstrated that the development and transformation of feline gonocytes to testicular germ cells was a slow process when compared to rodent model (de Rooij, 1998). At birth, cat seminiferous cords contained abundance of intracytoplasmically DDX-4 expressing gonocytes. The subpopulation of testicular cells with cell-surface DDX-4 pattern as previously reported in pig however was not demonstrated in this study (Kakiuchi et al., 2014). According to our results, the gonocytes in group I testes (less than 4 months) had high proliferation rate prior entering to the G/SSC phase. The relative percentage of gonocytes per tubule gradually decreased with an advancement of spermatogenesis as a consequence of an increase apoptosis and decrease in proliferation activity of gonocytes observed in group II testes. This topographic transformation of gonocytes or G/SSC transition is similar to other studies previously reported (Helal et al., 2002; Orwig et al., 2002a; Zogbi et al., 2012). The gonocytes become quiescence (mitotic arrest with apoptosis) before mitotic resumption and G/SSC transition (Hoei-Hansen et al., 2005; Rajpert-De Meyts, 2006; Culty, 2009). Although the phase at G/SSC transition precisely occurs in mouse and rat (Drumond et al., 2011). The quiescent period of gonocytes and G/SSC phase have been variable and controversial among species (Huckins and Clermont, 1968; Vergouwen et al., 1991). This study demonstrated that the process for G/SSC transition in cat was found to be asynchronously phenomenon throughout the seminiferous cord/tubule which was dissimilar to rodents (de Rooij and Grootegoed, 1998). Recently, the mechanisms that control the quiescent period of G/SCC transition is still unknown but this period is required for marked molecular changes (such as downstream of pluripotent markers) (Mitchell et al., 2008; Gangemi et al., 2009). The active gonocytes migrated down to basement membrane of seminiferous cord by KITL/KIT signaling prior the SSC transformation (Yoshida et al., 2006). The c-kit which is well known as the plasma membrane receptor localized at the peri-nuclear area (golgi pattern) of the gonocytes and then, translocalized to plasma membrane of spermatogonia. This golgi pattern of gonocytes was usually found in germ cells but this pattern also could be seen

expressed in gastrointestinal stromal tumor (GIST) cells of human (Sandlow et al., 1996; Gaskell et al., 2004; Gonzalez-Campora et al., 2011). The morphology and ultrastructure of gonocytes within the seminiferous cords were remarkably distinguished from the SSC, in terms of morphology and the numbers of mitochondria. These enriched mitochondria in the SSCs appeared to associate with the darkened cytoplasm on H&E sections and helped to maintain high level of mitochrondrial activity in rat and mouse SSC (Ryu et al., 2004; Lo et al., 2005) and to stimulate proliferation of the early phase of SSCs (Ryu et al., 2003). These feline SSCs expressed mRNA (*GFRA1*, *ZBTB16*, *RET* and *POU5F1*) and proteins (DDX-4 and GFRα-1) essentially importance for SSC activities similar to other reports (Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004d; Tiptanavattana et al., 2013)

The pubertal age of cat in this study was precisely identified at around 9 month of age as indicated by complete spermatogenesis and the presentation of spermatozoa in caudal epididymis. This finding is different from previous studies indicating that the pubertal period of cat ranges from 8 to 12 months (Siemieniuch and Woclawek-Potocka, 2007). The difference in pubertal period may cause by differences in photo period, climate, animal care and nutritional status (Kane et al., 1981; MacDonald et al., 1984; Kirkpatrick, 1985; Swanson et al., 1996; Tsutsui et al., 2009). For meiosis phase, the apoptosis which was occurred in spermatogonia and primary spermatocytes was intensively observed in the first wave of spermatogenesis. The apoptosis at this point is essential for regulation the balance between the number of germ cells and Sertoli cells and also for the reduction of impaired germ cells during the meiosis (Giampietri et al., 2005; Tripathi et al., 2009). In this study, group II testes contained the highest percentage of morphological spermatogonia (referred to as SSCs) per cord/tubule as a result of the resumption of mitosis (de Rooij and Grootegoed, 1998). This stage of testicular development was therefore appropriate for enrichment of the GFRα-1^+ cells. The percentage of spermatogonia per cord/tubule observed from histology was closely similar to the percentage of GFRα-1^+ testicular cells from flow cytometry (17.66±2.20% vs. 14.89±5.66%). The high proportion of SSCs in the group II testes coincided with the improved success rate of SSC colony formation (74.33±2.64%) when compared with

more advanced stage of testicular development (group III testes; 23.33±2.23%, $p<0.001$). This result, therefore, confirm the importance of G/SSC phase transition and also possibly the negative effects of other testicular cells (differentiated germ cells and supporting testicular cells) on successful SSC culture (Kanatsu-Shinohara et al., 2008a). However, maintaining SSC activities *in vitro* remains a hallmark for long term culture of SSCs in domestic cat (Tiptanavattana et al., 2013; Han et al., 2014). Further study is required to underpin the mechanisms that essentially contribute to the multifaceted fate of SSC properties during SSC culture.

This study concludes that testicular development and G/SSC transition phase in cat testis are precisely time-specific dependence. This transition phase is a synchronously phenomenon throughout the seminiferous tubules. Determination of this G/SSC transition successfully promotes the efficiency of SSC isolation and culture of SSC *in vitro*.

CHAPTER 5

SIMPLIFIED ISOLATION AND ENRICHMENT OF SPERMATOGONIAL STEM-LIKE CELLS FROM PUBERTAL DOMESTIC CATS (*FELIS CATUS*)

Narong Tiptanavattana, Mongkol Techakumphu, Theerawat Tharasanit

Journal of Veterinary Medical Science
Vol. 76, No 11, November 2015 (In press)

5.1 Abstract

The efficiency of spermatogonial stem cells (SSC) isolation and culture from pubertal donor is currently poor primarily because of the contamination of other testicular cells. This study aimed at purifying SSC-like cells using different extracellular matrixes and the discontinuous gradient density. In Exp.1, the testes (n=6) were analyzed for histology and SSC related protein expressions (laminin, SSEA-4, DDX-4 and GFRα-1). Cell suspension after enzymatic digestion was plated onto either laminin or gelatin coated dish. The number of SSC-like cells were determined at 15, 30 and 60 min of culture (Exp.2). The Exp.3 was performed to test whether or not the additional step of Percoll gradient density could really improve purification of SSC-like cells. Testicular histology represented the complete spermatogenesis with laminin expression essentially at the basal lamina of the seminiferous tubules. SSEA-4 and GFRα-1 co-localized with DDX-4 were present in the spermatogonia. The relative percentage of SSC-like cells, as determined by SSEA-4 (59.42±2.18%) and GFRα-1 (42.70±1.28%) expressing cells revealed that the highest SSC-like cells purity was obtained from the 15-min laminin coated dish compared with other incubation times and gelatin treatment (p<0.05). The Percoll treatment prior to laminin selection (15 min) significantly improved SSC-like cells recovery (91.33±0.14%, p<0.001) and purity (83.82±2.05% for SSEA-4 and 64.39±1.51% for GFRα-1, p<0.05). These attached cells

demonstrated a typical SSC-like cells morphology and also expressed *POU5F1*, *RET* and *ZBTB16* mRNA. In conclusion, the double enrichment with Percoll gradient density and laminin plating highly enriched the SSC-like cells population.

Keywords: Cat, Extracellular matrix, Purity, Spermatogonial stem cell

5.2 Introduction

Spermatogenesis is a complex process of male germ cell production, in which diploid spermatogonia or spermatogonial stem cells (SSCs) transform and differentiate into haploid spermatozoa within seminiferous tubules. This process is regulated by intra- and extra-testicular factors and continues throughout pubertal period of men and animals. Although the SSCs hold a great promise for the treatment of infertility problems in man due, for example, to premature loss of male germ cells following cytotoxic chemotherapy. These SSCs can also be used for propagation and preservation of the genetic profiles of valuable male animals such as endangered species (Kim et al., 2006). In addition, the SSCs have recently been reported to capable of differentiation into three germ layers of embryos including cardiomyocytes, smooth muscle cells, neural cells, endothelial cells, hepatocytes and renal tubular cells (Guan et al., 2006; Guan et al., 2009; Ko et al., 2009; Heer et al., 2013; De Chiara et al., 2014). The SSCs have therefore become an emerging model for regenerative medicine.

The SSCs are subpopulation of spermatogonia type A that settle on the basal lamina of the seminiferous tubule. The numbers of SSCs, however, have been estimated to be only 0.03% of the total testicular cells in adult rat testis (Tegelenbosch and de Rooij, 1993; van Pelt et al., 1996). Thus, purification of the SSCs from digested pubertal testis has therefore become an important step for isolation of SSCs since this technique eliminates the somatic testicular cells that interfere the proliferation of the SSCs *in vitro* (van Pelt et al., 1996; Oatley and Brinster, 2006; Kubota and Brinster, 2008). Until recently, several studies in mouse, rat and bull reported efficient techniques for SSC enrichment including plating with different coating substances, discontinuous Percoll™ gradient density and Fluorescent-Activated Cell/Magnetic-Activated Cell Sorting (FACs/MACs) translating spermatogonial stem cell transplantation to the clinic

(Shinohara et al., 1999; Izadyar et al., 2002; Kent Hamra et al., 2004; Kubota et al., 2004d; Herrid et al., 2009). The SSCs surrounded with Sertoli cells adhere on the basal lamina of seminiferous tubules by various extracellular matrixes (ECMs) (Shinohara et al., 2000; Oatley et al., 2011). These ECMs are important for attachment of the testicular cells to basal lamina of seminiferous tubules and also for the formation of the SSC niche (Shinohara et al., 1999). Various types of ECMs have been used to purify the SSC population such as laminin, fibronectin, collagen type I and IV and gelatin (Kanatsu-Shinohara et al., 2008n; Kim et al., 2010; Lim et al., 2014). Of substrates used to coat the culture dish, gelatin has generally been used because it is cost-effective for optimization of cell attachment in various cell types, such as fibroblasts. The gelatin plays a role in denaturing collagen, as connective tissue, and is also interacted with laminin and fibronectin. Although the efficiency of laminin in selecting SSCs in domestic cat has yet to be examined, the laminin-coated plate has been demonstrated to improve purifying efficacy of SSC isolation by 3.3, 5-7 and 8.5 folds in bull, mouse and rat SSCs, respectively (Shinohara et al., 2000; Orwig et al., 2002e; Herrid et al., 2009). This high efficiency of laminin for SSC selection has been postulated to associate with its receptors on the SSCs (Shinohara et al., 1999). Although the attachment of SSCs to laminin involves with integrin proteins, a laminin receptor (Hynes, 1992), α_6-integrin was only the specific surface marker of SSCs in mouse (Shinohara et al., 1999). In addition to plating selection, Percoll™ purification, a non-toxic gradient density, has been performed to recover the specific populations of testicular cells via different gradient density and centrifugation. This technique recovered approximately 80%, 72% and 96% for rat, buffalo and pig SSCs, respectively (van Pelt et al., 1996; Rafeeqi and Kaul, 2013; Han et al., 2014). Moreover, the viability of SSCs recovered from Percoll™ was also improved (van Pelt et al., 1996).

The objectives of this study was to examine the effects of types of ECM substrates and Percoll™ gradient density on the enrichment of SSC-like cells in pubertal domestic cats.

5.3 Materials and methods

5.3.1 Experimental designs

5.3.1.1 Experiment 1: The localization and immunolabelling of spermatogonial stem cells (SSCs) in pubertal cat testes

The cat testes (n=6) were collected from pubertal domestic cats. The testes were fixed and sectioned for the routine histology, immunohistochemistry (laminin) and immunofluorescence for SSC markers. Immunofluorescence was performed to demonstrate the co-expressions of SSEA-4/DDX-4 and GFRα-1/DDX-4 as SSC makers. The localization and expression pattern for each marker was descriptively analyzed.

5.3.1.2 Experiment 2: The enrichment efficiency of spermatogonial stem-like cells (SSCs) using different types of extracellular matrixes (ECMs)

The testes (n=6) were dissociated into single cells by a modified 2-step enzymatic digestion (Tiptanavattana et al., 2013). The digested testicular cells (0.5 x 10^6 cell/cm^2) were plated onto laminin (20 µg/ml) and gelatin (0.1% (w/v)) coated dishes for 15, 30 and 60 min. The attached testicular cells were fixed and examined for SSEA-4 and GFRα-1 expression using fluorescent microscopy. The percentages of SSEA-4^+ and GFRα-1^+ from different types of ECMs versus time points was analyzed.

5.3.1.3 Experiment 3: Double enrichment of spermatogonial stem-like cell (SSC) population with Percoll™ and laminin plating

The suspension of dissociated testicular cells (n=6, 2x10^6 cell/ml) was first layered onto discontinuous Percoll™ gradient density as previously described (Kubota et al., 2004a) with some modifications. The Percoll™ layers containing SSC-like cells were subsequently plated onto laminin coated dish for 15 min. The SSEA-4^+ and GFRα-1^+ cells were determined. In addition, the attached cells were collected for study of SSC-related gene expression (*POU5F1*, *RET* and *ZBTB16*) and differentiation marker (*KIT*).

5.3.2 Animals and sample preparation

Cats (aged between 1-2 years old) were used in this study. Cat testes were consentingly collected following routine castration at the Veterinary Public Health

Division of the Bangkok Metropolitan Administration, Bangkok, Thailand. The testes were transported in saline solution supplemented with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) at room temperature to the laboratory. The testes were dissected from extraneous testicular tissues prior to use. The testes were divided for fixation with 4% (w/v) paraformaldehyde in PBS (Exp. 1) and for dissociation with enzymatic digestion (Exp. 2 and 3).

5.3.3 Histology, immunohistochemistry and immunofluorescence

For histology and immunohistochemistry, testicular tissue was fixed with 4% (w/v) paraformaldehyde (VWR BDH Prolabo, Poole, UK) in Phosphate-buffered saline (PBS) at 4 °C (for overnight). The fixed testes were embedded in paraffin and cut at 4 µm thickness. The hematoxylin and eosin staining was used to study the structures of testis.

To detection the expression of laminin, a 3-step indirect immunoperoxidase immunohistochemistry (IHC) was performed by Leica microsystems Bond maX System (Leica Miosystems, Bannockburn, IL, USA). Briefly, the epitopes of antigen were retrieved by Bond Epitope retrieval Solution 2 (Leica Miosystems, Bannockburn, IL, USA) for 20 min at 100 °C. The slides were incubated with anti-mouse monoclonal of laminin antibody with dilution 1:100 (Novocastra™, Leica Miosystems, Bannockburn, IL, USA) at 25 °C for 45 min. Post Primary Polymer (Leica Miosystems, Bannockburn, IL, USA) was applied for 9 min and followed with Polymer Poly-HRP IgG (Leica Miosystems, Bannockburn, IL, USA) for 7 min. Mouse IgG (PP54, Millipore, Darmstadt, Germany) was used instead of primary antibody as for a negative control.

For immunofluorescence (IF) on paraffin embedded sections, the epitope was unmasked by microwave treatment at 900 watts for 15 min in citric acid buffer (VWR BDH Prolabo, Poole, UK; pH=6.0) adding 0.03% (v/v) Triton X-100. The non-specific staining was blocked using 3% (w/v) bovine serum albumin (BSA) in PBS. The sections were firstly incubated at 4 °C for overnight with either mouse monoclonal SSEA-4 (1:200, Abcam, MA, USA) or mouse monoclonal GFRα-1 (1:200, sc-10716, Santa Cruz Biotechnology, CA, USA). They were further incubated with corresponding secondary

antibody (goat anti-mouse IgG TRITC, 1:200). Subsequently, the sections were co-stained with primary antibody (rabbit polyclonal DDX-4, 1:100, Abcam, MA, USA) and followed by goat anti-rabbit IgG FITC (1:100, Abcam, MA, USA). For negative control, the staining procedures were identically performed as described above except that the primary antibodies were replaced with mouse IgG (PP54, Millipore, Darmstadt, Germany) or rabbit IgG (PP64, Millipore, Darmstadt, Germany). The co-expression of SSEA-4/DDX-4 and GFRα-1/DDX-4 markers was visualized with a fluorescent microscope (BX5, Olympus, Shinjuku, Japan).

The photomicrographs obtained from histology, IHC and IF were recorded using the cellSens program (Olympus, Shinjuku, Japan) and Adobe Photoshop CS6 Version 13.0.1 (Adobe systems, CA, USA).

For digested testicular cells, the cell suspension or attached cells were fixed with 4% (w/v) paraformaldehyde in PBS for 24 hr at 4 °C and then labelled with primary and secondary antibodies as aforementioned above (SSEA-4/TRITC, GFRα-1/TRITC). The fixed cells were also blocked with 3% (w/v) BSA in PBS to reduce non-specific background.

5.3.4 Viability test and enrichment of SSC-like cells

Testes were enzymatically dissociated to obtain single testicular cells as previous described (Tiptanavattana et al., 2013). The testicular cells were examined for the viability in terms of esterase enzyme activity (calcein AM staining) and plasma membrane integrity (ethidium homodimer-1, Molecular Probes, Invitrogen, CA, USA).

For differential plating selection, the culture dishes were first coated with either 20 µg/ml laminin or 0.1% (w/v) gelatin at 37 °C for 4 h before cell plating. The dissociated testicular cells (0.5x 10^6 cells/cm^2) were plated onto laminin or gelatin coated dishes. The cells were further incubated for 15, 30 and 60 min where the samples were fixed and collected for immunostaining. The culture condition was performed at 37 °C in a moisture incubator with 5% CO_2 in air.

Percoll™ gradient density treatment was performed by layering the cell suspension (2x10^6 cells/ml) onto 30%, 45%, 60% and 90% (v/v) isotonic Percoll™

solution, respectively. The cells were then centrifuged at 800x g for 30 min at 25 °C. The thin layers of cell suspension at interfaces between the two concentrations of Percoll™ were gently collected.

5.3.5 RT-PCR for SSC-related gene expression

The attached testicular cells were collected and extracted for total RNA using an Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, CA, USA). The total RNA (2 ng/µl) was reversely transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) for cDNA systhesis (RT+). Removal of Superscript™ III reverse transcriptase was performed for Negative control (RT-). PCR was performed with RT+ and RT- of cDNA using GoTag® Green Master Mix (Promega, WI, USA). Shortly, the PCR conditions consisted of denaturation (2 min at 95 °C), 30 cycles of 30 sec at 95 °C, 30 sec at annealing temperature for each primer and 30 sec at 72 °C) and final extension (2 min at 72 °C). The PCR products were electrophoresed in 1% (w/v) agarose gel (Bio-Rad, CA, USA) adding 5% (v/v) RedSafe™ nucleic acid stain (iNtRON Biotechnology, Gyeonggi-do, Korea) in TBE buffer. The products were detected by a Gel Documentation system (Syngene, CB, UK).

The primers and annealing temperature used in this study are as the following: *POU5F1* (5'- TGAGAGGCAACCTGGAGAAC-3' and 5'-AACCACACTCGGACCACATC-3', 55 °C, 112 bp) (Filliers et al., 2012); *RET* (5'-TGTGCATGACTACAGGCTGG-3' and 5'-CCTGCTCACAGTGAAGGTGT-3', 63 °C, 193 bp); *ZBTB16* (5'-GCAAGAAGTTCAGCCTCAAGC-3' and 5'- GCTTGATCATGGCCGAGTAGTC-3', 63 °C, 119 bp) and *KIT* (5'-TCCTGCT CCGCGTCCAGACA-3' and 5'-CTTGCCCTTCCGGTCCGCAG-3', 60 °C, 533 bp). *GAPDH* was used as a house keeping gene (Sano et al., 2005).

5.3.6 Statistical analysis

The percentages of SSEA-4$^+$ and GFRα-1$^+$ testicular cells and viability were expressed as mean ± SEM. The data were analyzed with SPSS version 20.0.0 (IBM corporation, Armonk, NY, USA). The statistical differences between the groups were

tested using the Analysis of Variance (ANOVA) followed by Bonferroni post hoc test. Different values with *p*-value ($p<0.05$) were considered statistical significance.

5.4 Results

5.4.1 The localization and immunolabelling of spermatogonial stem cells (SSCs) in pubertal cat testes

The histology of pubertal cat testes demonstrated fully-differentiation of male germ cells in the seminiferous tubules. Spermatogonia including SSCs resided on basal lamina of the seminiferous tubule and surrounded by Sertoli cells. The spermatogonia differentiated into spermatocytes, spermatids and spermatozoa (Fig. 11a). For IHC of laminin, the expression of this protein was observed at spermatogonia resided at the basal lamina of the seminiferous tubules. The localization of laminin was also essentially expressed at the extracellular matrixes of testis such as basal lamina of seminiferous tubules and blood vessels (Fig. 11b). The spermatogonia (SSCs) expressed SSEA-4 at the plasma membrane and cytoplasm. These cells co-expressed with DDX-4 (Fig. 11c). However, the DDX-4 was also detected in other testicular germ cells such as spermatocytes and round spermatids. Similar to SSEA-4^+, the co-expression of GFRα-1 and DDX-4 was precisely presented at the spermatogonia located on the basal lamina (Fig. 11d).

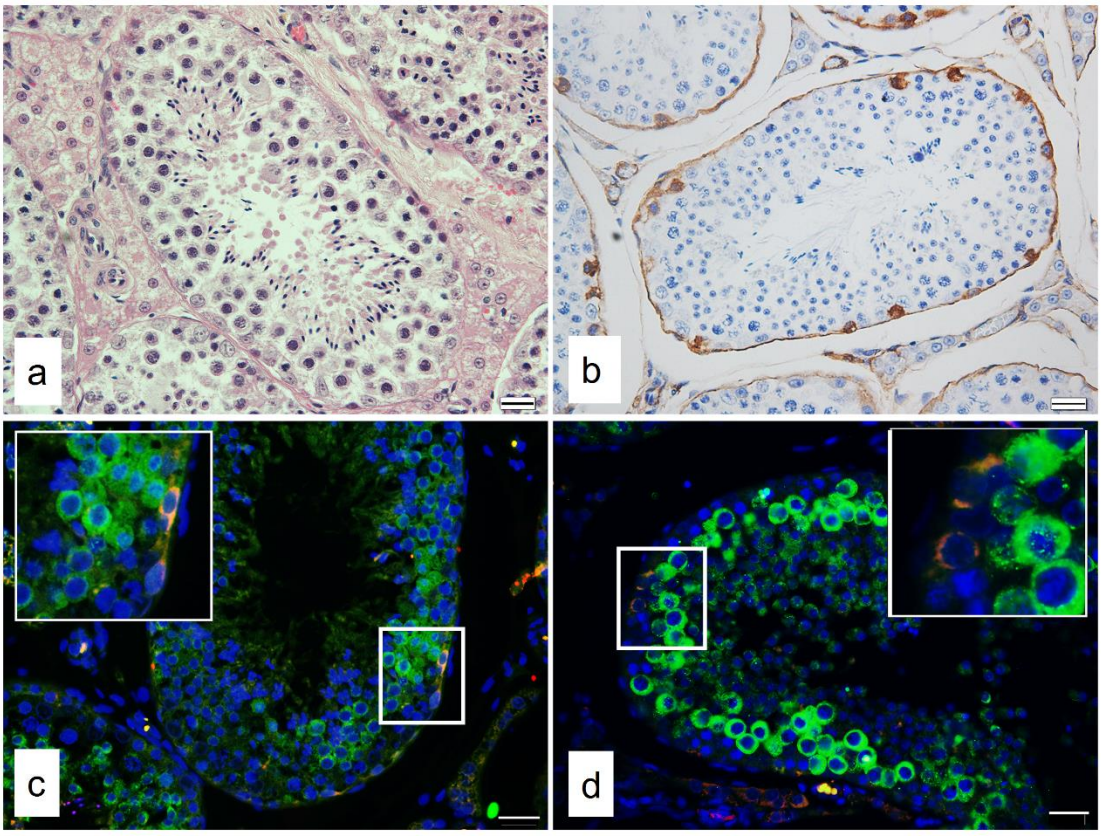

Figure 11 Histology of testicular sections.

(a) The pubertal cat testicular histology contains spermatogonia, primary and secondary spermatocytes, round and elongated spermatids and spermatozoa in the seminiferous tubule indicating the complete spermatogenic differentiation spermatogonia. (b) The cat testes expressed laminin at basal lamina of the seminiferous tubules, blood vessels and interstitial parts. The laminin expression also presented at the extracellular matrixes surrounded the SSCs. (c and d) The SSEA-4 (closed up, c) and GFRα-1 (closed up, d) were co-expressed with DDX-4 at the spermatogonia resided on basal lamina of the seminiferous tubules. The DDX-4 (green), as universal germ cell lineage marker, also presented with high intensity at secondary spermatocytes and round spermatids. Negative controls were not expressed the immunoreaction (data was not shown). Scale bar = 20 μm.

Table 3 Efficiency of differential plating with laminin and gelatin at 15, 30 and 60 min of incubation.

Marker (%)	Before	Differential plating					
		15 min		30 min		60 min	
		Laminin	Gelatin	Laminin	Gelatin	Laminin	Gelatin
SSEA-4$^+$	27.24±1.29†	59.42±2.18^{§,a,A}	9.05±1.07^{§,b,A,B}	53.37±1.08^{§,a,B}	14.68±0.94^{§,b,A}	16.77±2.59^{§,a,C}	5.34±0.22^{§,b,B}
GFRα-1$^+$	37.11±1.61†,§	42.70±1.28^{†,a,A}	10.97±0.84^{§,b,A}	41.71±2.17^{‡,a,A}	5.30±1.18^{§,b,A,B}	20.90±1.06^{§,a,B}	2.00±0.17^{§,b,B}

Different superscripts within the same row indicate values that are significantly different (p<0.05).

†,§,‡ All groups of differential plating compared with before group

^{a,b} Laminin compared with gelatin within the same time.

^{A,B} Times of incubation compared within the same substrate.

Table 4 Efficiency (mean ± SEM.) of single (Laminin) and double (Percoll^{TM}+Laminin) enrichment techniques.

Enrichment technique	SSEA-4$^+$ (%)	GFRα-1$^+$ (%)
Laminin	59.42±2.18^a	42.70±1.28^a
Percoll^{TM}+Laminin (30%/45%)	83.82±2.05^b	64.39±1.51^b

^{a,b} Different superscripts indicate values that differ significantly (p<0.05)

5.4.2 The enrichment efficiency of spermatogonial stem-like cells (SSCs) using different types of extracellular matrixes (ECMs)

After enzymatic isolation, viability of isolated testicular cells was 66.56±1.43%. The percentage of SSEA-4$^+$ and GFRα-1$^+$ testicular cells attached on different ECMs were expressed in Table 3. On laminin coated surface, adhered SSC-like cells had typical SSC-like morphology as previous study. They were round to oval cells (ranged 8-13 µm of size) with increased nucleus/cytoplasm ratio (Fig. 2b). However, all attached testicular cells were contaminated with mix populations of other testicular cells such as Sertoli cells, fibroblasts, spermatids and other testicular cells. Laminin-coated plate technique significantly enriched the SSEA-4$^+$ population

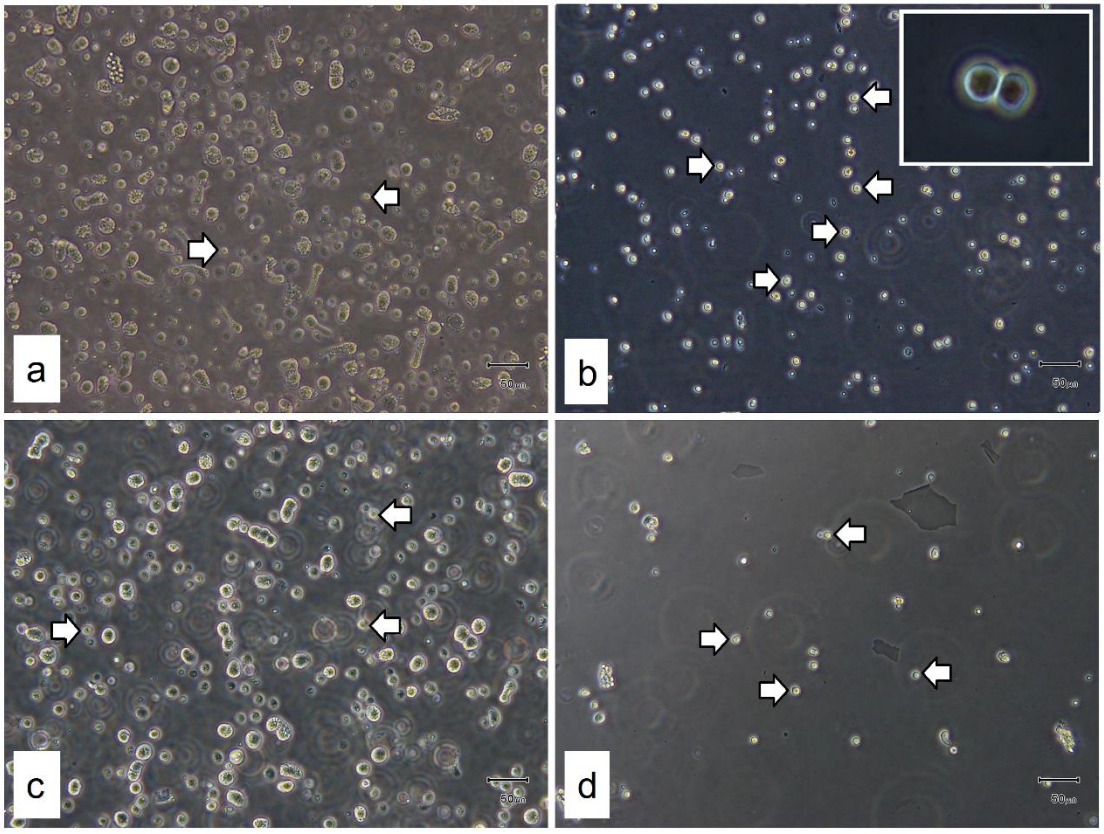

Figure 12 Morphology of testicular cells after testicular cell digestion and enrichment.

Following a modified 2-step enzymatic digestion, testicular cells contain several stages of spermatogenesis (a). Laminin was successfully used to enrich morphologically undifferentiated spermatogonia (or SSC-like cells (arrow)) (b). The SSC-like cells were round to oval shape with high nucleus/cytoplasm ratio (closed up, b) Percoll™ gradient density treatment (at 30% layer, c and 30%/45% interface, d), improved cell viability and cell purity. Scale bar = 50 µm.

of testicular cells when examined at 15 (59.42±2.18%) and 30 min (53.37±1.08%) of incubation. These percentages were significantly higher than non-treatment (27.24±1.29%, $p<0.001$). GFRα-1 testicular cells tended to increase in cell number of cells at 15 (42.70±1.28%) and 30 (41.71±2.17%) min of incubation ($p>0.05$). However, the number of SSEA-4^+ and GFRα-1^+ testicular cells examined at 60 min of incubation significantly diminished to 16.77±2.59% and 20.90±1.06%, respectively ($p<0.001$). The numbers of SSEA-4^+ and GFRα-1^+ cells attached onto gelatin was significantly lower compared with laminin, in all incubation times (Table 3, $p<0.001$). The attached SSEA-4^+ and GFRα-1^+ cells on gelatin coated dish were also significantly decreased from 9.05±1.07% to 5.34±0.22% and 10.97±0.84% to 2.00±0.17% when examined at 15 and 30 min, respectively ($p<0.001$). The gelatin bounded cells at 60 min of incubation were the highly contaminated with attached fibroblasts.

5.4.3 Double enrichment of spermatogonial stem-like cells (SSCs) with Percoll™ and laminin plating

After discontinuous Percoll™ gradient density was performed, majority of testicular cells was presented at the two Percoll™ densities (at 30% and interface between 30% and 45% of solutions (30%/45%)). The spermatozoa, red blood cells and other cell debris were observed at others of Percoll™ interface. Thin layer of testicular cells at 30% Percoll™ yielded higher cell numbers than that obtained from the 30%/45% interface (Fig 12c and d). However, the viability rate of testicular cells at 30%/45% (91.33±0.14%) was significantly higher than 30% layer (78.40±0.23%) and non-treatment (66.56±1.43%) ($p<0.001$). Double enrichment using (Percoll™+Laminin) significantly improved SSEA-4^+ and GFRα-1^+ cells when compared with only laminin treatment ($p<0.001$, Table 4). This double enrichment also improved cell uniformity. However, some of sperm heads were also presented on laminin coated dishes (Fig. 12b). We confirmed that the attached cells derived from Percoll™+Laminin expressed the SSC- related genes (*POU5F1*, *RET* and *ZBTB16* mRNA) but differentiated gene (*KIT* mRNA) was absent (Fig 13).

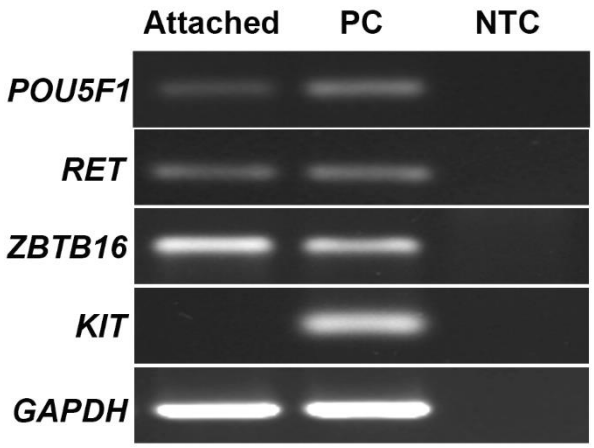

Figure 13 The mRNA expression of *POU5F1, RET, ZBTB16* and *KIT*.

The RT-PCR products of Laminin attached testicular cells from 30%/40% interface (Attached), Positive control of testis (PC) and No template control (NTC).

5.5 Discussion

This study revealed that only small numbers of testicular cells demonstrated morphologically and phenotypically SSC characteristics within pubertal testes of domestic cats. The limited numbers of SSC-like cells have been proposed to attenuate the success of SSC-like cells isolation and culture. The enrichment step has therefore become a critical part for establishment of SSC *in vitro*, especially for which species that well-characterized SSCs have yet to be reported. In domestic cat, the criterion for characterization of SSC have been limited, and functional tests of the SSCs by means of colonization with proliferative activity following transplantation remained unsuccessful (Shinohara et al., 2000; Kim et al., 2006). The indirect assay for cat SSC characterization such as expression of SSC markers at the levels of mRNA and proteins was used in this study. Until recently, the definitive markers for SSC characterization have not been entirely addressed. However, many proteins as putative SSC markers have universally used for studying and enrichment of the SSCs such as GDNF family receptor α-1 (GFRα-1), $\alpha_6\beta_1$-integrins, epithelial cell adhesion molecule (EpCAM), promyelocytic leukemia zinc finger (PLZF or ZBTB16), thymus cell antigen-1 (Thy-1 or CD90), stage-specific embryonic antigen-4 (SSEA-4) (Shinohara et al., 1999; Kanatsu-Shinohara et al., 2003a; Kubota et al., 2003; Hermann et al., 2007; Grisanti et al., 2009;

Maki et al., 2009; Morimoto et al., 2009; Reding et al., 2010; Kokkinaki et al., 2011; Li et al., 2012). Although there is no single marker that can ultimately be used to determine the SSC population, our study (experiment 1) confirmed that the SSEA-4 and GFRα-1 were co-localized specifically at the spermatogonia located onto the basal lamina of seminiferous tubules. Moreover, both SSEA-4 and GFRα-1 markers were co-expressed with DDX-4, defining the specific marker for germ cell lineage (Raz, 2000; Toyooka et al., 2000). The glial cell line derived neurotrophic growth factor (GDNF) binds and signals via glycosyl-phosphatidyl inositol GFRα-1 surface receptors and its ret tyrosine kinase co-receptors in the cell plasma membrane (Meng et al., 2000; Creemers et al., 2002b; Yomogida et al., 2003; Hofmann, 2008). This GDNF has been demonstrated to play a central role for *in vivo* and *in vitro* activities of spermatogonial stem cells (SSCs) (Sorrentino et al., 1991; Sariola and Saarma, 2003; Kubota et al., 2004a; Kubota et al., 2004d). Our study also demonstrated that laminin, an extracellular matrix, localized surrounding the spermatogonia and at the basal lamina of the seminiferous tubules. The attachment of SSCs to laminin involves with integrin proteins, a laminin receptor (Hynes, 1992), α_6- and β_1-integrin was only the specific surface marker of SSCs in mouse (Shinohara et al., 1999). In this study, we found that purified SSC like-cells rapidly adhered to laminin coated surface within 15 min of incubation (Table 3). This short incubation maximized the SSC-like cells with minimal contaminated cells and therefore is recommended for SSC isolation as similar to previous report in mouse (Shinohara et al., 2000). Prolonged incubation of cell suspension from 15 to 60 min increased the numbers of several types including fibroblasts, spermatids and sperm (Shinohara et al., 2000). By contrast to the properties of laminin, the proportion of SSEA4$^+$ and GFRα-1$^+$ testicular cells bound to gelatin decreased with advanced incubation time (Table 3). This gelatin substrate would therefore be suitable for negative selection rather than purifying the SSCs (Kent Hamra et al., 2004). However, it is worth noting that the efficiency of specific substrate for SSC enrichment in terms of time and adhesive force between the cells and extracellular matrixes may solely rely on the number of its receptors on the surface of SSCs and the concentration of substrate used (Luo et al., 2006; de Barros et al., 2012). This also appeared to be cell

type and species specific. For example, buffalo SSCs poorly selected using this technique (Ahmad et al., 2013). Although the differential plating with laminin could eliminate other contaminated testicular cells, the contaminated cells remained high (Table 3 and 4). We therefore further tested whether or not the enrichment with Percoll™ gradient density prior to laminin treatment would really enhance SSC purity. We found in the current study that the Percoll™ gradient could select the cells based on their cell size and shape as previously reported in bull and boar (Luo et al., 2006; Herrid et al., 2009; Izadyar et al., 2011). The double enrichment technique used significantly improved the purity of the SSC-like cells to 91.33±0.14% similar to other reports (Izadyar et al., 2002; Herrid et al., 2009). Although we did not further culture of selected cells for longer period, the obtained cells had morphologically and phenotypically similar to SSCs as previously reported (Tiptanavattana et al., 2013). Furthermore, the attached SSC-like cells obtained from the double enrichment (Percoll™+Laminin) expressed *POU5F1*, *RET* and *ZBTB16* mRNA (SSC markers) but absence the differentiation marker, *KIT* (Fig. 12b and 13).

In conclusion, we demonstrated that the SSC-like cells of domestic cat expressed SSEA-4 and GFRα-1, as SSC markers. The SSC-like cells preferentially attached onto laminin but the purity of cells was in a manner of time dependence. This technique combined with the discontinuous Percoll™ gradient density significantly improved the viability and also the purification rate of SSC-like cells in domestic cat. The double enrichment can be applied as a prerequisite tool for *in vitro* culture of cat SSC-like cell in particular to enrich the SSC-like cell population.

CHAPTER 6

GENERAL DISSCUSSION AND CONCLUSION

Stem cells are self-renewal cells demonstrating both asymmetrical and symmetrical cytokinesis (Weissman et al., 2001). These cells have capability to reserve the undifferentiation property while they remain the potential upon cell activation to become specialized cells of three germ lineages. These properties lead the stem cells to be a new promising tool for incurable diseases and conditions via regenerative medicine. The spermatogonial stem cell (SSC) is the only adult stem cell that can transfer the male genetic profiles to next generation. The SSC controls the spermatogenesis process by production of the gametes throughout adulthood. Surprisingly, the SSCs have also been documented to have pluripotency *in vitro* (Kanatsu-Shinohara et al., 2004a; Kossack et al., 2009). These cells fully differentiated into cellular lineages of the three germ layers *i.e*. endoderm (hepatic cells), mesoderm (smooth muscle cells and endothelial cells), ectoderm (neural cells) (Guan et al., 2006; Durcova-Hills et al., 2008; Geijsen and Hochedlinger, 2009; Golestaneh et al., 2009; Guan et al., 2009; Huang et al., 2009; Ko et al., 2009; Kossack et al., 2009; Guan et al., 2012; Im et al., 2012). However, successful research of *in vitro* culture of SSCs have been restricted in a few species studied such as mouse and rat (Shinohara et al., 1999; Kanatsu-Shinohara et al., 2003a; Kubota et al., 2003). This chapter discusses the results presented in this dissertation in particular to the obstacles that potentially influence the establishment and development of *in vitro* culture system of SSC in domestic cat. Moreover, this chapter also provides major findings and suggestions for prospects of further research.

6.1 Development of primordial germ cells to spermatogonial stem cells: Fundamental aspects of SSC isolation

Generally, the gonocytes transform to spermatogonial stem cells (SSCs) at concise and specific time after birth in rodents (ranged 3-8 days of birth) (de Rooij,

1998). However, the timing of transition phase of gonocyte to spermatogonial stem cell (G/SSC) is different in other species. For example, this phase takes place with a longer period from 3 to 12 months of childhood testes (Su et al., 2014). Thus, the quiescent period of gonocytes is different and referred to the species-specific on the germ cell development. The Chapter 4 has convincingly demonstrated that the cat gonocytes reconcile from the quiescent period at the 4 month of age, and the G/SSC transition completely occurs at the 6 months of age in domestic cat. This findings also refer to asynchronized development of male germ cells along the seminiferous tubules of the testis.

The diversity of male germ cell stages and other testicular cells such as Sertoli cells, Leydig cells, peritubular myoid cells within the seminiferous tubules is age and testicular stage dependent. This diverse cell types hamper the efficient isolation and of SSC *in vitro*. Previous studies described that the undefined growth factors secreted from contaminated cells disturbed and negatively affect to the establishment of SSC culture (Creemers et al., 2002a). This was likely the cause why the SSC-colony forming of pubertal cats (chapter 3) was poor and long term culture of the SSC *in vitro* was unsuccessful. According to chapter 4, the efficiency of SSC forming was significantly increased from 23.33±2.23% to 74.33±2.64% when isolated the SSC at G/SSC transition (4-6 month of age). This therefore confirms the importance of G/SSC transition of testicular development at SSC isolation especially when experiment set up would need to be performed for such species that have limited information on establishment of the SSCs.

6.2 Phenotypic and molecular signature of cat SSC: Remaining trouble

The complexity of SSC populations has been demonstrated in terms of their morphology and expression patterns at the levels of mRNA and proteins. The morphology of the SSC has been demonstrated to be variably described among the species studied (Chiarini-Garcia and Russell, 2002; Chiarini-Garcia et al., 2003; Hermann et al., 2010). The mouse and rat SSCs are round to oval shape with fine condensation of nucleus (heterogeneous of euchromatin) (Chiarini-Garcia and Russell, 2002).

Although rodent SSCs topographically are described as A_{paired} to $A_{aligned}$ spermatogonia, the morphology of *in vitro* SSCs has been found to be different (a grape-like colony). For non-human primate (NPH) SSC, the SSCs are histologically defined by the staining density of nuclei i.e. A_{dark} and A_{pale} (Hermann et al., 2010). In chapter 3, 4 and 5, the histological and ultrastructure studies revealed the different morphology when compared with rodents and NHP. The cat SSCs presented only 2 types of SSC morphology including individual SSC (as A_{single}) and paired SSCs (as A_{paired}). The ultrastructure of cat SSCs reflected the special phenotype as the high intensity of cytoplasm staining which was coincided with the presence of uncommonly high numbers of mitochondria (Chapter 4).

To define the SSC properties, the protein and gene expressions are generally used to refer to the "true SSC" in most of species except that of mouse and rat. During the past two decades, several studies have used the definitive SSC markers to elucidate the SSC properties. Likewise, this thesis (Chapter 3-5), the combinations of GFRα-1, ZBTB16, RET, POU5F1 or SSEA-4 as putative SSC markers were used in domestic cats. However, previous studies evidentially demonstrated heterogeneous populations of SSC expressing GFRα-1 and SSEA-4 (Grisanti et al., 2009). It is therefore suggesting that these techniques for SSC identification may be inappropriate to fully determine the SSC properties. Theoretically, the transplantation of *in vitro* cultured SSCs is the most reliable and precise assay to guarantee the true SSC activity. However, transplantation technique has strictly limitation, such as the distance of the phylogenetic evolution between the donor and recipient of SSCs. It is therefore essential to seek a novel SSC markers that is associated with the potential of SSC to be colonized and function following transplantation. Furthermore, development of further assays for SSC genotypes and phenotypes would also be important.

6.3 *In vitro* culture systems and growth factors: Species-specific requirement

The *in vitro* culture of mouse SSCs has been successfully changed from the cultivation with undefined SSC medium and STO (SIM mouse embryo derived thioguanine and ouabain resistant) feeder cells to the cultivation with semi-defined

SSC medium and MEF feeder cells (Nagano et al., 1998; Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004a). Until recently, the modest culture system developed in mouse SSC by Kanatsu-Shinohara et al. (2003a) has been generally used in other species but with some modifications. However, hamster SSCs differently preferred the TX-WES medium instead of SSC based medium (Kanatsu-Shinohara et al., 2008a). The mouse SSC is recognized as a GDNF-dependent cell type (He et al., 2008). The high concentration of GDNF is positively related and necessary for the *in vitro* proliferation. However, our results presented that cat SSC medium supplemented with 50 ng/µl of GDNF may be insufficient for maintaining long-term SSC activity (Chapter 3). Furthermore it is still required to study the interaction of GDNF with other growth factors. Evidentially, the FGF2 is a growth factor that acts synergistically with GDNF in hamster SSC culture system, irrespective the presence of EGF supplementation (Kanatsu-Shinohara et al., 2008a). In chapter 3, we revealed that cat Sertoli cells as feeder cells are more efficient to support proliferation of cat SSCs *in vitro* when compared with MEFs (Nagano et al., 2003; Han et al., 2014). However, defining factors secreted by Sertoli cells would need to be characterized and confirmed their functions.

6.4 Purification of SSC from pubertal testis: The strategy

Our studies pointed out the advantage of using testes from the SSC donor during pre-pubertal age as it increased the efficiency of SSC colony forming. In addition, this period of SSC donor is suitable for establishment of SSC culture system because testes of the pre-pubertal age can minimize any confounding factors from other testicular cells. However, the enrichment assay would be available for advanced stages of testicular development (pubertal testes) in order to eliminate unwanted testicular cells and to obtain the high purity of SSC population. The enrichment techniques previously described include induced cryptorchidism, differential plating, discontinuous gradient density and FACs/MACs assay (Shinohara et al., 1999; Izadyar et al., 2002; Kent Hamra et al., 2004; Kubota et al., 2004d; Herrid et al., 2009). The differential plating and discontinuous gradient density were reported to have high efficiency of purification with high viability rate (van Pelt et al., 1996). Laminin coated

dish provided the high purification of pubertal cat SSC (Chapter 5) and as well as in other species (Shinohara et al., 2000; Orwig et al., 2002e; Herrid et al., 2009). The cat testicular histology expressed the laminin at the basement membrane of seminiferous tubules and SSC surrounded ECM (Chapter 5). However, Matrigel™, a special ECM substance consisted of laminin, collagen, entactin and heparin sulphate proteoglycan, could not purify the SSCs in bull (Giassetti et al., 2012). This is likely because of the complexity of laminin molecules and also time requirement. Our results demonstrated that the attached cells by 15 min of incubation were not only the SSCs but also contaminated with the Sertoli cells and spermatids. Therefore, the double enrichment with differential plating and discontinuous gradient density would provide the higher efficiency of SSC purification with higher viability rate (Table 4, Chapter 5). Although this double enrichment significantly improved SSC purification, the functions of selected cells would be further elucidated in terms of their potentials to proliferate and colonize after cell transplantation. Furthermore development and further characterization of selectable markers for SSC isolation will play an important aspects in the future.

6.5 Concluding remarks

This dissertation defines and demonstrates the effects of donor age and stages of testicular development as one of the important factors for establishment of *in vitro* SSC culture. The appropriate time at enrichment of SSC populations in cat is determined at the G/SSC transition. In chapter 3, the study demonstrate that we successfully isolated the pubertal cat SSCs and could maintain SSC activities *in vitro*. In chapter 4 provides the information on the morphology and marker expressions of SSC during the G/SSC transition (4-6 months of age) and other testicular stages in domestic cat. This study also reveals that the SSCs preferred with the specific extracellular matrixes as laminin (Chapter 5). Furthermore, the laminin enrichment combined with Percoll™ isotonic solutions provided high rate of viability and promoted the purification of the SSC population.

6.6 Suggestions for further investigation

The establishment of *in vitro* SSC is essential for the basic to clinical proposes as restoration of spermatogenesis in testicular cell-depleting patients, male germ cell banking and biomedical model for inherited and infectious diseases.

As the aforementioned, the G/SSC transition period in cat is advantage to obtain the high efficiency of SSC-forming colony derivation. And, the pre-treatment with enrichment assays of puberty derived testicular cells is alternative method to enrich the SSC population. For the future study, the establishment of the long-term culture for with enriched SSC should be investigated and addressed because the *in vitro* culture for long term will provide the mechanistic pathways insight the stimulation and inhibition effects. The defined culture system and important growth factors are required and will be elucidate in further study. *In vitro* SSC can also be used to study the drugs or targeting models to test the contraception and fertility devices. Moreover, further study should be involved with the *in vitro* spermatogenesis in cat combined with other techniques of assistant reproductive technologies (ARTs) in order to study the possibility of offspring production. However, the cat SSC transplantation should be studied and developed to define the "true" function of SSC. The impact of new technology related the stem cells to drive from the pluripotent stem cell to specialized cells have been more impact for the researchers. The study related SSC is the alternative means for pluripotent stem cell production with less ethical concern. Therefore, the further study about cat SSC will also be used as the differentiation model for diseases model in cat and human.

REFERENCES

Aeckerle N, Eildermann K, Drummer C, Ehmcke J, Schweyer S, Lerchl A, Bergmann M, Kliesch S, Gromoll J, Schlatt S and Behr R 2012. The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells? Mol Hum Reprod. 18(10): 477-488.

Ahmad S, Xiao Y, Han L, Hua H, Riaz H, Liang A and Yang LG 2013. Isolation, identification and enrichment of type a spermatogonia from the testis of chinese cross-bred buffaloes (swamp x river). Reprod Domest Anim. 48(3): 373-381.

Airaksinen MS and Saarma M 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 3(5): 383-394.

Anakwe OO and Gerton GL 1990. Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. Biol Reprod. 42(2): 317-328.

Anway MD, Folmer J, Wright WW and Zirkin BR 2003. Isolation of sertoli cells from adult rat testes: an approach to ex vivo studies of Sertoli cell function. Biol Reprod. 68(3): 996-1002.

Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ and de Rooij DG 2008. Propagation of bovine spermatogonial stem cells in vitro. Reproduction. 136(5): 543-557.

Aponte PM, Soda T, van de Kant HJ and de Rooij DG 2006. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. Theriogenology. 65(9): 1828-1847.

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM and Dym M 1977. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol. 74(1): 68-85.

Braydich-Stolle L, Kostereva N, Dym M and Hofmann MC 2007. Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. Dev Biol. 304(1): 34-45.

Brinster RL and Avarbock MR 1994. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A. 91(24): 11303-11307.

Burgos MH and Fawcett DW 1955. Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (Felis domestica). J Biophys Biochem Cytol. 1(4): 287-300.

Cheng CY and Mruk DD 2002. Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev. 82(4): 825-874.

Cheng CY and Mruk DD 2011. Regulation of spermiogenesis, spermiation and blood-testis barrier dynamics: novel insights from studies on Eps8 and Arp3. Biochem J. 435(3): 553-562.

Chiarini-Garcia H, Hornick JR, Griswold MD and Russell LD 2001. Distribution of type A spermatogonia in the mouse is not random. Biol Reprod. 65(4): 1179-1185.

Chiarini-Garcia H, Raymer A and Russell L 2003. Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction. 126(5): 669-680.

Chiarini-Garcia H and Russell L 2002. Characterization of mouse spermatogonia by transmission electron microscopy. Reproduction. 123(4): 567-577.

Clermont Y and Leblond CP 1959. Differentiation and renewal of spermatogonia in the monkey, Macacus rhesus. Am J Anat. 104: 237-273.

Creemers LB, den Ouden K, van Pelt AM and de Rooij DG 2002a. Maintenance of adult mouse type A spermatogonia in vitro: influence of serum and growth factors and comparison with prepubertal spermatogonial cell culture. Reproduction. 124(6): 791-799.

Creemers LB, Meng X, den Ouden K, van Pelt AM, Izadyar F, Santoro M, Sariola H and de Rooij DG 2002b. Transplantation of germ cells from glial cell line-derived neurotrophic factor-overexpressing mice to host testes depleted of endogenous spermatogenesis by fractionated irradiation. Biol Reprod. 66(6): 1579-1584.

Culty M 2009. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today. 87(1): 1-26.

Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL and Porteus MH 2008. Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. Stem Cells. 26(11): 2928-2937.

de Barros FRO, Worst RA, Saurin GCP, Mendes CM, Assumpção M and Visintin JA 2012. α-6 Integrin Expression in Bovine Spermatogonial Cells Purified by Discontinuous Percoll Density Gradient. Reprod Domest Ani. 47(6): 887-890.

De Chiara L, Fagoonee S, Ranghino A, Bruno S, Camussi G, Tolosano E, Silengo L and Altruda F 2014. Renal cells from spermatogonial germline stem cells protect against kidney injury. J Am Soc Nephrol. 25(2): 316-328.

de Rooij DG 1998. Stem cells in the testis. Int J Exp Pathol. 79(2): 67-80.

de Rooij DG and Grootegoed JA 1998. Spermatogonial stem cells. Curr Opin Cell Biol. 10(6): 694-701.

de Rooij DG, Repping S and van Pelt AM 2008. Role for adhesion molecules in the spermatogonial stem cell niche. Cell Stem Cell. 3(5): 467-468.

De Rooij DG and Russell LD 2000. All You Wanted to Know About Spermatogonia but Were Afraid to Ask. J Androl. 21(6): 776-798.

Drumond AL, Meistrich ML and Chiarini-Garcia H 2011. Spermatogonial morphology and kinetics during testis development in mice: a high-resolution light microscopy approach. Reproduction. 142(1): 145-155.

Durcova-Hills G, Tang F, Doody G, Tooze R and Surani MA 2008. Reprogramming primordial germ cells into pluripotent stem cells. PLoS One. 3(10): e3531.

Escalier D, Gallo JM, Albert M, Meduri G, Bermudez D, David G and Schrevel J 1991. Human acrosome biogenesis: immunodetection of proacrosin in primary spermatocytes and of its partitioning pattern during meiosis. Development. 113(3): 779-788.

Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M and Vicini E 2004. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. Faseb j. 18(2): 376-378.

Filliers M, Goossens K, Van Soom A, Merlo B, Pope CE, de Rooster H, Smits K, Vandaele L and Peelman LJ 2012. Gene expression profiling of pluripotency and differentiation-related markers in cat oocytes and preimplantation embryos. Reprod Fertil Dev. 24(5): 691-703.

Franca LR and Godinho CL 2003. Testis morphometry, seminiferous epithelium cycle length, and daily sperm production in domestic cats (Felis catus). Biol Reprod. 68(5): 1554-1561.

Gangemi RMR, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, Ravetti GL, Zona GL, Daga A and Corte G 2009. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells. 27(1): 40-48.

Garcia TX, DeFalco T, Capel B and Hofmann MC 2013. Constitutive activation of NOTCH1 signaling in Sertoli cells causes gonocyte exit from quiescence. Dev Biol. 377(1): 188-201.

Gaskell TL, Esnal A, Robinson LL, Anderson RA and Saunders PT 2004. Immunohistochemical profiling of germ cells within the human fetal testis: identification of three subpopulations. Biol Reprod. 71(6): 2012-2021.

Geijsen N and Hochedlinger K 2009. gPS navigates germ cells to pluripotency. Cell Stem Cell. 5(1): 3-4.

Giampietri C, Petrungaro S, Coluccia P, D'Alessio A, Starace D, Riccioli A, Padula F, Palombi F, Ziparo E, Filippini A and De Cesaris P 2005. Germ cell apoptosis control during spermatogenesis. Contraception. 72(4): 298-302.

Giassetti MI, Barros FRO, Worst RA, Saurin GCP, Bruno AH, Moreira PV, Hamilton TR, Nichi M, D'Ávila Assumpção MEO and Visintin JA 2012. 292 Comparison of differential plating methods to obtain enriched populations of bovine spermatogonial stem cells. Reprod Fertil Dev. 25(1): 293-294.

Gillis AJ, Stoop H, Biermann K, van Gurp RJ, Swartzman E, Cribbes S, Ferlinz A, Shannon M, Oosterhuis JW and Looijenga LH 2011. Expression and interdependencies of pluripotency factors LIN28, OCT3/4, NANOG and SOX2 in human testicular germ cells and tumours of the testis. Int J Androl. 34(4 Pt 2): e160-174.

Goertz MJ, Wu Z, Gallardo TD, Hamra FK and Castrillon DH 2011. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest. 121(9): 3456-3466.

Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI and Dym M 2009. Pluripotent stem cells derived from adult human testes. Stem Cells Dev. 18(8): 1115-1126.

Gonzalez-Campora R, Delgado MD, Amate AH, Gallardo SP, Leon MS and Beltran AL 2011. Old and new immunohistochemical markers for the diagnosis of gastrointestinal stromal tumors. Anal Quant Cytol Histol. 33(1): 1-11.

Grisanti L, Falciatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Berno V, Boitani C, Stefanini M and Vicini E 2009. Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells. 27(12): 3043-3052.

Griswold MD, Morales C and Sylvester SR 1988. Molecular biology of the Sertoli cell. Oxf Rev Reprod Biol. 10: 124-161.

Guan K, Cheng IF and Baazm M 2012. Human spermatagonial stem cells: a novel therapeutic hope for cardiac regeneration and repair? Future Cardiol. 8(1): 39-51.

Guan K, Nayernia K, Maier L, Wagner S, Dressel R, Lee J, Nolte J, Wolf F, Li M, Engel W and Hasenfuss G 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature. 440: 1199 - 1203.

Guan K, Wagner S, Unsold B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W and Hasenfuss G 2007. Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res. 100(11): 1615-1625.

Guan K, Wolf F, Becker A, Engel W, Nayernia K and Hasenfuss G 2009. Isolation and cultivation of stem cells from adult mouse testes. Nat Protoc. 4(2): 143-154.

Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB and De Rooij DG 2003. Intercellular bridges and apoptosis in clones of male germ cells. Int J Androl. 26(6): 348-353.

Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE and Garbers DL 2005. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A. 102(48): 17430-17435.

Han N, Park Y, Yun J, Park H, Park M, Kim M, Choi J, Lee E, Gong S, Lim J and Lee S 2014. Determination of Feeder Cell-Based Cellular Niches Supporting the Colonization and Maintenance of Spermatogonial Stem Cells from Prepubertal Domestic Cat Testes. Reprod Domest Anim. 49(5): 705-710.

Hayashi K, de Sousa Lopes SM and Surani MA 2007. Germ cell specification in mice. Science. 316(5823): 394-396.

He Z, Jiang J, Kokkinaki M, Golestaneh N, Hofmann MC and Dym M 2008. Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells. 26(1): 266-278.

Heer R, Hepburn AC, Williamson SC, Kennedy A, El-Sherif A, Soomro NA, Brown CD and Robson CN 2013. Renal differentiation from adult spermatogonial stem cells. Ren Fail. 35(10): 1387-1391.

Helal MA, Mehmet H, Thomas NS, Cox PM, Ralph DJ, Bajoria R and Chatterjee R 2002. Ontogeny of human fetal testicular apoptosis during first, second, and third trimesters of pregnancy. J Clin Endocrinol Metab. 87(3): 1189-1193.

Hermann B and Orwig K 2011. Translating Spermatogonial Stem Cell Transplantation to the Clinic. In: Male Germline Stem Cells: Developmental and Regenerative Potential. Kyle E. Orwig and Brian P. Hermann (eds). Humana Press. 227-253.

Hermann BP, Sukhwani M, Hansel MC and Orwig KE 2010. Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction. 139(3): 479-493.

Hermann BP, Sukhwani M, Lin C-C, Sheng Y, Tomko J, Rodriguez M, Shuttleworth JJ, McFarland D, Hobbs RM, Pandolfi PP, Schatten GP and Orwig KE 2007. Characterization, Cryopreservation, and Ablation of Spermatogonial Stem Cells in Adult Rhesus Macaques. Stem Cells. 25(9): 2330-2338.

Hermann BP, Sukhwani M, Simorangkir DR, Chu T, Plant TM and Orwig KE 2009. Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. Hum Reprod. 24(7): 1704-1716.

Herrid M, Davey RJ, Hutton K, Colditz IG and Hill JR 2009. A comparison of methods for preparing enriched populations of bovine spermatogonia. Reprod Fertil Dev. 21(3): 393-399.

Hoei-Hansen CE, Almstrup K, Nielsen JE, Brask Sonne S, Graem N, Skakkebaek NE, Leffers H and Rajpert-De Meyts E 2005. Stem cell pluripotency factor NANOG is expressed in human fetal gonocytes, testicular carcinoma in situ and germ cell tumours. Histopathology. 47(1): 48-56.

Hofmann MC 2008. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol Cell Endocrinol. 288(1-2): 95-103.

Hofmann MC, Braydich-Stolle L and Dym M 2005. Isolation of male germ-line stem cells; influence of GDNF. Dev Biol. 279(1): 114-124.

Huang YH, Chin CC, Ho HN, Chou CK, Shen CN, Kuo HC, Wu TJ, Wu YC, Hung YC, Chang CC and Ling TY 2009. Pluripotency of mouse spermatogonial stem cells maintained by IGF-1- dependent pathway. Faseb j. 23(7): 2076-2087.

Huckins C 1971. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec. 169(3): 533-557.

Huckins C and Clermont Y 1968. Evolution of gonocytes in the rat testis during late embryonic and early post-natal life. Arch Anat Histol Embryol. 51(1): 341-354.

Hynes RO 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 69(1): 11-25.

Im JE, Song SH, Kim JY, Kim KL, Baek SH, Lee DR and Suh W 2012. Vascular differentiation of multipotent spermatogonial stem cells derived from neonatal mouse testis. Exp Mol Med. 44(4): 303-309.

Izadyar F, Spierenberg GT, Creemers LB, den Ouden K and de Rooij DG 2002. Isolation and purification of type A spermatogonia from the bovine testis. Reproduction. 124(1): 85-94.

Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K, Yuen C, Greilach S, Zhao H, Chow M, Chow Y, Rao J, Barritt J, Bar-Chama N and Copperman A 2011. Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod. 26: 1296 - 1306.

Kakiuchi K, Tsuda A, Goto Y, Shimada T, Taniguchi K, Takagishi K and Kubota H 2014. Cell-Surface DEAD-Box Polypeptide 4-Immunoreactive Cells and Gonocytes Are Two Distinct Populations in Postnatal Porcine Testes. Biol Reprod. 90(4): 82, 81-11.

Kanatsu-Shinohara M, Inoue K, Lee J, Miki H, Ogonuki N, Toyokuni S, Ogura A and Shinohara T 2006. Anchorage-independent growth of mouse male germline stem cells in vitro. Biol Reprod. 74(3): 522-529.

Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A and Shinohara T 2004a. Generation of pluripotent stem cells from neonatal mouse testis. Cell. 119(7): 1001-1012.

Kanatsu-Shinohara M, Inoue K, Ogonuki N, Morimoto H, Ogura A and Shinohara T 2011. Serum- and feeder-free culture of mouse germline stem cells. Biol Reprod. 84(1): 97-105.

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A and Shinohara T 2005. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod. 72(4): 985-991.

Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T and Shinohara T 2008a. Long-term culture of male germline stem cells from hamster testes. Biol Reprod. 78(4): 611-617.

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S and Shinohara T 2003a. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod. 69(2): 612-616.

Kanatsu-Shinohara M and Shinohara T 2013. Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol. 29: 163-187.

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fässler R and Shinohara T 2008n. Homing of Mouse Spermatogonial Stem Cells to Germline Niche Depends on β1-Integrin. Cell Stem Cell. 3(5): 533-542.

Kanatsu-Shinohara M, Toyokuni S, Morimoto T, Matsui S, Honjo T and Shinohara T 2003q. Functional assessment of self-renewal activity of male germline stem cells following cytotoxic damage and serial transplantation. Biol Reprod. 68(5): 1801-1807.

Kanatsu-Shinohara M, Toyokuni S and Shinohara T 2004b. CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod. 70(1): 70-75.

Kane E, Morris JG, Rogers QR, Ihrke PJ and Cupps PT 1981. Zinc deficiency in the cat. J Nutr. 111(3): 488-495.

Kee K, Pera RA and Turek PJ 2010. Testicular germline stem cells. Nat Rev Urol. 7(2): 94-100.

Kent Hamra F, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE and Garbers DL 2004. Defining the spermatogonial stem cell. Dev Biol. 269(2): 393-410.

Kim B-G, Cho CM, Lee Y-A, Kim B-J, Kim K-J, Kim Y-H, Min K-S, Kim CG and Ryu B-Y 2010. Enrichment of Testicular Gonocytes and Genetic Modification Using Lentiviral Transduction in Pigs. Biol Reprod. 82(6): 1162-1169.

Kim Y, Selvaraj V, Dobrinski I, Lee H, McEntee MC and Travis AJ 2006. Recipient Preparation and Mixed Germ Cell Isolation for Spermatogonial Stem Cell Transplantation in Domestic Cats. J Androl. 27(2): 248-256.

Kim Y, Selvaraj V, Pukazhenthi B and Travis AJ 2007. Effect of donor age on success of spermatogenesis in feline testis xenografts. Reprod Fertil Dev. 19(7): 869-876.

Kim Y, Turner D, Nelson J, Dobrinski I, McEntee M and Travis AJ 2008. Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction. 136(6): 823-831.

Kirkpatrick JF 1985. Seasonal testosterone levels, testosterone clearance, and testicular weights in male domestic cats. Can J Zool. 63(6): 1285-1287.

Ko K, Tapia N, Wu G, Kim JB, Bravo MJ, Sasse P, Glaser T, Ruau D, Han DW, Greber B, Hausdorfer K, Sebastiano V, Stehling M, Fleischmann BK, Brustle O, Zenke M and Scholer HR 2009. Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell. 5(1): 87-96.

Kokkinaki M, Djourabtchi A and Golestaneh N 2011. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). J Stem Cell Res Ther. 2(2): 2488.

Kossack N, Meneses J, Shefi S, Nguyen H, Chavez S, Nicholas C, Gromoll J, Turek P and Reijo-Pera R 2009. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells. 27: 138 - 149.

Kubota H, Avarbock MR and Brinster RL 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci USA. 100(11): 6487-6492.

Kubota H, Avarbock MR and Brinster RL 2004a. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod. 71(3): 722-731.

Kubota H, Avarbock MR and Brinster RL 2004d. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA. 101(47): 16489-16494.

Kubota H and Brinster RL 2008. Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. Methods Cell Biol. 86: 59-84.

Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P and Allemand I 2004. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development. 131(2): 479-487.

Lawson KA and Pedersen RA 1992. Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. Ciba Found Symp. 165: 3-21; discussion 21-26.

Lee K, Haugen HS, Clegg CH and Braun RE 1995. Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci U S A. 92(26): 12451-12455.

Li S, Shi R, Wang Q, Cai J and Zhang S 2012. Nanostructure and β1-integrin distribution analysis of pig's spermatogonial stem cell by atomic force microscopy. Gene. 495(2): 189-193.

Lim JJ, Seol DW, Choi KH, Shin DH, Kim HJ, Song S-H and Lee DR 2014. Spermatogonial stem cell enrichment using simple grafting of testis and in vitro cultivation. Sci. Rep. 4: 5923 DOI: 5910.1038/srep05923.

Liu S, Tang Z, Xiong T and Tang W 2011. Isolation and characterization of human spermatogonial stem cells. Reprod Biol Endocrinol. 9(1): 141.

Lo KC, Brugh VM, Parker M and Lamb DJ 2005. Isolation and Enrichment of Murine Spermatogonial Stem Cells Using Rhodamine 123 Mitochondrial Dye. Biol Reprod. 72(3): 767-771.

Luo J, Megee S, Rathi R and Dobrinski I 2006. Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. Mol Reprod Dev. 73(12): 1531-1540.

MacDonald ML, Rogers QR, Morris JG and Cupps PT 1984. Effects of linoleate and arachidonate deficiencies on reproduction and spermatogenesis in the cat. J Nutr. 114(4): 719-726.

Maki CB, Pacchiarotti J, Ramos T, Pascual M, Pham J, Kinjo J, Anorve S and Izadyar F 2009. Phenotypic and molecular characterization of spermatogonial stem cells in adult primate testes. Hum Reprod. 24(6): 1480-1491.

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M and Sariola H 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science. 287(5457): 1489-1493.

Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T and Andersson M 2006. Transplantation of normal boar testicular cells resulted in complete focal

spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim. 41(2): 124-128.

Mitchell R, Cowan G, Morris K, Anderson R, Fraser H, Mckenzie K, Wallace W, Kelnar C, Saunders P and Sharpe R 2008. Germ cell differentiation in the marmoset (Callithrix jacchus) during fetal and neonatal life closely parallels that in the human. Hum Reprod. 23(12): 2755-2765.

Morimoto H, Kanatsu-Shinohara M, Takashima S, Chuma S, Nakatsuji N, Takehashi M and Shinohara T 2009. Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One. 4(11): e7909.

Mruk DD and Cheng CY 2004. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev. 25(5): 747-806.

Nagano M, Avarbock MR, Leonida EB, Brinster CJ and Brinster RL 1998. Culture of mouse spermatogonial stem cells. Tissue Cell. 30(4): 389-397.

Nagano M, Ryu BY, Brinster CJ, Avarbock MR and Brinster RL 2003. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod. 68(6): 2207-2214.

Nagano MC 2011. Techniques for culturing spermatogonial stem cells continue to improve. Biol Reprod. 84(1): 5-6.

Nagano R, Tabata S, Nakanishi Y, Ohsako S, Kurohmaru M and Hayashi Y 2000. Reproliferation and relocation of mouse male germ cells (gonocytes) during prespermatogenesis. Anat Rec. 258(2): 210-220.

Nakagawa T, Sharma M, Nabeshima Y, Braun RE and Yoshida S 2010. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science. 328(5974): 62-67.

Oakberg EF 1971. Spermatogonial stem-cell renewal in the mouse. Anat Rec. 169(3): 515-531.

Oatley JM and Brinster RL 2006. Spermatogonial stem cells. Methods Enzymol. 419: 259-282.

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW and Brinster RL 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development. 136(7): 1191-1199.

Oatley JM, Reeves JJ and McLean DJ 2004. Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. Biol Reprod. 71(3): 942-947.

Oatley MJ, Racicot KE and Oatley JM 2011. Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod. 84(4): 639-645.

Ogawa T, Arechaga JM, Avarbock MR and Brinster RL 1997. Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol. 41(1): 111-122.

Ogawa T, Dobrinski I, Avarbock MR and Brinster RL 2000. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med. 6(1): 29-34.

Ogawa T, Dobrinski I and Brinster RL 1999. Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell. 31(5): 461-472.

Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T and Ohbo K 2004. Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol. 67(4): 285-296.

Ooba T, Ishikawa T, Yamaguchi K, Kondo Y, Sakamoto Y and Fujisawa M 2008. Expression and distribution of laminin chains in the testis for patients with azoospermia. J Androl. 29(2): 147-152.

Orth JM, Jester WF, Li L-H and Laslett AL 2000. Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. In: Current Topics in Developmental Biology. P. Schatten Gerald (ed). Academic Press. 103-124.

Orth JM, Qiu J, Jester WF, Jr. and Pilder S 1997. Expression of the c-kit gene is critical for migration of neonatal rat gonocytes in vitro. Biol Reprod. 57(3): 676-683.

Orwig KE, Ryu BY, Avarbock MR and Brinster RL 2002a. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. Proc Natl Acad Sci U S A. 99(18): 11706-11711.

Orwig KE, Shinohara T, Avarbock MR and Brinster RL 2002e. Functional analysis of stem cells in the adult rat testis. Biol Reprod. 66(4): 944-949.

Rafeeqi T and Kaul G 2013. Isolation and enrichment of type A spermatogonia from pre-pubertal buffalo (Bubalus bubalis) testis. Andrologia. 45(3): 195-203.

Rajpert-De Meyts E 2006. Developmental model for the pathogenesis of testicular carcinoma in situ: genetic and environmental aspects. Hum Reprod Update. 12(3): 303-323.

Raz E 2000. The function and regulation of vasa-like genes in germ-cell development. Genome Biol. 1(3): reviews1017.1011-reviews1017.1016.

Reding SC, Stepnoski AL, Cloninger EW and Oatley JM 2010. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. Reproduction. 139(5): 893-903.

Resnick JL, Bixler LS, Cheng L and Donovan PJ 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature. 359(6395): 550-551.

Ryu BY, Kubota H, Avarbock MR and Brinster RL 2005. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A. 102(40): 14302-14307.

Ryu BY, Orwig KE, Avarbock MR and Brinster RL 2003. Stem cell and niche development in the postnatal rat testis. Dev Biol. 263(2): 253-263.

Ryu BY, Orwig KE, Kubota H, Avarbock MR and Brinster RL 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol. 274(1): 158-170.

Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, Hovingh S, de Reijke TM, de la Rosette JJ, van der Veen F, de Rooij DG, Repping S and van Pelt AM 2009. Propagation of human spermatogonial stem cells in vitro. JAMA. 302(19): 2127-2134.

Sandlow JI, Feng HL, Cohen MB and Sandra A 1996. Expression of c-KIT and its ligand, stem cell factor, in normal and subfertile human testicular tissue. J Androl. 17(4): 403-408.

Sano J, Nagafuchi S, Yamazaki J, Oguma K, Kano R and Hasegawa A 2005. Effect of antineoplastic drugs on the expression of Bcl-2 and Bcl-xL genes in the feline T-cell leukemia cell line. Res Vet Sci. 79(3): 197-201.

Sariola H and Immonen T 2008. GDNF Maintains Mouse Spermatogonial Stem Cells In Vivo and In Vitro. In: Germline Stem Cells. StevenX Hou and ShreeRam Singh (eds). Humana Press. 127-135.

Sariola H and Saarma M 2003. Novel functions and signalling pathways for GDNF. J Cell Sci. 116(Pt 19): 3855-3862.

Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y and Ogawa T 2011. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 471(7339): 504-507.

Sato T, Katagiri K, Kubota Y and Ogawa T 2013. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc. 8(11): 2098-2104.

Schrans-Stassen BH, van de Kant HJ, de Rooij DG and van Pelt AM 1999. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology. 140(12): 5894-5900.

Schulze C 1988. Response of the human testis to long-term estrogen treatment: morphology of Sertoli cells, Leydig cells and spermatogonial stem cells. Cell Tissue Res. 251(1): 31-43.

Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP and Rafii S 2007. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature. 449(7160): 346-350.

Shetty G and Meistrich ML 2007. The Missing Niche for Spermatogonial Stem Cells: Do Blood Vessels Point the Way? Cell Stem Cell. 1(4): 361-363.

Shinohara T, Avarbock MR and Brinster RL 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 96(10): 5504-5509.

Shinohara T, Avarbock MR and Brinster RL 2000. Functional Analysis of Spermatogonial Stem Cells in Steel and Cryptorchid Infertile Mouse Models. Dev Biol. 220(2): 401-411.

Siemieniuch MJ and Woclawek-Potocka I 2007. Morphological features of the seminiferous epithelium in cat (Felis catus, L. 1758) testes. J Reprod Dev. 53(5): 1125-1130.

Silva RC, Costa GM, Lacerda SM, Batlouni SR, Soares JM, Avelar GF, Bottger KB, Silva SF, Jr., Nogueira MS, Andrade LM and Franca LR 2012. Germ cell transplantation in felids: a potential approach to preserving endangered species. J Androl. 33(2): 264-276.

Simon L, Ekman GC, Garcia T, Carnes K, Zhang Z, Murphy T, Murphy KM, Hess RA, Cooke PS and Hofmann MC 2010. ETV5 regulates sertoli cell chemokines involved in mouse stem/progenitor spermatogonia maintenance. Stem Cells. 28(10): 1882-1892.

Sorrentino V, Giorgi M, Geremia R, Besmer P and Rossi P 1991. Expression of the c-kit proto-oncogene in the murine male germ cells. Oncogene. 6(1): 149-151.

Stevens LC 1967. The biology of teratomas. Adv Morphog. 6: 1-31.

Su S, Szarek M, Vooght A, Hutson J and Li R 2014. Gonocyte transformation to spermatogonial stem cells occurs earlier in patients with undervirilisation syndromes. J Pediatr Surg. 49(2): 323-327.

Suzuki H, Sada A, Yoshida S and Saga Y 2009. The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev Biol. 336(2): 222-231.

Swanson WF, Brown JL and Wildt DE 1996. Influence of seasonality on reproductive traits of the male Pallas' cat (Felis manul) and implications for captive management. J Zoo Wildl Med. 234-240.

Tagelenbosch RAJ and de Rooij DG 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res. 290(2): 193-200.

Tegelenbosch RA and de Rooij DG 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res. 290(2): 193-200.

Tiptanavattana N, Thongkittidilok C, Techakumphu M and Tharasanit T 2013. Characterization and in vitro culture of putative spermatogonial stem cells derived from feline testicular tissue. J Reprod Dev. 59(2): 189-195.

Tokuda M, Kadokawa Y, Kurahashi H and Marunouchi T 2007. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod. 76(1): 130-141.

Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M and Noce T 2000. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech Dev. 93(1-2): 139-149.

Tripathi R, Mishra DP and Shaha C 2009. Male germ cell development: turning on the apoptotic pathways. J Reprod Immunol. 83(1-2): 31-35.

Tsutsui T, Onodera F, Oba H, Mizutani T and Hori T 2009. Plasma hormone levels and semen quality in male cats during non-breeding and breeding seasons. Reprod Domest Anim. 44 Suppl 2: 291-293.

van Alphen MM and de Rooij DG 1986. Depletion of the seminiferous epithelium of the rhesus monkey, Macaca mulatta, after X-irradiation. Br J Cancer Suppl. 7: 102-104.

van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG and Stefanini M 1996. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol Reprod. 55(2): 439-444.

van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG and van Dissel-Emiliani FM 2002. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology. 143(5): 1845-1850.

Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA and de Rooij DG 1991. Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil. 93(1): 233-243.

Vester BM, Beloshapka AN, Middelbos IS, Burke SL, Dikeman CL, Simmons LG and Swanson KS 2010. Evaluation of nutrient digestibility and fecal characteristics of exotic felids fed horse- or beef-based diets: use of the domestic cat as a model for exotic felids. Zoo Biol. 29(4): 432-448.

Weissman IL, Anderson DJ and Gage F 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annual review of cell and developmental biology. 17(1): 387-403.

Wongsrikeao P, Saenz D, Rinkoski T, Otoi T and Poeschla E 2011. Antiviral restriction factor transgenesis in the domestic cat. Nat Meth. 8(10): 853-859.

Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR and Brinster RL 2010. The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. Biol Reprod. 82(6): 1103-1111.

Yan HH and Cheng CY 2006. Laminin alpha 3 forms a complex with beta3 and gamma3 chains that serves as the ligand for alpha 6beta1-integrin at the apical ectoplasmic specialization in adult rat testes. J Biol Chem. 281(25): 17286-17303.

Yomogida K, Yagura Y, Tadokoro Y and Nishimune Y 2003. Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. Biol Reprod. 69(4): 1303-1307.

Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T and Nabeshima Y 2006. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development. 133(8): 1495-1505.

Zheng K, Wu X, Kaestner KH and Wang PJ 2009. The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol. 9: 38.

Zogbi C, Tesser RB, Encinas G, Miraglia SM and Stumpp T 2012. Gonocyte development in rats: proliferation, distribution and death revisited. Histochem Cell Biol. 138(2): 305-322.

APPENDIX

APPENDIX

List of publications and conference proceedings

Full Articles

1. **Tiptanavattana, N**., Techakumphu, M., Tharasanit, T. 2015. Simplified isolation and enrichment of Spermatogonial stem-like cells from pubertal domestic cats (*Felis catus*). J Vet Med Sci. (Accepted). [IF= 0.875, Q2(Veterinary)]
2. Klincumhom, N., Tharasanit, T., Thongkittidilok, C., **Tiptanavattana, N.**, Rungarunlert, S., Dinnyés, A., Techakumphu, M. 2014. Selective TGF-β1/ALK inhibitors improves neuronal differentiation of mouse embryonic stem cells. Neurosci Lett. 1-6. doi: 10.1016/j.neulet.2014.06.001. [IF= 2.05, Q2(Neuroscience)]
3. **Tiptanavattana, N.**, Thongkittidilok, C., Techakumphu, M., Tharasanit, T. 2013. Characterization and *in vitro* culture of putative spermatogonial stem cells derived from feline testicular tissue. J Reprod Dev. 59(2): 189-95. [IF=1.76, Q1(Animal science and zoology)]
4. Klincumhom, N., Tharasanit, T., Thongkittidilok, C., **Tiptanavattana, N.**, Dinnyes, A., Techakumphu, M. 2013. Modulating neurogenesis in embryoid body using a selective TGF beta1/ALK inhibitor affects gene expression of embryonic stem cell-derived motor neurons. Thai J Vet Med. 43(1): 49-56. [IF=0.15, Q3(Veterinary)]
5. Tharasanit, T., Buarpung, S., Manee-In, S., Thongkittidilok, C, **Tiptanavattana, N.**, Comizzoli, P., Techakumphu, M. 2012. Birth of Kittens After the Transfer of Frozen–Thawed Embryos Produced by Intracytoplasmic Sperm Injection with Spermatozoa Collected from Cryopreserved Testicular Tissue. Reprod Dom Anim (suppl 6). 47: 305-308. [IF=1.39, Q1(Animal science and zoology)]
6. Tharasanit, T., Savasu, W., Suwimonteerabutr, J., **Tiptanavattana, N.**, Techakumphu, M., Siriaroonrat, B., Sommanustweechai, A., Lohachit, C., Kongkum, W., Kamolnorranath, S. Conservation of genetic potentials of Eastern Sarus Crane (*Grus antigone sharpii*): effects of season and preservation

techniques on semen quality. Reprod Dom Anim (suppl 6). 47: 305-308. [IF=1.39, Q1(Animal science and zoology)]

Conference Proceedings

1. **Tiptanavattana, N.**, Phakdeedindan, P., Techakumphu, M., Tharasanit, T. Generation of sertoli cells derived induced pluripotent stem cells in pre-pubertal cat. The International Society of Stem Cell Research 2015, Stockholm, Sweden, June 24-27, 2015. (*poster presentation*)
2. **Tiptanavattana, N.**, Radtanakatikanon A., Buranapraditkun S., Hyttel P., Holmes H.M., Setthawong P., Techakumphu M., Tharasanit, T.. Chronological transition of gonocytes to spermatogonial stem cells during prepubertal and pubertal periods in domestic cats. The International Embryo Transfer Society, Versailles, France, January 10-13, 2015. *(poster presentation)*
3. Tharasanit, T., **Tiptanavattana, N.**, Phakdeedindan, P., Techakumphu M. Feline embryonic stem-like cells derived from *in vitro*-produced blastocysts retain *in vitro* differentiation potential. The International Embryo Transfer Society, Versailles, France, January 10-13, 2015. *(poster presentation)*
4. **Tiptanavattana, N.**, Radtanakatikanon, A., Buranapraditkun, S., Techakumphu, M., Tharasanit, T. Dynamic Changes of GFR**α**-1 Expressing Testicular cells in Pre- and Post- Pubertal Cat. Proceedings of the 2nd Symposium of the Thai Society for Animal reproduction, Bangkok, Thailand, March 20-21, 2014, Thai J. Vet. Med. Suppl. 1, 2014 44:135-136. *(oral presentation)*
5. Buarpung, S., Tharasanit, T., Manee-In, S., Thongkittidilok, C., **Tiptanavattana, N.**, Techakumphu, M. Intracytoplasmic sperm injection using spermatozoa from preserved testicular tissue: An alternative Method for embryo production in felid species. Proceedings RGJ Seminar Series XCIX “Innovative Reproductive Technology for Wildlife”, Chonburi, Thailand, November 20, 2013. *(oral presentation)*
6. **Tiptanavattana, N.**, Techakumphu, M., Tharasanit, T. Frontier approach for male genetic rescue using spermatogonial stem cell technique. Proceedings

RGJ Seminar Series XCIX “Innovative Reproductive Technology for Wildlife”, Chonburi, Thailand, November 20, 2013. *(oral presentation)*

7. Tharasanit, T., Savasu, W., Suwimonteerabutr, J., **Tiptanavattana, N.**, Techakumphu, M., Siriaroonrat, B., Sommanustweechai, A., Lohachit, C., Kongkum, W., Kamolnorranath, S. Conservation of genetic potentials of Eastern Sarus Crane (*Grus antigone sharpii*): effects of season and preservation techniques on semen quality. Proceedings 17th The International Congress on Animal Reproduction (ICAR), Vancouver, British Columbia, Canada, July 29 - Aug. 2, 2012. (*poster presentation*)
8. Tharasanit, T., Buarpung, S., Manee-In, S., Thongkittidilok, C., **Tiptanavattana, N.**, Comizzoli, P., Techakumphu, M. Kittens born following transfer of frozen-thawed embryos produced by intracytoplasmic sperm injection using sperm recovered from cryopreserved testicular tissue. The 7th International Symposium on Canine and Feline Reproduction (ISCFR). Whistler, British Columbia, Canada 26-29 July, 2012 *(oral presentation)*
9. **Tiptanavattana, N.**, Techakumphu, M., Tharasanit, T. 2012. Establishment of *In vitro* Culture of Spermatogonial Stem Cell-like Colony in Pubertal Domestic Cat (*Felis catus*). Proceedings 37th International Conference on Veterinary Science (ICVS), Bangkok, Thailand, 29th February – 2nd March, 2012. *(oral presentation)*
10. Tharasanit, T., Savasu, W., Suwimonteerabutr, J., **Tiptanavattana, N.**, Techakumphu, M., Siriaroonrat, B., Sommanustweechai, A., Lohachit, C., Kamolnorranath, S. 2011. Cold storage and cryopreservation of eastern Sarus crane (*Grus antigone sharpii*) semen. Proceedings 19th The South east asian zoos association (SEAZA) Annual conference, Pattaya, Thailand, 12th -14th September, 2011. P.41-4. (*oral presentation*)
11. Tharasanit, T., Savasu, W., Suwimonteerabutr, J., **Tiptanavattana, N.**, Techakumphu, M., Siriaroonrat, B., Sommanustweechai, A., Lohachit, C., Kamolnorranath, S. 2010. Preserving genetic potentials of Eastern Sarus Crane (*Grus antigone sharpii*) using semen preservation techniques. Proceedings 36th International Conference on Veterinary Science (ICVS), Bangkok, Thailand, 3rd - 5th November, 2010. (*poster presentation*)

VITA

Narong Tiptanavattana was born on October 20th, 1986 in Bangkok province, Thailand. He graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 1st honor from Faculty of Veterinary Science, Chulalongkorn University, in 2010. In the same year, 2010, he received a scholarship from the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej to perform a PhD program of Theriogenology at Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. His research is focusing on the development of spermatogenesis and spermatogonial stem cell (SSC) in pre-pubertal and post-pubertal cat. His PhD thesis title is "CHARACTERIZATION AND CULTURE OF SPERMATOGONIAL STEM CELLS IN PRE- AND POST-PUBERTAL CATS". Defending his thesis was completed in June 2015 and he was appointed as instructor at Faculty of Veterinary Science, Prince of Songkla University.