การผลิต รูปแบบ และการปรับอิพิเจเนติกส์ของตัวอ่อนโคลนแมว
จากการย้ายฝากนิวเคลียสแบบข้ามสปีชีส์โดยใช้โอโอไซต์ของโคเป็นตัวรับ

นางสาวมานิตา วิทยารัตน์

PRODUCTION, EPIGENETIC PATTERNS AND MODULATION

OF INTER-SPECIES CLONED CAT EMBRYOS USING THE BOVINE OOCYTES

AS RECIPIENT CYTOPLASTS

Miss Manita Wittayarat

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 2012

Thesis Title   PRODUCTION, EPIGENETIC PATTERNS AND MODULATION OF INTER-SPECIES

CLONED CAT EMBRYOS USING THE BOVINE OOCYTES AS RECIPIENT CYTOPLASTS

By   Miss Manita Wittayarat

Field of Study   Theriogenology

Thesis Advisor   Professor Mongkol Techakumphu, D.V.M., Doctorate de 3^e cycle

Thesis Co-advisor   Associate Professor Kaywalee Chatdarong, D.V.M., M.Sc., Ph.D.

Professor Takeshige Otoi, D.V.M., Ph.D.

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 Mongkol Techakumphu, D.V.M., Doctorate de 3^e cycle)

THESIS COMMITTEE

.... Chairman

(Associate Professor Padet Tummaruk, D.V.M., M.Sc., Ph.D.)

....Thesis Advisor

(Professor Mongkol Techakumphu, D.V.M., Doctorate de 3^e cycle)

....Thesis Co-advisor

(Associate Professor Kaywalee Chatdarong, D.V.M., M.Sc., Ph.D.)

....Thesis Co-advisor

(Professor Takeshige Otoi, D.V.M., Ph.D.)

....Examiner

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

....Examiner

(Associate Professor Kumthorn Pruksananonda, M.D.)

....External Examiner

(Ampika Thongphakdee, D.V.M., Ph.D.)

มานิตา วิทยารัตน์: การผลิต รูปแบบ และการปรับอิพิเจเนติกส์ของตัวอ่อนโคลนแมว จากการย้ายฝากนิวเคลียสแบบข้ามสปีชีส์โดยใช้โอโอไซต์ของโคเป็นตัวรับ (PRODUCTION, EPIGENETIC PATTERNS AND MODULATION OF INTER-SPECIES CLONED CAT EMBRYOS USING THE BOVINE OOCYTES AS RECIPIENT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.สพ.ญ.ดร. เกวลี ฉัตรดรงค์, ศ.นส.พ.ดร. ทาเคชิเกะ โอโตอิ, 119 หน้า

การทดลองที่ 1 ศึกษาหาวิธีการที่เหมาะสมของการปรับปรุงเซลล์ต้นกำเนิด โดยวิธีการเหนี่ยวนำเซลล์ให้อยู่ในระยะพักด้วยวิธีการต่างๆ เซลล์ไฟโบรบลาสท์ที่ได้จากผิวหนังของแมวบ้านสามารถถูกเหนี่ยวนำให้อยู่ในระยะพักได้หลายวิธี ทั้งวิธีการลดปริมาณซีรัมในน้ำยาเลี้ยงเซลล์ลงเป็นเวลา 3 วัน การเลี้ยงเซลล์ให้มีความหนาแน่นร้อยละ 100 การใช้โรสโควิทีนที่ความเข้มข้น 15 ไมโครโมลาร์ แต่วิธีการลดปริมาณซีรัมนั้นสามารถทำให้เกิดเซลล์ตายที่ค่อนข้างมากเมื่อเทียบกับวิธีอื่นๆ ดังนั้นวิธีการที่เหมาะสมที่สุดในการปรับปรุงเซลล์แมวต้นกำเนิดในการศึกษานี้คือ การเลี้ยงเซลล์ให้มีความหนาแน่นร้อยละ 100 เป็นเวลา 5 วัน หรือการใช้โรสโควิทีนที่ความเข้มข้น 15 ไมโครโมลาร์ เป็นเวลา 24 ชั่วโมง

การทดลองที่ 2 การศึกษาแรกเพื่อสังเกตการพัฒนาของตัวอ่อนโคลนแมว จากการย้ายฝากนิวเคลียสแบบข้ามสปีชีส์โดยใช้โอโอไซต์ของโคเป็นตัวรับและใช้เซลล์แมวต้นกำเนิดที่ได้รับการปรับปรุงด้วยวิธีการเลี้ยงให้มีความหนาแน่นร้อยละ 100 เป็นเวลา 5 วัน หรือการใช้โรสโควิทีนที่ความเข้มข้น 15 ไมโครโมลาร์ เป็นเวลา 24 ชั่วโมง ประสิทธิภาพเชื่อมติดของเซลล์แมวที่ได้จากการใช้โรสโควิทีนและโอโอไซต์ของโคสูงกว่าในกลุ่มของตัวอ่อนที่ใช้เซลล์แมวจากการเลี้ยงเซลล์ให้มีความหนาแน่นร้อยละ 100 อย่างมีนัยสำคัญ (77.3 และ 57.9%, P<0.05) แต่อย่างไรก็ตาม ในทั้งสองกลุ่มการทดลอง ตัวอ่อนโคลนแมวแบบข้ามสปีชีส์ส่วนมากหยุดการพัฒนาที่ระยะ 2-4 เซลล์ และไม่มีตัวอ่อนใดเลยที่สามารถพัฒนาไปจนถึงระยะโมรูลาและบลาสโตซิส การศึกษาที่สองเพื่อศึกษาการพัฒนาของตัวอ่อนโคลนแมวแบบข้ามสปีชีส์ที่มีการปรับปรุงระบบการเลี้ยงโดยการเติมสารทริโคสแตตินเอเพื่อปรับอิพิเจเนติกส์ที่ความเข้มข้นต่างๆ เป็นเวลา 24 ชั่วโมงภายหลังการเชื่อมติดเซลล์ การเติมสารทริโคสแตตินเอที่ความเข้มข้น 50 นาโนโมลาร์ช่วยเพิ่มร้อยละของการแบ่งตัวและการพัฒนาของตัวอ่อนไปยังระยะบลาสโตซิส (84.3 และ 4..6%, P<0.05) เมื่อเทียบกับกลุ่มการควบคุม (63.8 และ 0%, P<0.05) และกลุ่มที่มีการเติมสารทริโคสแตตินเอที่ความเข้มข้น 100 นาโนโมลาร์อย่างมีนัยสำคัญ (71.4 และ 0%, P<0.05)

การทดลองที่ 3 ศึกษารูปแบบของการเกิดอะเซทิลเลชั่นหรือเมทิลเลชั่นบนโปรตีนฮีสโตนของเซลล์ต้นกำเนิดแมวและตัวอ่อนโคลนแมวแบบข้ามสปีชีส์ระหว่างกลุ่มที่มีการเติมสารทริโคสแตตินเป็นเวลา 24 ชั่วโมงเปรียบเทียบกับกลุ่มควบคุม การเกิดอะเซทิลเลชั่นบนโปรตีนฮีสโตนที่ตำแหน่งไลซีน 9, 18 และ 23 ของเซลล์ต้นกำเนิดในกลุ่มที่มีการเติมสารทริโคสแตตินเอมีระดับสูงกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญ ในขณะที่ระดับของโปรตีนฮีสโตนไม่แตกต่างกันระหว่างกลุ่มทดลอง เช่นเดียวกับตัวอ่อนโคลนแมวข้ามสปีชีส์ ในกลุ่มที่มีการเติมสารทริโคสแตตินเอเป็นเวลา 24 ชั่วโมงภายหลังการเชื่อมติดเซลล์มีระดับการเกิดอะเซทิลเลชั่นบนโปรตีนฮีสโตนที่ตำแหน่งไลซีน 9, 18 และ 23 ที่สูงกว่ากลุ่มควบคุมในตัวอ่อนระยะภายหลังการเชื่อมติดเซลล์ 2 ชั่วโมง ระยะโปรนิวเคลียส ระยะ 2 เซลล์ ระยะ 4 เซลล์ และระยะ 8 เซลล์ อย่างมีนัยสำคัญ ยกเว้นในตัวอ่อนระยะภายหลังการเชื่อมติดเซลล์ 6 ชั่วโมง ที่ระดับของการเกิดอะเซทิลเลชั่นบนโปรตีนฮีสโตนที่ตำแหน่งไลซีน 9 และ 23 ต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ในระยะโปรนิวเคลียส ตัวอ่อนในกลุ่มที่มีการเติมสารทริโคสแตตินเอแสดงการเกิดเมทิลเลชั่นบนโปรตีนฮีสโตนที่ตำแหน่งไลซีน 9 ที่สูงกว่าตัวอ่อนกลุ่มควบคุม ซึ่งรูปแบบการเกิดอะเซทิลเลชั่นและเมทิลเลชั่นดังกล่าวของกลุ่มที่มีการเติมสารทริโคสแตตินเอมีความคล้ายคลึงกับตัวอ่อนโคที่เกิดจากกระบวนการปฏิสนธิภายนอกร่างกาย

การทดลองที่ 4 ศึกษาการพัฒนาภายนอกร่างกายของตัวอ่อนโคลนแมวข้ามสปีชีส์ที่มีการปรับปรุงระบบการเลี้ยงโดยการเติมสารทริโคสแตตินที่ความเข้มข้น 50 นาโนโมลาร์ โดยการย้ายฝากตัวอ่อนที่มีการแบ่งตัวที่ระยะ 2-4เซลล์จำนวนทั้งหมด 224 ตัวอ่อน สู่แมวตัวรับ 5 ตัว อย่างไรก็ตามไม่มีแมวตัวรับใดตั้งท้อง ภายหลังการตรวจด้วยอัลตร้าชาวน์ในวันที่ 30 หลังการย้ายฝาก

สรุปว่าตัวอ่อนโคลนแมวข้ามสปีชีส์สามารถพัฒนาไปถึงระยะบลาสโตซิสได้ ภายหลังการปรับปรุงระบบการเลี้ยงด้วยการเติมสารปรับอิพิเจเนติกส์ทริโคสแตตินเอที่ความเข้มข้น 50 นาโนโมลาร์เป็นเวลา 24 ชั่วโมง ภายหลังการเชื่อมติดเซลล์ ซึ่งสารดังกล่าวมีความสามารถในการปรับรูปแบบอิพิเจเนติกส์ให้มีความใกล้เคียงกับตัวอ่อนที่เกิดจากการปฏิสนธิภายนอกร่างกาย อย่างไรก็ตามตัวอ่อนที่ได้รับการรักษาด้วยสารดังกล่าวไม่สามารถพัฒนาต่อภายหลังการย้ายฝากสู่แม่ตัวรับ

ภาควิชา ...สูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์...   ลายมือชื่อนิสิต…………………………….………………….............

สาขาวิชา ...วิทยาการสืบพันธุ์สัตว์ …………………………   ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…….………………………….......

ปีการศึกษา ...2555…………. …………………………   ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ……………….....…….

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม …………………......…….

5175957531: MAJOR THERIOGENOLOGY

KEYWORDS: CELL CYCLE SYNCHRONIZATION / HISTONE ACETYLATION / HISTONE METHYLATION / INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER / EMBRYO DEVELOPMENT / TRICHOSTATIN A / CAT / BOVINE

MANITA WITTAYARAT: PRODUCTION, EPIGENETIC PATTERNS AND MODULATION OF INTERSPECIES CLONED CAT EMBRYOS USING THE BOVINE OOCYTES AS RECIPIENT CYTOPLASTS. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT DE 3e CYCLE. CO-ADVISOR: ASSOC. PROF. KAYWALEE CHATDARONG, D.V.M., M.Sc., Ph.D., PROF. TAKESHIGE OTOI, D.V.M., Ph.D., 119 pp.

Experiment I was purposed to find the appropriate treatment of donor cell modification that gave the highest percentage of G0/G1 phase cells. Skin fibroblast cells from domestic cat were cultured and treated with either serum starvation for 1-5 days, cell confluency-contact inhibition for 5 days or roscovitine at various concentrations (7.5-30 µM) for 24 h. Results show that cells were successfully synchronized to G0/G1 stage using the serum starvation for 3 d, confluency-contact inhibition and roscovitine treatment at concentration 15 µM. However, the serum starvation method also increased the number of apoptotic cells. Therefore, the confluency-contact inhibition or roscovitine treatment at concentration 15 µM may be valuable for preparing cat donor cells for SCNT.

Experiment II was conducted to evaluate the effect of modification of donor cell by cell cycle synchronization and modification of cultured procedure modification by treatment of histone deacetylase inhibitor TSA on the developmental ability of cat iSCNT embryos using bovine oocytes matured *in vitro*. First study was aimed to observe the development of interspecies embryos reconstructed from enucleated bovine oocytes and modified cat donor cells by cell cycle synchronization (confluency-contact inhibition or 15 µM roscovitine) in comparison with intraspecies cat and bovine NT. Results show that the fusion rate of interspecies couplets was significantly greater in the roscovitine group than in the contact inhibition group. In both of treatment groups, most embryos stopped the development at the 2- or 4-cell stage, and none of the iSCNT cat embryos developed to the morula or blastocyst stage. Second study was conducted to compare the effect of TSA at different concentrations on the *in vitro* development of iSCNT cat embryos. Reconstructed cat–bovine embryos were treated with 0, 25, 50, and 100 nM concentrations of TSA for 24 h following fusion. The results showed that 50 nM TSA treatment contributed significantly higher rates of cleavage and blastocyst formation in iSCNT cat embryos compared with untreated embryos and embryos treated with 100 nM TSA.

Experiment III was aimed to determine the differential acetylation on histone H3 lysine 9 (K9), 18 (K18), 23 (K23) and di-methylation on histone H3 lysine 9 (K9) in the cat donor cell and iSCNT cat embryos at the early stage between with and without 50 nM TSA treatment compared to bovine IVF embryos. Results show that the acetylation levels on H3K9, H3K18 and H3K23 of TSA-treated cat cells were significant higher than those of non-TSA treated cells. The acetylation levels of AcH3K9, AcH3K18 and AcH3K23 in TSA-treated embryos and bovine IVF embryos were higher than that of control embryos at all examined stages (2 h PF, PN, 2-cell, 4-cell and 8-cell). Exceptionally, in 6 h PN stage, the levels of AcH3K9 and AcH3K23 in embryos treated with 50 nM of TSA and bovine IVF embryos were significant lower than that of control embryos. At PN stage, the significantly higher intensity levels of Me2H3K9 were found in embryos treated with TSA and bovine IVF than that of control embryos. This suggest that the treatment of 50 nM TSA for 24 h after fusion in iSCNT cat embryos contribute the beneficial effects on the modification of acetylation levels of lysine residues (K9, K18 and K23) on histone H3 and di-methylation levels on histone H3K9 during the early embryogenesis.

Experiment IV, The total of 224 TSA-treated iSCNT cat embryos at 2- to 4-cell stages was successfully transferred into five recipients. The pregnancy was assessed at day 30 after the embryo transfer by using real-time, B-mode ultrasonography. However, none of the recipients receiving TSA-treated iSCNT cat became pregnant.

In conclusion, cat cells can be reprogrammed in bovine oocytes, which the reconstructed iSCNT embryos could successfully developed to the blastocyst stages when TSA was supplemented. However, the production of offspring has not been achieved.

Department: _____ Obstetrics Gynaecology and Reproduction _____ Student's Signature _____

Field of Study: __ Theriogenology _____ Advisor's Signature _____

Academic Year: __ 2012 _____ Co-advisor's Signature _____

Co-advisor's Signature _____

ACKNOWLEDGEMENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ac	acetylation
ANOVA	one-way analysis of variance
ARTs	assisted reproductive technologies
BSA	bovine serum albumin
CH	corpus haemorrhagicum
CHx	cycloheximide
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
CT	cell confluency-contact inhibition
°C	Celsius
d	day
DAPI	diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ET	embryo transfer
EGF	epidermal growth factor
FBS	fetal bovine serum
FSH	follicular stimulating hormone
GLM	general linear model
h	hour
hCG	human chorionic gonadotropin
HDACs	histone deacetylases
hMG	human menopausal gonadotropin
H3K9	histone H3 lysine 9
H3K18	histone H3 lysine 18
H3K23	histone H3 lysine 23
ICSI	intracytoplasmic sperm injection

iSCNT	interspecies somatic cell nuclear transfer
IVF	*in vitro* fertilization
kV/cm	kilo volt per centimeter
LOS	large offspring syndrome
MAPK	mitogen activated protein kinase
Me2	di-methylation
mg/kg	milligram/kilogram
min	minute
mL	milliliter
mm	millimeter
MPF	maturation promoting factor
mSOF	modified synthetic oviductal fluid
mtDNA	mitochondrial deoxyribonucleic acid
MII	metaphase of mitosis II
nM	nanomolar
NT	nuclear transfer
PBS	phosphate buffer saline
PF	post-fusion
PI	post-insemination
PLSD	Fisher's protected least significant difference
PMSG	pregnant mare serum gonadotropin
PN	Pronuclear
RT	room temperature
s/c	subcutaneous
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
TSA	Trichostatin A
v/v	volume/volume
w/v	weight/volume

μg/mL	microgram/milliliter
μl	microliter
μM	micromolar
μsec	microsecond

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Nine species of wild cats in Thailand are classified as three genera (1) *Felis,* (2) *Neofelis* and (3) *Panthera*. The genus *Felis* is composed of Asian golden cat (*Pardofelis temminckii*), marbled cat (*Pardofelis marmorata*), leopard cat *(Felis bengalensis)*, fishing cat *(Felis viverrina)*, jungle cat *(Felis chaus)* and flat-headed cat *(Prionailurus planiceps)*. The only one wild cat classified in genus *Neofelis* is clouded leopard *(Neofelis nebulosa)*, while the genus *Panthera* is composed of tiger *(Panthera tigris)* and leopard *(Panthera pardus)*. Most of them are specified in the International Union for Conservation of Nature and Natural Resources red lists (IUCN, 2009) of threatened animals as endangered, vulnerable or near threatened. Throughout its range it is thought to be rare, although it has also been suggested that the perception of the wild bovid's rarity may be caused by its secretive nature and its preference for remote forest areas. Because of its depending on forest habitat, the major threat is habitat destruction caused by felling of trees and shifting cultivation. Moreover, they are also thought to be intolerant of human disturbance, abandoning a forest that is even moderately disturbed. Poaching for skins, bones and meat may also be a threat.

One of alternative ways for their rescue from extinction is the use of somatic cell nuclear transfer (SCNT), which is a technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm. Moreover, it is possible to produce their offspring. This technique has been extensively investigated in many species either amphibian or mammalian since 1952. The pioneer report was released by Briggs and King (1952) in the topic of the production of adult frog clones using embryonic nuclei, which lighted up the researchers working with mammalian eggs to be interested until now (Keefer, 2008). The SCNT has progressed from being a novel technology to (1) a widely used for generating identical individuals in case of endangered species conservation, (2) a model for

understanding the cellular and molecular aspects of nuclear reprogramming and (3) a means to produce embryonic stem cells for their potential use in cell-based therapies (Gómez et al., 2006). To date, SCNT is not only a valuable tool for producing animals with the same genetic traits, but also an alternative technique to help for preserving endangered animals such as African wild cat *(Felis silvestris libica)* (Gómez et al., 2003), giant panda *(Ailuropoda melanoleuca)* (Han et al., 2003), banteng *(Bos javanicus)* (Sansinena et al., 2005), leopard cat *(Prionailurus bengalensis)* (Yin et al., 2006), goral *(Naemorhedus caudatus)* (Hashem et al., 2006) and Cantabric brown bear *(Ursus arctos pyrenaicus)* (Caamano et al., 2008). Attempts through SCNT in wild animals provides the opportunity to developing "inter-species/generic SCNT", which involves the transfer of donor cell nuclei from one species/genus to enucleated oocytes of another related species/genus (Yin et al., 2006).

The bovine ooplasm has shown the abilities for supporting the *in vitro* development of embryos to blastocyst stage produced by iSCNT of somatic cells from various unrelated mammalian species, including sheep, pig, monkey, dog and yak. However, no pregnancy was carried to full-term after the embryo transfer to recipients (Dominko et al., 1999; Murakami et al., 2005). According to our knowledge, there are very few reports concerning the ability of bovine oocytes to reprogram the nucleus of felid species. Owing to the similarities between the domestic cat and wild cats, the domestic cat (*Felis catus*) is widely used as a model organism for development of assisted reproductive technologies (ARTs) in all felids (Waurich et al., 2010), such as intracytoplasmic sperm injections (ICSI) (Pope et al., 1998) and SCNT (Yin et al., 2008). The iSCNT cloned cat embryo reconstructed from cat somatic cell and bovine ooplasm has been created by Thongphakdee et al. (2008), however, no any iSCNT cloned cat embryos developed beyond the 8-cell stage. Developmental block of iSCNT cloned cat embryos in this previous study was suggested to be associated with a developmental cell block and mitochondrial incompatibility between the recipient oocytes and donor cells (Thongphakdee et al., 2008).

The SCNT technologies have been significantly improved so far. Morulae and blastocysts can be produced with reasonable efficiency, but the *in vivo* situation still cannot be mimicked sufficiently well. The underlying mechanisms are largely unknown at present, but changes in epigenetic modifications occurring during preimplantation development resulting in perturbed embryonic and fetal gene expression patterns are thought to be involved (Wrenzycki et al., 2004). Although differentiated cells contain complete genetic information, they are modified epigenetically by methylation of DNA and modification of histones. Any faults in a donor cell will be amplified in subsequent development, as failure to alter its epigenetic marks will lead to an embryo not fully totipotent and after further development and differentiation (Enright et al., 2003).

The transfer of somatic cells to enucleated oocytes requires a process termed nuclear reprogramming to transform the differentiated cell nuclei to dedifferentiated in the ooplasm before convert to a totipotent state and induce somatic gene expression (Wu et al., 2010). Incomplete donor nuclei reprogramming and abnormal epigenetic reprogramming (DNA methylation or histone modifications) are thought to be related to the low efficiencies in SCNT- and iSCNT cloned embryos (Arat et al., 2003; Chen et al., 2006; Lee et al., 2010). Histone acetylation provides the greatest potential for unfolding chromatin to recruit different transcriptional factors, and thus acetylation is related to the active genes, and removal of acetylated groups by histone deacetylases (HDACs) is generally associated with gene silencing (Shi et al., 2008). Therefore, abnormal patterns of histone acetylation could seriously affect the development of cloned embryos (Shi et al., 2008). Previous studies has suggested that the *in vitro* embryo development and full-term development of cloned embryos have been improved by epigenetic modification of donor cells or early cloned embryos with trichostatin A (TSA), a HDACs inhibitor that increases histone acetylation (Ding et al., 2008; Kishigami et al., 2006). Moreover, the histone acetylation patterns of TSA treated cloned embryos also appeared to be similar to those of normal embryos (Lee et al., 2010).

This study is proposed to improve the quality of the inter-species cloned cat embryos by modified condition of donor cells and recipient cytoplasts. The lack of

species-specificity between oocytes and donor cells is one of the major problems for endangered species conservation, so the inter-species nuclear transfer was developed so far. However, the possibility to get the live offspring is still quite low due to many unknown factors. Then, improvement of the SCNT procedures should be performed either modification of donor cells or epigenetical modulation of both donor cells and recipient cytoplasts. The subsequent development after nuclear transfer is controlled by many factors including epigenetic program, which lead to an embryo not fully totipotent and after further development and differentiation in case of abnormal regulation. This demonstrated that the epigenetic modulation might be necessary as same as the modification of donor cells.

The inter-species cloned cat *(Felis catus)* embryos using the bovine *(Bos taurus)* oocytes as recipient cytoplasts will be produced and used in this study. This model could be applied for endangered animal conservation in the future.

1.2 Literature review

1.2.1 *Somatic cell nuclear transfer (SCNT)*

Somatic cell nuclear transfer (SCNT), the one of assisted reproductive technologies (ART's), is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm (Fig. 1). SCNT in mammals was not achieved until over four decades after the initial reports from Briggs and King of the production of adult frog clones using embryonic nuclei (Briggs and King, 1952), which lighted up the researchers working with mammalian eggs to be interested until now. This technique has been accomplished in an ever-growing list of species. In each case, an enucleated oocyte has successfully reset the nucleus of a somatic cell such that the embryonic program could progress to the production of a live offspring (Keefer, 2008). The first live cloned offspring produced from the differentiated cell populations were two lambs born in 1995 using cultured embryonic cells as nuclear donors and enucleated unfertilized eggs (metaphase II oocytes, MII) as recipient cytoplasts (Campbell et al., 1996). In the

following year, offspring were produced using the cultured cell populations derived from fetal and adult tissues (Wilmut et al., 1997).

Since this time, SCNT has been successfully applied to many species including cattle (Cibelli et al., 1998), mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999), pig (Polejaeva et al., 2000), cat (Shin et al., 2002), rabbit (Chesne et al., 2002), horse (Galli et al., 2003), rat (Zhou et al., 2003), dog (Lee et al., 2005), and ferret (Li et al., 2006) using a range of cell types. To date the frequency of development has not dramatically increased, although, the modification and improvements in techniques still ongoing investigation including: (1) simplifying the methodology, (2) reducing costs and (3) improving survival following birth. The production of cloned embryos involves many steps and each of these may influence the successful outcome (Campbell et al., 2007).
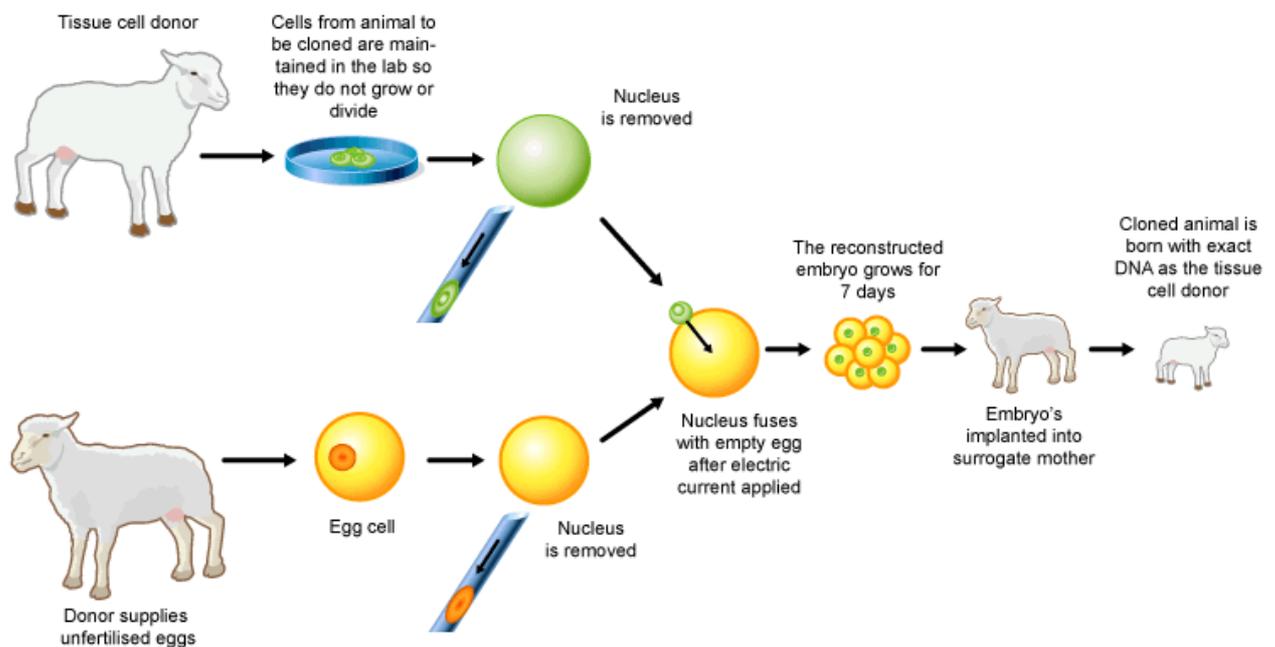
Figure 1. The SCNT animal is produced by transferring the nucleus from a diploid to an unfertilized oocyte from which the chromosomes have been removed. The chromosomes are remodeled and developed to a certain stage before being transferred to a surrogate mother. The cloned offspring is born with the exact DNA of the donor cell used (www.biotechnologyonline.gov.au/.../img_scnt.cfm).

1.2.2 Factors improving the SCNT efficiency

To improving the efficiency of SCNT prospects for the future, modification of donor oocytes, modification of the donor cells prior to use and modification of culture procedures following embryo reconstruction should be considered.

1.2.2.1 Modification of oocyte recipient

The quality and source of oocytes is a key factor in determining the proportion of oocytes developing to the blastocyst stage and the efficiency at which live offspring are produced (Gómez et al., 2006). In the domestic animal, both *in vivo*-and *in vitro*-matured oocytes have been used as recipient cytoplasts for production of cloned embryos. In domestic cat, when *in vivo* matured oocytes were used as recipient cytoplasts, a higher percentage of fusion was observed (97%) as compared to *in vitro* matured oocytes (90%). In contrast, cleavage frequency after reconstruction using *in vitro* matured oocytes (85%) was higher than that after reconstruction with *in vivo* matured oocytes (79%). Nonetheless, no significant differences were observed in frequency of blastocyst development between embryos reconstructed with in vivo or *in vitro* matured cytoplasts (27% versus 23%, respectively) (Gómez et al., 2003). In case of domestic cattle, since their ovaries are easily accessible from slaughterhouse and are available throughout the year, the use of *in vitro* matured bovine oocytes as donor cytoplasts is a reliable alternative.

Mammalian oocytes become matured and are in general fertilized at metaphase of the second meiotic division (MII), controlled by the cytoplasmic protein kinases maturation promotion factor (MPF) and mitogen activated protein kinase (MAPK). The use of MII oocytes may improve the development and increase nuclear reprogramming (Campbell, 1999; Campbell and Alberio, 2003). Transfer of a donor nucleus into an ooplasm containing high levels of MPF and MAPK theoretically causes nuclear envelope breakdown and premature chromosome condensation as the donor nucleus undergoes a pseudomeiotic/mitotic event. However, the occurrence of this phenomenon is variable in each recipient oocytes (Campbell et al., 1993). Some studies suggested that the treatment of oocytes with the non-specific phosphatase inhibitor caffeine could increase

the levels of MPF and MAPK. Moreover, they found that SCNT embryos could be produced using caffeine treated oocytes as recipient cytoplast, an increase in cell number at the blastocyst stage was observed. When transferred to the synchronized recipient, an increase in maintenance of pregnancy was also observed (Lee et al., 2006).

1.2.2.2 Modification of the donor cells

Cell cycle synchronization plays a crucial role affecting the success of the somatic cell nuclear transfer. Most of the experimental findings suggested that the G0/G1 stages of the cell cycle provided better results in term of development of reconstructed embryo (Boquest et al., 1999; Gómez et al., 2003; Hashem et al., 2006; Liu et al., 2004). When a metaphase II (MII) oocyte with a high level of MPF is used as recipient ooplasm, a G0/G1 or pre-S phase donor nucleus is preferred (Campbell et al., 1996). Furthermore, (Wilmut et al., 1997), the first who reported the production of cloned sheep from mammary cells, which were synchronized into the presumptive G0 phase (quiescent phase). This result suggested that the chromatin at this phase is amenable to nuclear reprogramming. These are the reasons why synchronization of the cell cycle stages in the G0/G1 phase is one of the key factors determining the success of the SCNT technique (Hashem et al., 2006)

There are several methods to synchronize cell cycles using serum starvation, cell confluency-contact inhibition and chemical inhibition. Serum starvation is performed by replacing tissue culture medium containing low level of serum. It provides 81.3-96% of cells arresting at G0/G1 after treatment for 3 days in giant pandas (Han et al., 2003), 4 days in sheep (Wilmut et al., 1997), 5 days in cattle (Han et al., 2003), brown bears (Caamano et al., 2008), African wild cats and domestic cats (Gómez et al., 2003) and 5-10 days in rabbits (Mitalipov et al., 1999). However, it could reduce cell survival with subsequent low development of embryos caused by DNA fragmentation (Gómez et al., 2003; Khammanit et al., 2008). Using the contact inhibition by an additional 5 days in culture after confluency reached to 100%, a high percentage of G0/G1 cells were obtained in porcine fetal fibroblasts (Boquest et al., 1999). This method has been

successfully used for producing cloned dogs (Kim et al., 2007) as well as transgenic dairy goats (Melican et al., 2005). Synchronizing cell cycle by culturing donor cells in culture medium contained chemical inhibitor such as roscovitine, also provides a high potency for arresting bovine granulosa cells at G0/G1, resulting in an increase in cloning efficiency (Gibbons et al., 2002). However, with high concentration of roscovitine, a decrease in the proportion of G0/G1 cells and an increase in the percentage of cells underwent apoptosis were observed in dogs (Khammanit et al., 2008).

1.2.2.3 Modification of cultured procedure

In SCNT embryos, it has been suggested that the use of culture medium suited for the donor cell may improve development (Gao et al., 2003) and also the culture environment can influence gene expression (Moreira et al., 2006). It has been suggested that supplementation of the culture medium with the factors which may aid this process may be beneficial. In a number of studies the addition of trichostatin A, an inhibitor of histone deacetylase, to the culture medium of murine or bovine SCNT embryos until the blastocyst stage has been shown to increase the frequency of blastocyst formation (Kishigami et al., 2007) and to increase the efficiency of production of live offspring from murine cumulus cells but not murine ES cells (Iwamoto et al., 2007).

1.2.3 Interspecies/generic SCNT

The SCNT is not only a valuable tool for producing animals with the same genetic traits, it is also an alternative technique to help for preserving endangered animals such as gaur *(Bos frontalis gaurus)* (Lanza et al., 2000), mouflon *(Ovis orientalis musimom)* (Loi et al., 2001), African wild cat *(Felis silvestris libica)* (Gómez et al., 2003), argali *(Ovis ammon)* (Janssen et al., 2004), giant panda *(Ailuropoda melanoleuca)* (Han et al., 2003), marbled cats *(Pardofelis marmorata)* (Thongphakdee et al., 2007), leopard cat *(Prionailurus bengalensis)* (Yin et al., 2006), goral *(Naemorhedus caudatus)* (Hashem et al., 2006), flat-headed cats *(Prionailurus planiceps)* (Thongphakdee et al., 2010), and Cantabric brown bear *(Ursus arctos pyrenaicus)* (Caamano et al., 2008). Attempts through SCNT in wild animals provide the opportunity to developing "inter-

species/generic SCNT", which involves the transfer of donor cell nuclei from one species/genus to enucleated oocytes of another species/genus (Yin et al., 2006).

1.2.3.1 Interspecies SCNT (iSCNT)

Live offspring from a few endangered mammalian species have been produced by using inter-species SCNT. Mouflon *(Ovis orientalis musimom)* and Argali *(Ovis ammon)* cloned embryos have been derived by transferring somatic cells into enucleated domestic sheep oocytes *(Ovis aries),* and gaur cloned embryos *(Bos frontalis gaurus)* were obtained after transfer of cells into enucleated domestic cow oocytes *(Bos taurus).* Cloned embryos of these three species were transferred into domestic recipient animals (sheep or cow) and could be able to maintain pregnancies to term (Janssen et al., 2004; Lanza et al., 2000; Loi et al., 2001). Moreover, the domestic cow oocytes were also used as recipients to supported the development of reconstructed banteng *(Bos javanicus)* (Sansinena et al., 2005) and yak *(Bos gruniens)* (Li et al., 2007) embryos after transferred their cells into perivitelline space (Table 1). Similarly, the cloned felid embryos developed after transfer African wild cat *(Felis silvestris lybica)* or black-footed cat *(Felis nigripes)* somatic cells into enucleated domestic cat oocytes (Table 2). Moreover, pregnancies were established after transfer black-footed cloned embryos and live offspring were born after the transfer of African wild cloned embryos into domestic cat recipients (Gómez et al., 2006). These experiments were performed by using the donor nuclei and recipient oocytes, which came from the animals in the same genus but difference species. Although the pregnancies or the live offspring were obtained from the inter-species SCNT but the abortions or respiratory failure are frequently observed in the few days. It appears that placental atrophy may be the principal cause of losses, which resulted in premature separation before delivery and, secondarily, respiratory failure due to lung immaturity (Gómez et al., 2006).

Table 1. *In vitro* development of inter-species and inter-generic cloned embryos reconstructed by fusion of somatic cells of non-domestic bovids with enucleated domestic bovine oocytes

Species	No. of fused (%)	No. of cleaved (%)	No. of blastocysts (%)	References
Buffalo *(Bubalus bubalis)*	-	100/151 (66.2)	20/151 (13.3)	(Lu et al., 2005)
Camel *(Camelus bactrianus)*	N/A (53)	N/A (34)	-	(Zhou and Guo, 2006)
Gaur *(Bos gaurus)*	-	-	N/A (12)	(Lanza et al., 2000)
Takin *(Budorcas taxicolor)*	-	-	N/A (5)	(Li et al., 2006)
Yak *(Bos gruniens)*	N/A (72)	N/A (84)	N/A (34)	(Li et al., 2007)
Banteng *(Bos javanicus)*	91/143 (68)	60/91 (67)	25/91 (28)	(Sansinena et al., 2005)

N/A: numbers not available.

1.2.3.2 Intergeneric SCNT

The only vertebrate offspring that have been obtained by inter-generic SCNT is carp fish *(Cyprinus carpio)* which was reconstructed by transferring embryonic carp cells into enucleated goldfish oocytes *(Carassius auratus)* (Sun et al., 2005). Even though no live mammals have been produced by inter-generic SCNT, successful *in vitro* embryo development to the blastocyst stage has been achieved after inter-generic SCNT of somatic cells from different non-domestic felids into domestic cat *(Felis catus)* oocytes such as leopard cat *(Prionailurus bengalensis)* (Lorthongpanich et al., 2004), rusty spotted cat *(Prionailurus rubiginosus)* (Gómez et al., 2004) and Korean tiger

(Panthera tigris altaica) (Hwang et al., 2001) (Table 2). Similarly to bovine species, the inter-generic SCNT embryos have been obtained after transferred the mountain bongo antelope (Lee et al., 2003), swamp buffalo *(Bubalus bubalis)* (Lu et al., 2005), camel *(Camelus bactrianus)* (Zhou and Guo, 2006) and takin *(Budorcus taxicolor)* (Li et al., 2006) cells into enucleated bovine oocytes (Table 1).

Table 2. *In vitro* development of inter-species and inter-generic cloned embryos reconstructed by fusion of somatic cells of non-domestic felids with enucleated domestic cat oocytes

Species	No. of fused/injected (%)	No. of cleaved (%)	No. of blastocysts (%)	References
Korean tiger *(Panthera tigris altaica)*	N/A[a] (54)	N/A (65)	N/A (9)	(Hwang et al., 2001)
African wild cat *(Felis silvestris lybica)*	425/484 (88)	357/425 (84)	101/357 (28)	(Gómez et al., 2006)
Leopard cat *(Prionailurus bengalensis)*	55/80 (69)	45/54 (83)	3/45 (7)	(Lorthongpanich et al., 2004)
Rusty spotted cat *(Prionailurus rubiginosus)*	52/52 (100)	42/52 (80.8)	1/42 (2.4)	(Gómez et al., 2004)
Black-footed cat *(Felis nigripes)*	170/170 (100)	144/170 (84.7)	3/144 (2.1)	Gómez (unpublished data)

N/A: numbers not available.

The technique inter-generic SCNT was also conducted for producing the cloned leopard cat in Korean. The results demonstrated that transferring of the leopard cat fibroblast cells into enucleated oocytes of the domestic cat were able to develop to the blastocyst stage *in vitro* as well as the six fetuses, but no viable offspring were obtained after embryo transfer (Yin et al., 2006). Similarly, in Thailand, the inter-generic SCNT were reported in the endanger animals which are the marbled cat *(Pardofelis mamorata)* and flat-headed cat *(Prionailurus planiceps)*. They founded that the marbled cat and flat-headed cat cloned embryos have potentially developed *in vitro*; however, a lower rate of blastocyst development of inter-generic SCNT embryos than that observed in *in vitro* fertilization (IVF) embryos was observed. Furthermore, no pregnancy of recipients receiving flat-headed cat and domestic cat SCNT embryo was observed in their study (Thongphakdee et al., 2007).

It has been demonstrated that some factors present in the oocyte cytoplasm are responsible for distinguishing the duration of cell division. The first three cycles of cat-rabbit embryo development is the same as those for rabbit-rabbit, as is the duration to form the cat-rabbit blastocysts which is similar to cat-cat embryos (Wen et al., 2003). The good model for inter-generic SCNT was established by the implantation of panda-rabbit cloned embryo in domestic cat oviduct (Chen et al., 2002).

The major problems that effect to development of inter-generic SCNT embryos are chromosomal abnormalities derived from karyotypically abnormal somatic cells, inadequate nuclear reprogramming and inter-generic difference. Furthermore, other external factors such methylation and co-existing mitochondrial DNA (mtDNA) may have been involved. Chromosomal abnormalities, i.e. hypoploidy, are major defects of donor fibroblasts, which increase progressively during culture *in vitro*. Moreover, the use of fibroblast cells at the late of passage as donor cell results an increased incidence of chromosomal abnormalities in reconstructed cloned embryos (Gómez et al., 2006). However, the recipient oocytes from unrelated genetic animals are feasible for *in vitro* studies in both cellular and molecular aspects in addition to oocytes from closely related animals. Nowadays, lived endangered cloned animals produced from closely genetic

oocytes, different in species, have been achieved. This shows that more attempts are necessary to find the proper conditions for the production of other endangered animals where their closely genetic host oocytes are not available. Furthermore, in-depth examination of the mechanism of reprogramming of the cloned embryo reconstructed from species-unspecific oocytes is needed (Thongphakdee et al., 2007).

In summary, the low frequency of development to the blastocyst stage of inter-species and/or inter-generic SCNT embryos may be a result of disparity in cell lines, abnormal nuclear remodeling and reprogramming, mitochondrial heteroplasmy, as well as incompatibilities between the nucleus and cytoplasm. Further studies are required to understand other aspects of chromatin modification which, in turn, should provide significant insight into the mechanisms involved in nuclear reprogramming and the causes of embryo developmental arrest. The developmental significance of having a cloned embryo derived from the nucleus and oocyte of different genera remains to be determined. Lastly, for preserving endangered species, it is appropriate to consider the possibility of genetic incompatibility between the donor nucleus and the cytoplasmic mitochondria in reconstructed cloned embryos and it potential effect on successful generation of live offspring (Gómez et al., 2006).

1.2.4 *Gene expression patterns in in vitro-produced (IVP) and SCNT-derived embryos*

In vitro-produced (IVP) and SCNT technologies have been significantly improved so far. Morulae and blastocysts can be produced with reasonable efficiency, but the *in vivo* situation still cannot be mimicked sufficiently well. The quality of the embryos produced is impaired in comparison with their *in vivo* counterparts (Niemann et al., 2002). Pregnancy rates after transfer of IVP and SCNT-derived embryos can be markedly reduced and a considerable proportion of the offspring is affected by the "large offspring syndrome (LOS)". This syndrome is characterized by a great variety of abnormal phenotypes, including significant increases in birth weight, polyhydramnios, hydrops fetalis, altered organ growth, various placental and skeletal defects, immunological defects and increased perinatal death. The underlying mechanisms are

largely unknown at present, but changes in epigenetic modifications occurring during preimplantation development resulting in perturbed embryonic and fetal gene expression patterns are thought to be involved in the syndrome (Wrenzycki et al., 2004).

The ultimate test of the quality of an embryo is its ability to produce live and healthy offspring after transfer to a recipient. Morphology and the proportion developing to the blastocyst stage are used as criteria to assess developmental competence. In most mammalian species, evaluation of embryo morphology remains the method of choice for selection of viable embryos prior to transfer. However, with the advent of IVP and SCNT, it has become clear that embryo competence can be severely compromised without obvious changes in morphology. Thus, embryo morphology is not accurate enough to act as the sole criterion for the prediction of embryo developmental potential *in vivo*. Therefore, non-invasive techniques are required, such as cryoresistance, which can provide valuable information on embryo viability (Massip and Leibo, 2002). Other approaches are analysis of embryonic metabolism, ultrastructure, chromosomal abnormalities, and gene expression patterns (Wrenzycki et al., 2004). The products of the analyze genes play important roles during pre- and post-implantation development and, hence, have the potential to be used as genetic markers for embryo viability (Niemann and Wrenzycki, 2000).

1.2.5 Epigenetics

Over 15,000 genes are marked for expression throughout murine development. Many of these genes are expressed only during the pre-implantation period and are then shut down in somatic cells by epigenetic regulations of specific genes expression patterns. This implicates the crucial role that epigenetics plays during embryonic development. During pre-implantation, the fidelity of the expression levels and the timing of these genes expression critically influence normal embryonic development and differentiation (Suteevun, 2005). The vast majority of factors that switch on and off gene activity are epigenetic (Li, 2002). Epigenetics influence gene activity without affecting the DNA sequence and can also be modified by the environment and transmitted to

progeny. Two major mechanisms of epigenetic activity are DNA methylation and histone modifications. The structure of DNA and histones are shown in Fig. 2.

In bovine SCNT, although the efficiency of development to blastocyst can be comparable or even higher than that by IVF but fetal loss during gestation remains unclear. Even if the cloned fetuses are carried to term, incidence of developmental anomalies and neonatal deaths are common. These failures may be explained by incomplete reprogramming of the donor cell genome. Although differentiated cells contain complete genetic information, they are modified epigenetically by methylation of DNA and modification of histones. Any faults in a donor cell will be amplified in subsequent development, as failure to alter its epigenetic marks will lead to an embryo not fully totipotent and after further development and differentiation (Enright et al., 2003).

1.2.5.1 DNA methylation

DNA methylation is the major epigenetic mechanism, which can interact with and modulate the other regulatory mechanisms (Li, 2002). Methylation plays a role in MEPC), which results in the blocking of the binding of transcription factors on the DNA. Another mechanism of transcriptional regulation by methylation is the association of methylated DNA with the deacetylation of histones which leads to the condensation of the DNA into a chromatin conformation. This interrupts the binding of transcription factors on the DNA, resulting in gene repression. DNA methylation also interacts with other histone modifications to modulate gene repression and heterochromatic regions (Reik et al., 2003). DNA methylation takes place mainly on the 5'-cytosine residues at CpG dinucleotides. This mechanism is essential for normal development and is associated with a number of key process including genomic imprinting, X-chromosome inactivation, suppression of repetitive elements and carcinogenesis (Bestor, 2000). The demethylation/remethylation waves generally appear to coincide with the activation and transcription of the embryonic genome. In mammals, it is well known that the onset of genomic activation differs between species, occurring at the 2-cell stage in mice, 4-cell stage in pigs, and the 8- to 16-cell stage in cows (Campbell, 1999). It has been

proposed that the delayed or aberrant demethylation/remethylation wave observed in pre-implantation cloned embryos may impair the normal progression towards the activation of the genome (Shi et al., 2003).

The two main components of the epigenetic code

DNA methylation
Methyl marks added to certain DNA bases repress gene activity.

Histone modification
A combination of different molecules can attach to the 'tails' of proteins called histones. These alter the activity of the DNA wrapped around them.
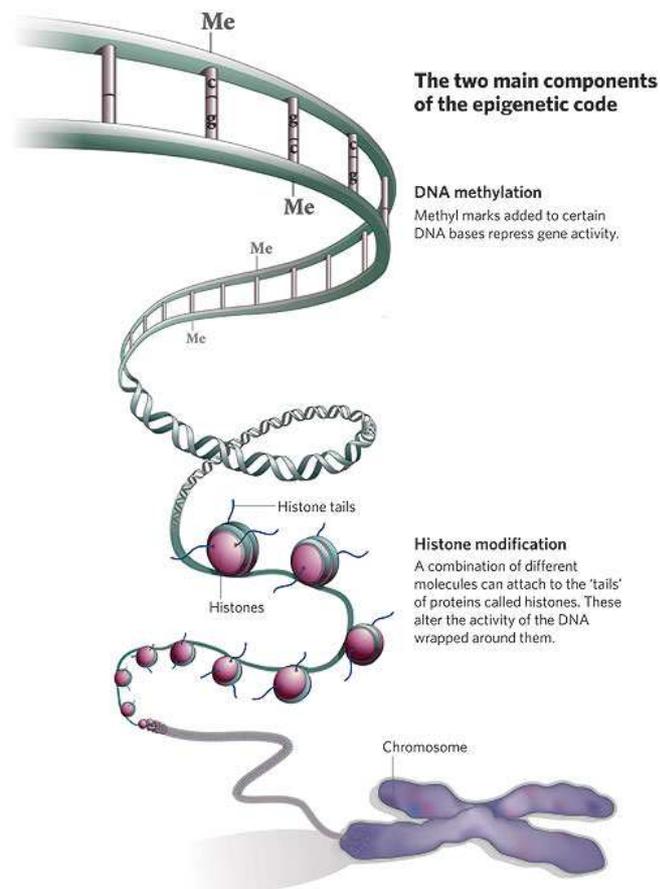
Figure 2. Structure of DNA and histones; histones are the major structural proteins of chromosomes that composed of two copies of each of histones H2A, H2B, H3 and H4. The DNA molecule is tightly wrapped twice around a histone octamer to make a nucleosome. (http://drugline.org/img/term/histone-7192_2.jpg)

In natural reproduction, relatively low levels of DNA methylation exist in male and female gametes, which are further demethylated during early development. However, somatic cells have been shown to have much higher levels of DNA methylation than either gametes or early embryos. In SCNT, highly methylated somatic donor cells are used to generate clone embryos, which in turn, have been shown to be abnormally hypermethylated (Enright et al., 2005). Therefore, the possibility of the cloned embryo

development and differentiation to term could be predicted by detection the epigenetic patterns in the blastocyst stage.

1.2.5.2 Histone modifications

Histone proteins play essential structural and functional roles in the transition between active and inactive chromatin states. Although histones have a high degree of conservation due to constraints to maintain the overall structure of the nucleosomal octameric core, variants have evolved to assume diverse roles in gene regulation and epigenetic silencing. Histone variants, post-translational modifications and interactions with chromatin remodeling complexes influence DNA replication, transcription, repair and recombination.

Histones are subject to a wide variety of posttranslational modifications including but not limited to, lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation (Fig. 3). These modifications occur primarily within the histone amino-terminal tails protruding from the surface of the nucleosome as well as on the globular core region (Bannister and Kouzarides, 2005). Histone modifications are proposed to affect chromosome function through at least two distinct mechanisms. The first mechanism suggests modifications may alter the electrostatic charge of the histone resulting in a structural change in histones or their binding to DNA. The second mechanism proposes that these modifications are binding sites for protein recognition modules, such as the bromodomains or chromodomains, which recognize acetylated lysines or methylated lysine, respectively (Sarma and Reinberg, 2005).

Histone acetylation

Histone acetylation is an important epigenetic modification. Modifications of lysine residues of core histones have the greatest potential for unfolding chromatin to recruit different transcriptional factors and it is almost invariably associated with activation of gene transcription (Wu et al., 2010). Presently, known site for acetylation include at least four highly conserved lysines (K) in histone H4 (K5, K8, K12, K16) and in

histone H3 (K9, K14, K18, K23) (Bjerling et al., 2002). Histone acetylation is reported to be regulated by histone acetyltransferase (HATs) and deacetylase (HDAC) (Bertos et al., 2001) which are classified into many subfamilies that are mostly conversed from yeast to human. Comprehension of the structure, function, and regulation of class II deacetylases including HDAC4, HDAC5, HDAC6, HDAC7, and HDAC9 is important for elucidating how acetylation regulates functions of histones and other proteins *in vivo* (Bertos et al., 2001).

Dynamic changes of deacetylation and re-acetylation during germ cell maturation and embryo development have been reported in mice (van der Heijden et al., 2006) and cattle (Wu et al., 2010). The high levels of acetylation on H3K9 and H3K18 were observed in1- and 2-cell bovine embryos; however, the satining intensity were significant decreased at the 8-cell stage and then partially resumed during subsequent developmental stages (Santos et al., 2003; Wu et al., 2010). Intersestingly, the fluorescence signals were stronger in cloned embryos than IVF embryos from pronuclear to 8-cell stages (Wu et al., 2010), which might be indicated that aberrant histone acetylation occurred before donor genome activation (Wu et al., 2010)

Histone methylation

Histone methylation is another important histone modification in the regulation of chromatin remodeling and gene expression. Histone methylation can signal either gene activation or repression, depending on the sites of methylation.   H3K4me3 is an indicator of gene transcription activation, while H3K9me2 is associated with heterochromatin where non-gene expression occurs (Wu et al., 2010).

(A)

(B)

Nature Reviews | Cancer
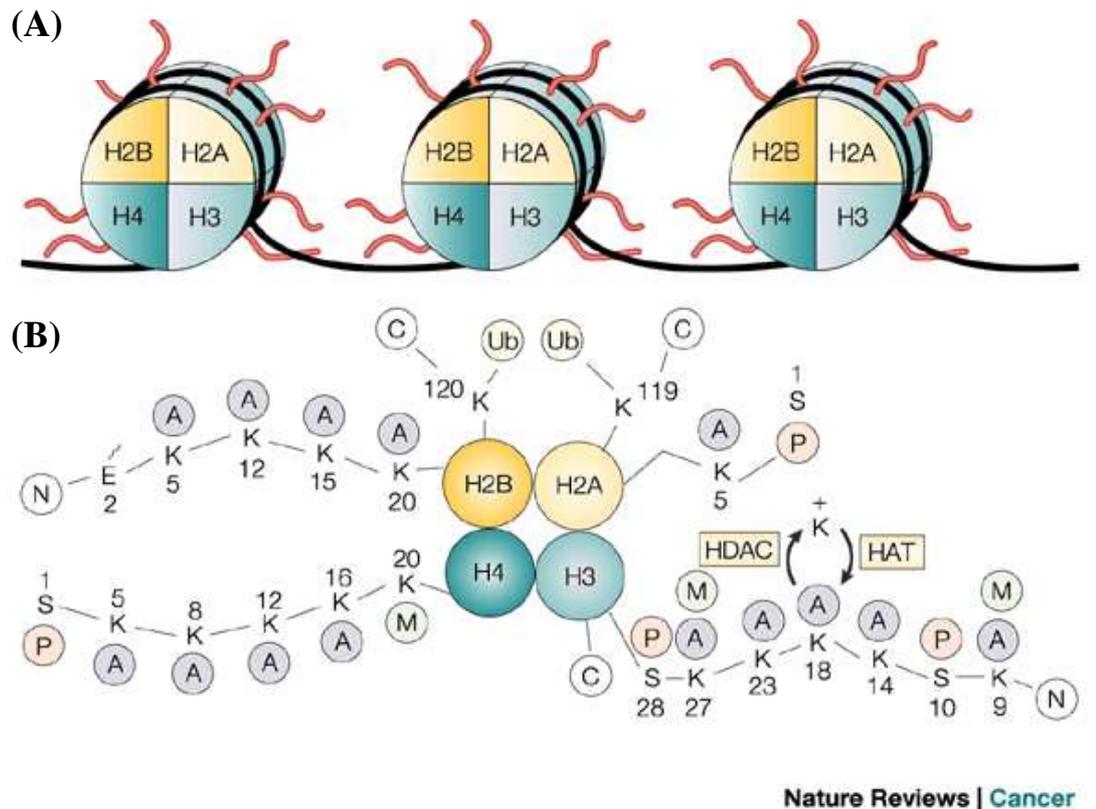
H2A, H2B, H3 and H4 are potential acetylation/deacetylation sites for histone acetyltransferases (HATs) and histone deacetylases (HDACs) (B). Acetylation neutralizes the charge on lysines. A, acetyl; C, carboxyl terminus; E, glutamic acid; M, methyl; N, amino terminus; P, phosphate; S, serine; Ub, ubiquitin. (www.nature.com/nrc/journal/v1/n3/images/.../.gif)

1.2.4.3 Epigenetic modulations

Several studies revealed that using the epigenetic modulation such as the DNA methyl-transferase inhibitor (5-aza-2′-deoxycytidine; 5-aza-dC) or histone-deacetylase inhibitor (Trichostatin A; TSA) may improve cloning efficiency of NT embryos (Lee et al., 2010). The beneficial effects of TSA treatment on improving the development of bovine cloned embryo were shown by Enright et al. (2003) and Lee et al. (2010). While treatment of the donor cell with 5-azadC was able to reduce histone acetylation and DNA methylation in cloned embryos to levels that are similar to those found in IVF-derived embryos (Enright et al., 2005). Ding et al. (2008) also founded that the

development of cloned bovine embryos could be enhanced by the 5-azadC and TSA; furthermore, the combination was more effective than either one alone.

According to the above reviews, the development of iSCNT will play an important role for conservation in endangered species. However, the incomplete nuclear reprogramming, the low blastocyst rate, and the abnormal epigenetic reprogramming are still the major problems for this technique. The bovine ooplasm have shown the abilities for supporting the *in vitro* development of embryos to blastocyst stage produced by iSCNT of somatic cells from various unrelated mammalian species, including the sheep, pig, monkey, dog and yak, however, no pregnancy had carried to full-term after the embryo transfer to recipients (Dominko et al., 1999; Murakami et al., 2005). To our knowledge, there are very few reports concerning the ability of bovine oocytes to reprogram the nucleus of felid species. Owing to the similarities between the domestic cat and wild cats, the domestic cat (*Felis catus*) is widely used as a model organism for development of assisted reproductive technologies (ARTs) in all felids (Waurich et al., 2010), such as intracytoplasmic sperm injections (ICSI) (Pope et al., 1998) and SCNT (Yin et al., 2008).

Histone acetylation provides important roles for cell growth, cell cycle control, development, differentiation and survival during the embryo development. Therefore, abnormal patterns of histone acetylation could seriously affect the development of cloned embryos. To alter the patterns of histone acetylation in cloned embryos to be similar to those of normal embryos, the treatment of early stage embryos with Trichostatin A (TSA), histone deacetylase (HDAC) inhibitor, has been suggested. Therefore, it could be possible to improve the *in vitro* development of iSCNT cloned cat embryos reconstructed from cat somatic cell and bovine cytoplast by the modification of histone acetylation level with the treatment of TSA. The data acquired from this study will be advantages for improving the iSCNT embryos efficiency in the future.

1.3 Objectives of the thesis

1. To establish an appropriate iSCNT protocol including donor cell cycle synchronization (modification of donor cell) and *in vitro* embryo culture (modification of

cultured procedure) for iSCNT cat embryos reconstructed from cat donor nuclei and bovine cytoplasm

2. To assess the effect of modified donor cell by cell cycle synchronization on *in vitro* development of iSCNT cat embryos

3. To investigate the effect of modified cultured procedure by supplementation of histone deacetyalse inhibitor TSA on *in vitro* development of iSCNT cat embryos

4. To compare the differential acetylation on histone H3 lysine 9 (K9), 18 (K18), 23 (K23), and di-methylation pattern on histone H3 lysine9 in the early stage of iSCNT cat embryos between with and without the treatment of TSA in comparison with bovine IVF embryos.

5. To investigate the pregnancy establishment of recipients receiving TSA-treated iSCNT cat embryos.

1.4 Hypothesis

1. The iSCNT cat embryos can be reconstructed from domestic cat nuclei and bovine recipient oocytes.

2. The modification of donor cell by cell cycle synchronization affects the efficiency of iSCNT cat embryo production

2. The *in vitro* embryo culture with TSA affects the efficiency of iSCNT cat embryo production.

3. The differential acetylation on histone H3K9, H3K18, H3K23 and Me2H3K9 in the early stage of iSCNT cat embryos can be modified by the treatment of TSA.

4. Recipients receiving the TSA-treated iSCNT cat embryos can become pregnant.

1.5 Key words: cell cycle synchronization, histone acetyaltion, histone methylation, interspecies somatic cell nuclear transfer, embryo development, trichostatin A, cat, bovine

1.6 Research merits:

1. Appropriate procedures of iSCNT including donor cell cycle synchronization and *in vitro* embryo culture for iSCNT cat embryos

2. Information of the TSA effect on *in vitro* development and diferretial acetylation and di-methylation on histone H3 of iSCNT cat embryos

3. Techniques of embryo transfer in domestic cat.

4. The possibility of producing domestic cat offspring by iSCNT

CHAPTER II

ESTABLISHMENT OF CULTURE PROTOCOL FOR
DOMESTIC CAT FIBROBLAST PREPARED AS DONOR CELL
IN SOMATIC CELL NUCLEAR TRANSFER

2.1 Abstract

This study was purposed to find the appropriate treatment of donor cell modification that gave the highest percentage of G0/G1 phase cells in the domestic cat (*Felis catus*). Skin tissues of two female domestic cats were obtained from the abdominal region at the time of ovariohysterectomy. Two cell lines from the domestic cats were generated. Skin fibroblast cells were cultured and treated with either serum starvation for 1-5 days (experiment I), cell confluency-contact inhibition for 5 days (experiment II) or roscovitine at various concentrations (7.5-30 µM) (experiment III). Flow cytometric analysis revealed that serum starvation for 3 days provided the highest cell population arrested at the G0/G1 stage when compared with cycling cells (86% vs. 66.9%, respectively). However, prolonging the serum starvation period for more than three days increased the rates of apoptosis. Induction of 100% confluency also gave a significantly higher percentage of cells arrested at the G0/G1 stage compared with the non-treated control cells ($P<0.05$) (78% vs. 66.9%, respectively). The treatment of fibroblast cells with different concentrations of roscovitine diluted with 30 µM DMSO gave the significant higher percentage of G0/G1 cells ($P<0.05$) than that of cycling cells. In conclusions, cat fibroblast cells were successfully synchronized to G0/G1 stage using the serum starvation, confluency-contact inhibition and roscovitine treatment. These findings may be valuable for preparing cat donor cells for somatic cell nuclear transfer.

2.2 Introduction

Somatic cell nuclear transfer (SCNT), one of the assisted reproductive technologies (ARTs), has been successfully used to produce cloned offspring in many mammalian species (Gómez et al., 2003; Jang et al., 2007; Melican et al., 2005; Wilmut

et al., 1997). This technique has been proposed to support the preservation of endangered felids such as the African wild cat *(Felis silvestris libica)* (Gómez et al., 2003), the leopard cat *(Prionailurus bengalensis)* (Yin et al., 2006), the marbled cat *(Pardofelis marmorata)* (Thongphakdee et al., 2006), and the flat-headed cat *(Prionailurus planiceps)* (Thongphakdee et al., 2010). However, the production of cloned embryos involves many steps and each of these has been suggested to influence the successful outcome (Campbell et al., 1996). The efficiency of cloning depends on four key parameters that influence the development of the reconstructed embryo: (1) the state and nature of the recipient cytoplast, (2) the genetics of the donor cell, (3) the type, differentiation stage and developmental potency of the donor cell, and (4) the cell cycle stage of the donor cell (Eggan and Jaenisch, 2002).

Cell cycle synchronization plays a crucial role in that it affects the success of the development of cloned embryos (Hashem et al., 2006). Previous studies have reported that the G0/G1 stage of the cell cycle provide better results in term of the development of the cloned embryo when a metaphase II (MII) oocyte with a high level of maturation promoting factor (MPF) is used as the recipient ooplasm (Boquest et al., 1999; Campbell et al., 1996; Gómez et al., 2003; Liu et al., 2004). Furthermore, a live cloned sheep has been successfully produced from mammary cells which were synchronized into the presumptive G0 phase (quiescent phase) (Wilmut et al., 1997). These results suggested that the chromatin at the G0/G1 phase is amenable to nuclear reprogramming for the cloned embryo (Hashem et al., 2006).

The synchronization of the cell cycle into the G0/G1 phase has been investigated in many species (Gómez et al., 2003; Han et al., 2003; Yin et al., 2006). Previous studies have suggested that the percentage of synchronization into the G0/G1 phase varies in the cell-cycle synchronization method and depends on the cell type and the mammalian species; therefore the suitable G0/G1 phase cell synchronization method should be examined before the use of donor cells for SCNT in all species. The present study was conducted to determine synchronization accuracy at the G0/G1 stage of the cell cycle through the confluent, serum starvation and roscovitine treatment

in domestic cat fibroblast cells. Moreover, the comparison between the effects of confluent treatment, the duration of serum starvation, and roscovitine concentration on cell-cycle synchronization for the use of donors in SCNT procedure was also investigated.

2.3 Materials and Methods

All chemicals were purchased from Sigma–Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise.

2.3.1 Establishment and culture of fibroblast cells

The skin tissues of two female domestic cats were obtained from the abdominal region at the time of ovariohysterectomy in the Small Animal Teaching Hospital at the Faculty of Veterinary Sciences, Chulalongkorn University. Two cell lines from the domestic cats were generated. The skin tissues were removed from the connective tissue using a scalpel blade and were then cut into 2x2x2 mm^2 pieces. They were washed three times in Phosphate buffered saline (PBS; Invitrogen, Grand Island, NY, USA) contaning antibiotics and re-washed three times with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. After washing, the skins were placed in 60 mm culture dishes (Falcon 3001, BD, Franklin Lakes, NJ, USA) containing DMEM supplemented with 20% FBS and antibiotics, and were then cultured at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every two days. After the cell confluency reached 70-80%, fibroblast cells were trypsinized by 0.5% trypsin for culture in the next passage. The 3-6 cells culture passages were used in this study.

2.3.2 Cell treatments and experimental designs

In a series of three cell cycle synchronization methods, we examined the effects of serum starvation, cell confluency-contact inhibition and chemical inhibitor (roscovitine) treatment on the cell cycle stages of fibroblast cells from domestic cat. In each treatment group, cells without any treatment were used as a control.

Expeiment I: Effect of serum starvation

Serum starvation was performed by replacing the tissue culture medium with DMEM supplemented with 0.5% FBS after the cell confluency reached 60-70% (Fig. 4A) in DMEM supplemented with 10% FBS. After the start of the serum starvation treatment (Day = 0), the stage of fibroblast cells from each animal was analyzed on Day 0, 1, 2, 3, 4, and 5.

Experiment II: Effect of cell confluency-contact inhibition

The effect of cell confluency-contact inhibition was investigated by an additional 5 days in culture after confluency had reached 100% (Fig. 4B). During the treatment of cell confluency-contact inhibition, the culture medium (DMEM + 10% FBS) was changed every two days.

Experiment III: Effect of concentration of roscovitine treatment

Roscovitine treatment was performed by 24 h of cell culture after the cell confluency reached 60-70% (Fig. 4A). The cells were cultured for 24 h in DMEM supplemented with 10% FBS and roscovitine with different concentrations (7.5, 15, and 30 µM), in which the roscovitine was diluted in 30 µM dimethylsulphoxide (DMSO). Cell toxicity was tested by using 30 µM DMSO treatment.
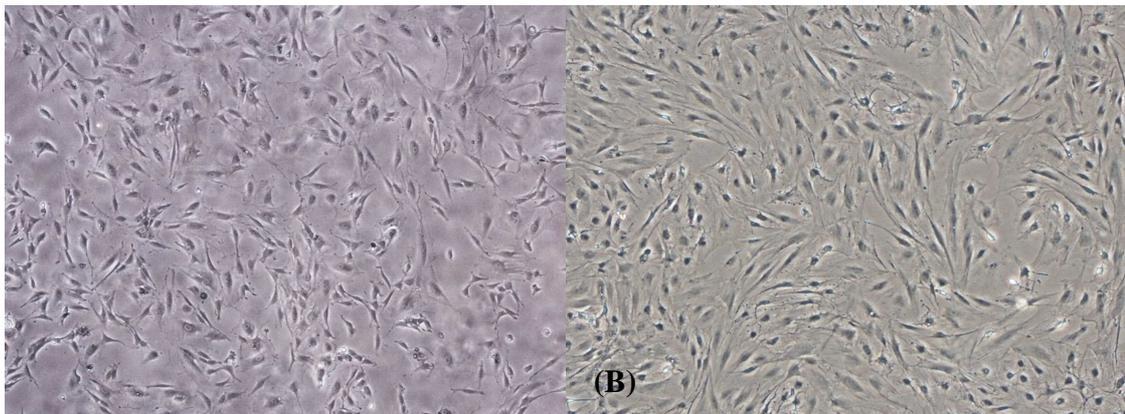
Figure 4. The monolayer of domestic cat fibroblast cells after the cell confluency reached 60-70% (A) and 100% (B) (×100).

2.3.3 Flow cytometric analysis

To analyze the cell cycle stages of the fibroblast cells, the cells were disaggregated with 0.5% trypsin prior to fixation. The disaggregated cells were re-suspended in DMEM supplemented with 5% FBS in centrifuge tubes, washed by centrifugation at 1500 × g for 5 min. After centrifugation, the cells were re-suspended in 0.2 mL PBS before fixation by drop-wise containing 0.8 mL cold methanol. The fixed cells were stored at -20°C for later analysis. After storage for 2-3 months, the fixed cells were centrifuged at 1500 × g for 5 min and then were re-centrifuged with PBS. The cells were re-suspended in 0.25 mL PBS supplemented with 0.6 mg/mL RNase and incubated at 37°C for 30 min. After incubation, the cells were stained with 0.25 mL of propidium iodide solution (PBS containing 50 μg/mL propidium iodide and 0.1% TritonX-100) and incubated at 37°C for 30 min. The cells were filtered through 41 μM nylon mesh (Spectrum, Los Angeles, CA, USA) for flow cytometric analysis, using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The cells were analyzed at a rate of 100-500 cells/sec as the sheath fluid and 5,000-10,000 events were recorded in each sample. The flow cytometer was standardized for each analytical session using Calibrite beads (Becton Dickinson). The percentage of cells within the various phases of the cell cycle was calculated using WinMDI version 2.8 software (J. Trotter, http://facs.scripps.edu/software.html) by gating G0/G1, S, and G2/M cell populations. Moreover, the numbers of apoptotic cells in samples were also detected by being represented in a sub-G_0/G_1 peak in the fluorescence histogram.

2.3.4 Statistical analysis

The experiments were repeated four times for each species. Data were presented as mean ± SEM. Generalized linear model (GLM) procedure (SAS version 9.0) was used to compare differences of percentages of G0/G1 cells and the percentages of apoptotic cells among treatment groups and among felid species. Differences among the means were analyzed using one-way analysis of variance (ANOVA). Results were considered statistically significant when $P<0.05$.

2.4 Results

2.4.1 Experiment I: Effect of serum starvation

The serum starvation treatment for one day resulted in a higher percentage (74.6%) of cells being arrested at the G0/G1 stage when compared with the percentages (66.9%) of the control cells (P<0.05). The proportion of G0/G1-phase cells increased further by prolonging serum starvation treatment to three days in the domestic cat (P<0.05) (Fig. 5A). However, the proportion of apoptotic cells also did increase after serum starvation treatment up to more than three days (Fig. 5B).

2.4.2 Experiment II: Effect of cell confluency-contact inhibition

The cell confluency treatment significantly increased the proportions of cells arrested at the G0/G1 stage (78%) (P<0.05) when compared to the control cells (66.9%) (Fig. 6A). There was no significant difference in the proportion of apoptotic cells between the treatment and control cycling cells (Fig. 6B).

2.4.3 Experiment III: Effect of roscovitine treatment

The roscovitine treatment with more than 7.5 µM significantly increased the proportion of G0/G1-phase cells compared with the control group (P<0.05). Cell toxicity was tested by 30 µM DMSO. Results indicated that the percentages of apoptotic cells were not affected by DMSO at this concentration compared to control cycling cells (Fig. 7B).
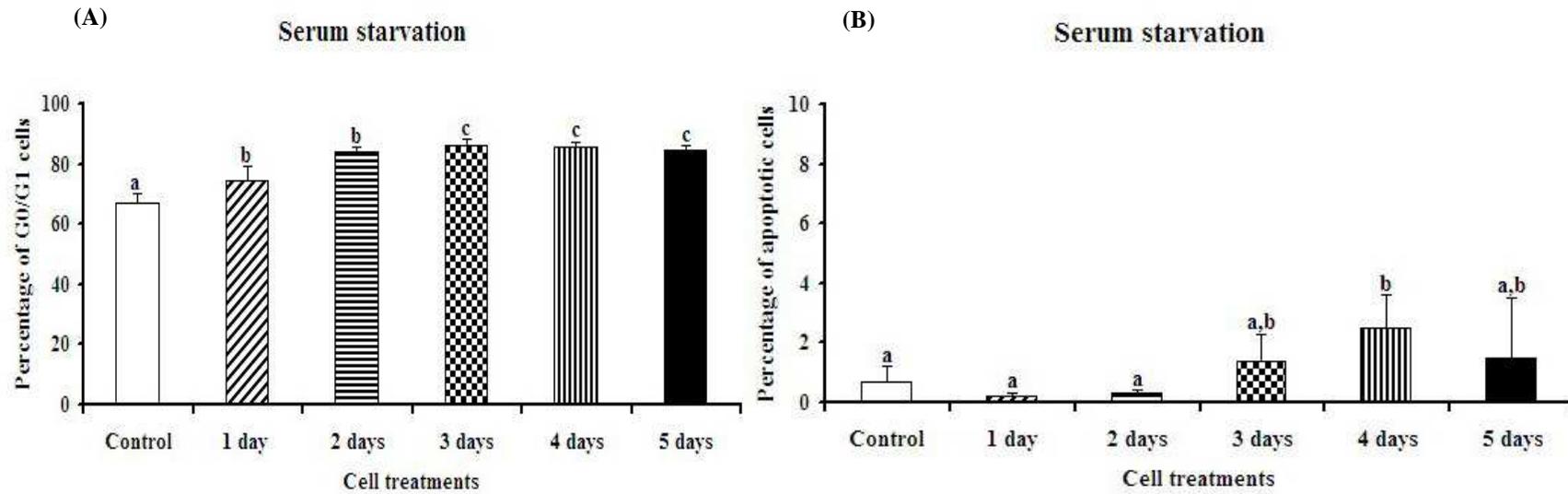
Figure 5. Mean ± SEM percentages of cells arrested at the G0/G1 phase of the cell cycle (A) and apoptotic cells (B) after the treatment of serum starvation for 0 (control), 1, 2, 3, 4, and 5 days. Fibroblast cells from the skin of the domestic cat were cultured in DMEM supplemented with 0.5% FBS. Bars with different letters differ significantly ($P<0.05$).
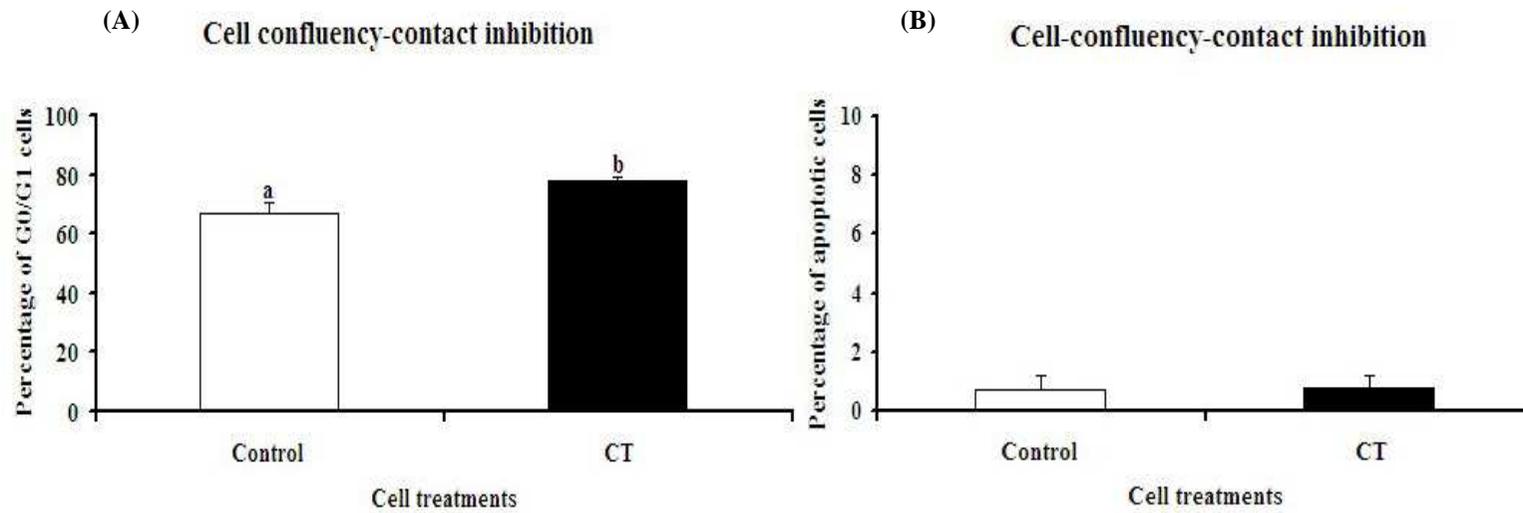
Figure 6. Mean ± SEM percentages of cells arrested at the G0/G1 phase of the cell cycle (A) and apoptotic cells (B) after the treatment of cell confluency-contact inhibition. Fibroblast cells from the skin of the domestic cat were investigated by an additional 5 days in culture after cell confluency reached 100%. Bars with different letters differ significantly (P<0.05).
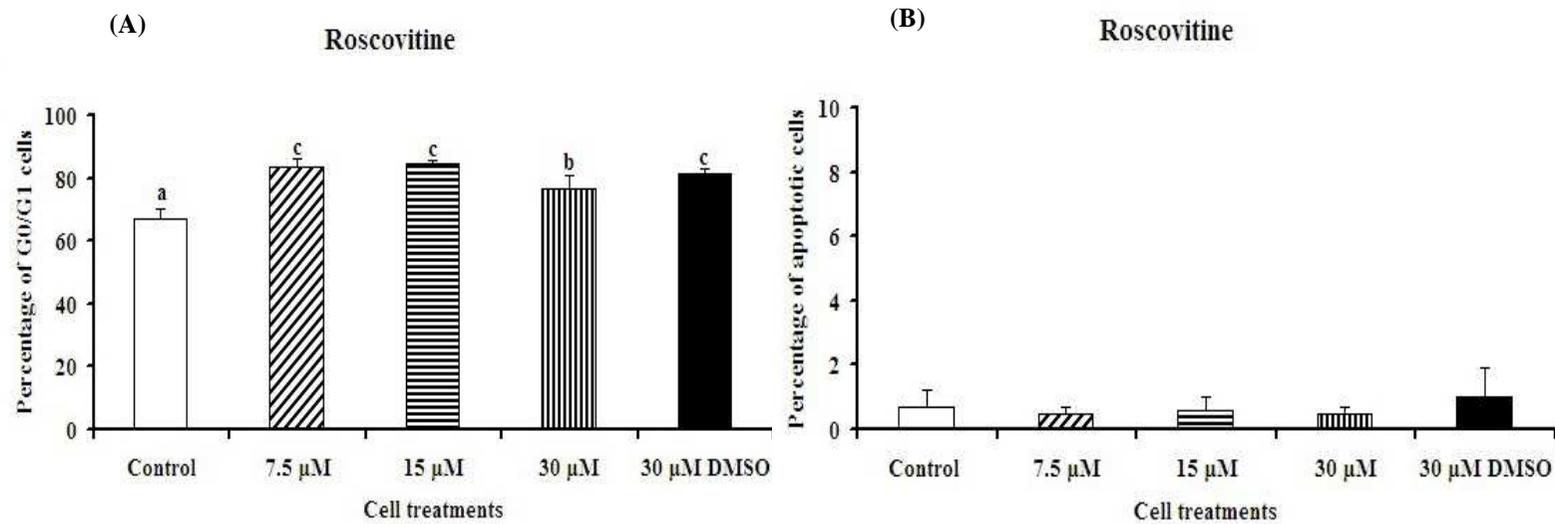
Figure 7. Mean ± SEM percentages of cells arrested at the G0/G1 phase of the cell cycle (A) and apoptotic cells (B) after the treatment of chemical inhibitor with different concentration (0 µM (control), 7.5 µM, 15 µM, and 30 µM). Cell toxicity was tested by using 30 µM DMSO. Fibroblast cells from the skin of the domestic cat were cultured in various concentrations of roscovitine for 24 h. Bars with different letters differ significantly (P<0.05).

2.5 Discussion

Dolly, the first cloned sheep was successfully produced in 1997 by transferring the adult somatic cell at the resting phase or G0 phase, which was synchronized by serum starvation for 4-5 days, into an enucleated oocyte (Wilmut et al., 1997). Since then cell-cycle synchronization has had a crucial role that affects the success of nuclear transfer. When a G0/G1 cell was transferred into metaphase (MII) oocyte, which contained of high level of maturation promoting factor (MPF) and mitogen activated protein kinases (MAPK), the breakdown of nuclear membrane (NEBD), early chromosome condensation (PCC), and the diploid nucleus still can conduct the normal DNA synthesis and cell division resulting to the normal development of reconstructed embryo. In case of the G2 or S phase-cell, it causes the damage or loss of DNA, which lead to chromosomal anomalies and precluding the subsequent embryo development. (Campbell et al., 1993; Campbell, 1999; Collas et al., 1992; Song et al., 2007; Stumpf et al., 1992; Wang et al., 2002)

In the present study, we compared the ability of the different methods (serum starvation, cell confluency-contact inhibition, and roscovitine) to synchronize the skin fibroblast cells of the domestic cat into the G0/G1 phase of the cell cycle. Flow cytometric analysis revealed that fibroblast cells from domestic cat could be synchronized at the G0/G1 phase by cell cycle inhibitor treatment. Serum starvation for more than three days provided the highest percentage of cells arrested at the G0/G1 stage rather than control cycling cells. Serum starvation is a successful method used to produce of the embryos of the Dolly (Wilmut et al., 1997). Gómez et al. (2003) also successfully generated a live cloned African wild kitten from fibroblast cells that had been synchronized by the serum starvation for five days. This method can induce the cell to be arrested at the G0/G1 stage of the cell cycle by dramatically reducing proliferating stimuli such as growth hormone, and the cell will be induced into a quiescent phase in a reversible fashion (G0 phase), when cultured for several days (Coller, 2007). However, we found that extended serum starvation periods more than three days did not increase the proportion of G0/G1 cells in this study and the apoptotic

cells increased. Our results are in agreement with Barros et al. (2010), which revealed that a lower percentage of G0/G1 cells were noticed after three days of serum starvation in domestic cat fetal fibroblasts. This method induced DNA fragmentation with some characteristics of apoptosis and necrosis and resulted in the reduction of cell survival (Khammanit et al., 2008; Kues et al., 2000; Stice et al., 2000). In cell confluency-contact inhibition for five days, a high percentage of G0/G1 cells have been obtained in porcine fetal fibroblast cells (Boquest et al., 1999). This method has been successfully used for producing cloned endangered wolves (Kim et al., 2007) as well as transgenic dairy goats (Melican et al., 2005). In the present study, the treatment of cell confluency-contact inhibition provided a significantly higher percentage of cells arrested at the G0/G1 phase without causing damage to the cells, compared to control cycling cells. Therefore, this method may be optional in felid cloning.

Alternatively, cells can be arrested at the G0/G1 phase of the cell cycle by the addition of chemical inhibitors such as roscovitine (a potent inhibitor of cyclin-dependent kinases). This method also provides a high potency for arresting bovine granulosa cells at the G0/G1 phase, resulting in an increase in cloning efficiency (Gibbons et al., 2002). Similarly, the success of G0/G1 cells induction in goldfish caudal fin-derived fibroblast cells has been obtained after treatment with the roscovitine (Choresca et al., 2009). The previous study suggested that treatment of donor cells with roscovitine resulted in a better blastocyst yield than serum starvation, and the blastocyst yields were equivalent to that of the confluent donor cells (Akshey et al., 2010). In the present study, roscovitine treatment with more than 7.5 µM significantly increased the proportion of G0/G1-phase cells in the domestic cat. Moreover, our findings support the report by Gómez et al. (2003) and Khammanit et al. (2008), who demonstrated that roscovitine treatment in fibroblast cells from African wild cats and dogs, respectively, significantly increased the percentage of cells that underwent apoptosis. In the present study, the roscovitine treatment with 30 µM did not damage the cultured domestic cat cells. Cell toxicity was tested by using 30 µM DMSO. Results indicated that domestic cat

cells were not affected by DMSO at this concentration and also gave a high percentage of G0/G1 cells.

Apart from flow cytometric analysis of the chromosomal number might be performed to ascertain the G0/G1-phase cells identification. In bovine foetal fibroblasts, a slightly higher incidence of chromosomal abnormalities was found in serum starvation compared with those in confluent and roscovitine (Cho et al., 2005). To ensure that our results are appropriate synchronization methods for domestic cat fibroblast cells, further investigation of chromosomal analysis should be performed as well as the ability of the synchronized cells to reprogramming after SCNT.

In conclusion, domestic cat fibroblast cells were successfully synchronized to G0/G1 stage using confluency-contact inhibition and roscovitine treatment, in which the apoptotic cells were in the acceptable level. These findings may be valuable for preparing cat donor cells for somatic cell nuclear transfer in this study.

CHAPTER III

EFFECTS OF DONOR CELL OR CULTURED PROCEDURE MODIFICATION
ON *IN VITRO* DEVELOPMENT OF INTERSPECIES NUCLEAR TRANSFER EMBRYO
RECONSTRUCTED FROM CAT DONOR CELL AND BOVINE CYTOPLASM

3.1 Abstract

Interspecies somatic cell nuclear transfer (iSCNT) is an invaluable tool for studying nucleus–cytoplasm interactions and may provide an alternative for cloning endangered animals whose oocytes are difficult to obtain. The developmental ability of iSCNT embryos decreases with increases in taxonomic distance between the donor and recipient species. The development of cat–bovine iSCNT embryos is reportedly blocked at the 8-cell stage. The present study was conducted to evaluate the effect of modification of donor cell by cell cycle synchronization and modification of cultured procedure modification by treatment of histone deacetylase inhibitor trichostatin A (TSA), previously used to enhance nuclear reprogramming following SCNT, on the developmental ability of cat iSCNT embryos using bovine oocytes matured *in vitro*. Experiment I was conducted to observe the development of interspecies embryos reconstructed from enucleated bovine oocytes and modified cat donor cells by cell cycle synchronization (confluency-contact inhibition or 15 µM roscovitine) in comparison with intraspecies cat and bovine NT. Results show that the fusion rate of interspecies couplets was significantly greater in the roscovitine group than in the contact inhibition group. In both of treatment groups, most embryos stopped the development at the 2- or 4-cell stage, and none of the iSCNT cat embryos developed to the morula or blastocyst stage. The significant higher percentages of total cleavage (the ≥ 2-cell stage) and development to the ≥ 8-cell stage were observed in the cat NT rather than bovine NT and iSCNT cat embryos. Experiment II was conducted to compare the effect of TSA at different concentrations on the *in vitro* development of iSCNT cat embryos. Reconstructed cat–bovine embryos were treated with 0, 25, 50, and 100 nM concentrations of TSA for 24 h following fusion. The results showed that 50 nM TSA

treatment contributed significantly higher rates of cleavage and blastocyst formation in iSCNT cat embryos (84.3% and 4.6%, respectively) compared with untreated embryos (63.8% and 0%, respectively) and embryos treated with 100 nM TSA (71.4%, and 0%, respectively). Development to the morula stage of iSCNT embryos was observed in the TSA treatment groups, whereas no embryos developed beyond the 16-cell stage in the untreated group. The parthenogenetic bovine embryos was served as control for iSCNT cat embryos development. The results demeonstrated that TSA treatment at concentration 50 nM of bovine parthenogenetic embryos also yielded the significant higher rate of cleavage and embryos developed to the blastocyst stage (94.8% and 18.6%, $P<0.05$) than those of untreated embryos (90.6% and 14.4%, $P<0.05$). In conclusion, our results indicate that the modification of donor cell by cell cycle synchronization did not overcome developmental arrest in interspecies cloned embryos. On the other hands, that modification of cultured procedure by TSA treatment for 24 h following fusion can improve the development of iSCNT embryos. Specifically, 50 nM TSA treatment provides a beneficial effect on cleavage and development to the blastocyst stage of cat iSCNT embryos using bovine cytoplasm as recipients.

3.2 Introduction

Somatic cell nuclear transfer (SCNT) is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm (Gómez et al., 2006). The SCNT provides not only a valuable tool for producing animals with the same genetic traits and also the opportunity to develop "interspecies SCNT (iSCNT)", which involves the transfer of donor cell nuclei from one species to enucleated oocytes of another species (Yin et al., 2006). In future, the iSCNT seems to be more valuable tool for many reasons including the production of embryos from species with limited availability of oocytes either their oocytes are difficult to obtain or where their collection is under restricted control (Thongphakdee et al., 2008; Yin et al., 2006).

Cell cycle synchronization plays a crucial role in that it affects the success of the development of cloned embryos (Hashem et al., 2006). Previous studies have reported that the G0/G1 stage of the cell cycle provide better results in term of the development of the cloned embryo when a metaphase II (MII) oocyte with a high level of maturation promoting factor (MPF) is used as the recipient ooplasm (Boquest et al., 1999; Campbell et al., 1996; Gómez et al., 2003; Liu et al., 2004). Furthermore, a live cloned sheep has been successfully produced from mammary cells which were synchronized into the presumptive G0 phase (quiescent phase) (Wilmut et al., 1997). These results suggested that the chromatin at the G0/G1 phase is amenable to nuclear reprogramming for the cloned embryo (Hashem et al., 2006).

The bovine ooplasm have shown the abilities for supporting the *in vitro* development of embryos to the blastocyst stage produced by iSCNT of somatic cells from various unrelated mammalian species, including the sheep, pig, monkey, dog, and yak. However, no pregnancy had reached full-term after the embryo transfer to recipients (Dominko et al., 1999; Murakami et al., 2005). According to our knowledge, there are very few reports concerning the ability of bovine oocytes to reprogram the nucleus of felid species. The iSCNT embryos reconstructed from cat somatic cell and bovine ooplasm has been created by Thongphakdee et al. (2008). However, they reported that no any iSCNT cat embryos had developed beyond the 8-cell stage. The developmental block of iSCNT cat embryos may be associated with a developmental cell block and mitochondrial incompatibility between the recipient oocytes and donor cells (Thongphakdee et al., 2008).

The transfer of somatic cells to enucleated oocytes requires a process termed nuclear reprogramming to transform the differentiated cell nuclei to dedifferentiated in the ooplasm before convert to a totipotent state and induce somatic gene expression (Wu et al., 2010). Incomplete donor nuclei reprogramming and abnormal epigenetic reprogramming (DNA methylation or histone modifications) are thought to be related to the low efficiencies in SCNT- and iSCNT cloned embryos (Arat et al., 2003; Chen et al., 2006; Lee et al., 2010). Histone acetylation provides the greatest potential for unfolding

chromatin to recruit different transcriptional factors, and thus acetylation is related to the active genes, and removal of acetylated groups by histone deacetylases (HDACs) is generally associated with gene silencing (Shi et al., 2008). Therefore, abnormal patterns of histone acetylation could seriously affect the development of cloned embryos (Shi et al., 2008). Previous studies have suggested that the *in vitro* embryo development and full-term development of cloned embryos have been improved by epigenetic modification of donor cells or early cloned embryos with trichostatin A (TSA), a HDACs inhibitor, that increases histone acetylation (Ding et al., 2008; Kishigami et al., 2006). Moreover, the histone acetylation patterns of SCNT embryos treated with TSA also appeared to be similar to those of normal embryos (Lee et al., 2010).

Therefore, it could be possible to improve the *in vitro* development of iSCNT embryos reconstructed from cat somatic cell and bovine cytoplast by the modification of donor cells by cell cycle synchronization or modification of cultured procedure by the treatment of TSA. The objectives of this study was 1) to bserve the development of interspecies embryos reconstructed from enucleated bovine oocytes and modified cat donor cells by cell cycle synchronization (confluency-contact inhibition or 15 μM roscovitine), 2) to determine the effect of TSA at different concentrations on the *in vitro* developmental competence of iSCNT cat embryos in comparison with the parthenogenetic bovine embryos.

3.3 Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise. Media were prepared weekly, filtered (0.2 μm, # 16534 Sartorius, Minisart) and kept in sterile tubes weekly. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air for overnight before use.

3.3.1 Recovery of recipient oocytes

3.3.1.1 Recovery of bovine oocytes

Bovine ovaries were obtained from a slaughter house and transported to the laboratory in 0.9% (w/v) physiological saline within 3 h after slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles (≤ 5 mm in diameter) using a 5-mL syringe fitted with an 18-gauge needle. COCs were collected in modified phosphate buffer saline (m-PBS; Nihonzenyaku, Japan) supplemented with 100 IU/mL of penicillin G potassium (Meiji, Japan) and 0.1 mg/mL of streptomycin sulfate (Meiji).

3.3.1.2 Recovery of cat oocytes

Cat ovaries were obtained from local veterinary clinics following routine ovariohysterectomy and kept in physiological saline at room temperature (RT) before oocyte recovery. Each ovary were sliced repeatedly with a scalpel blade to release COCs into a 90-mm culture dish containing m-PBS with 100 IU/mL of penicillin G potassium and 0.1 mg/mL of streptomycin sulfate.

3.3.2 Oocytes preparation

3.3.2.1 Preparation of bovine recipient cytoplasm

Bovine oocytes were matured according to procedures previously described by Mori et al. (2002). COCs were cultured in maturation medium that consisted of TCM 199 with Earle's salts (Invitrogen) supplemented with 0.05 g taurine, 0.02 IU/mL of follicle stimulating hormone (FSH; KawasakiMitaka K.K., Kawasaki, Japan), 5% FBS, 40 μg/mL of epidermal growth hormone (EGF) and 50 μg/mL of gentamicin. About 10 COCs were incubated for 22 h in the drop of maturation medium (100 μl) covered with mineral oil in a polystyrene culture dish at 38.5°C in a humidified atmosphere containing 5% CO_2 (Fig. 8A).

3.3.2.2 Preparation of domestic cat recipient cytoplasm

Cat oocytes were matured according to procedures previously described by Kaedei et al. (2010). Briefly, cat COCs were cultured in a maturation medium consisting

of TCM 199 with Earle's salts supplemented with 4 mg/mL of bovine serum albumin (BSA), 0.1 mg/mL of human menopausal gonadotropin (hMG, TeikokuZoki, Japan), 10 IU/mL of human chorionic gonadotropin (hCG, KawasakiMitaka K.K., Japan), 1 µg/mL of 17ß-oestradiol, and 50 µg/mL of gentamicin. The maturation cultures for cat oocytes were maintained at 38.0 °C for 24 h in an atmosphere of 5% CO_2 and 95 % air with high humidity (Fig. 8C).
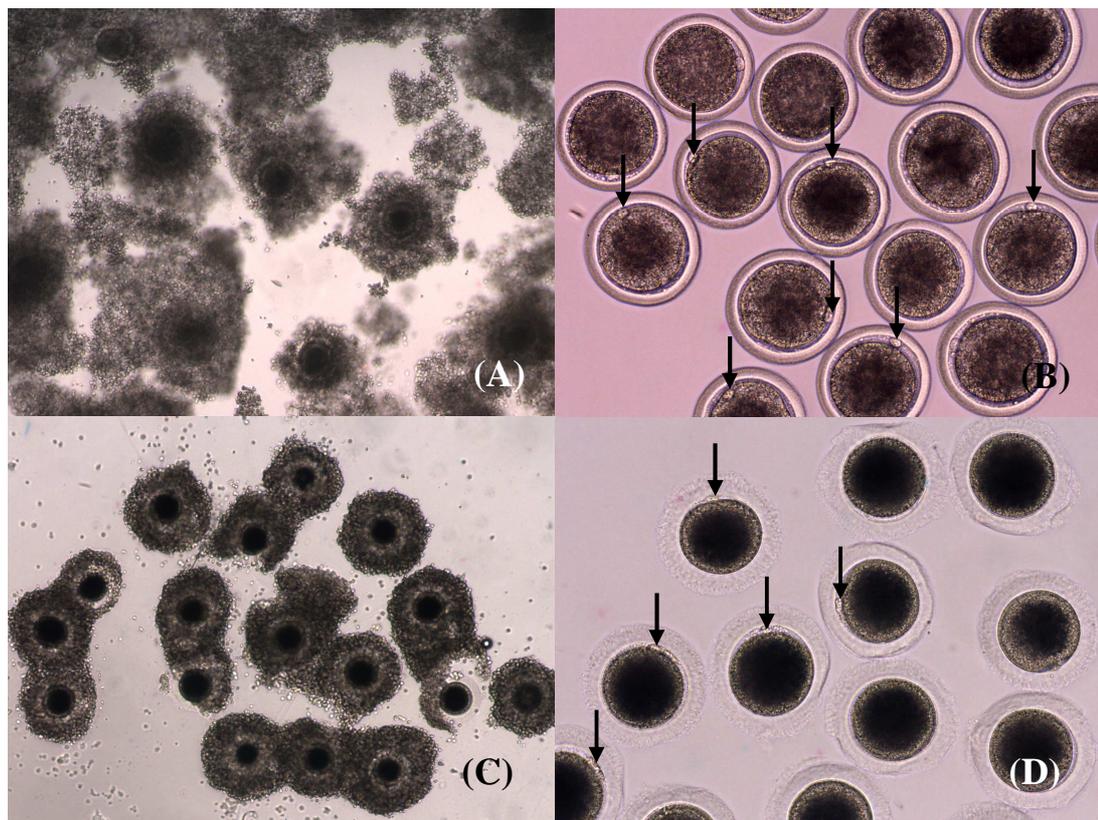
Figure 8. Bovine (A) and cat (C) oocytes with compact cumulus cells of more than 2 layers and homogenous dark ooplasm were selected and cultured for 22 h and 36 h, respectively. (X80). Mature oocytes presenting the 1st polar body (arrows) were selected for NT (B, D) (X100).

\

3.3.2.3 Enucleation of recipient oocytes

After maturation culture, bovine (experiments I, II and III) and cat COCs (experiments I) were mechanically denuded in a TCM199 medium supplemented with 0.1% (w/v) hyaluronidase. Oocytes with the first polar body extrusion were collected (Fig.

8B,D) and then incubated for 10 min in 3 µg/mL of Hoechst 33342 diluted in manipulation medium (PBS supplemented with 5% FBS and 5 µg/mL of cytochalasin B). Oocytes were then washed, transferred to a drop of manipulation medium, and enucleated after minimal exposure to low-light filtered fluorescence. Oocytes were secured with a holding pipette and rotated as needed into a position suitable for enucleation (Fig. 9A). The zona pellucida above the first polar body was cut with a glass needle (Fig. 9B), and a small volume (approximately 5%-10%) of cytoplasm underneath the first polar body was squeezed out (Fig. 9C). Complete enucleation was confirmed by staining the squeezed-out cytoplasm (Fig. 9D).
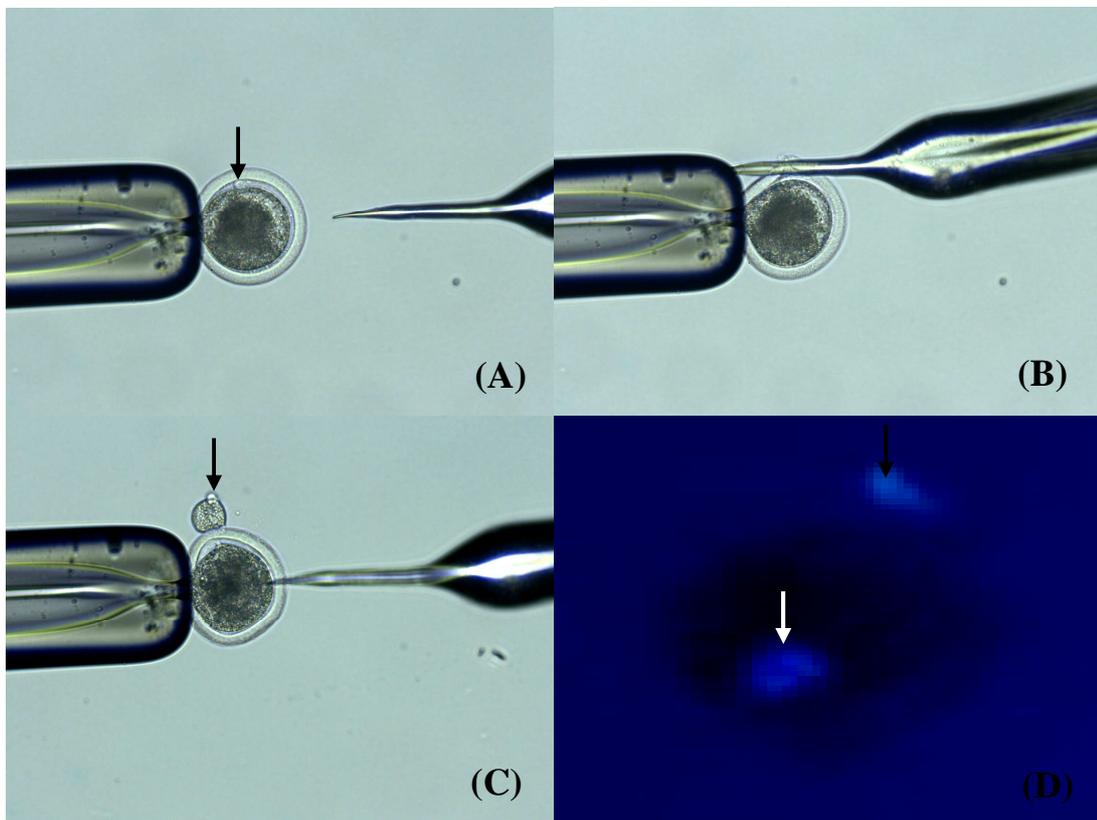
Figure 9. Enucleation of bovine oocyte; oocyte was secured with a holding pipette and rotated as needed into a position suitable for enucleation (A). The zona pellucida above the first polar body (black arrows) was cut with a glass needle (B) and a small volume of cytoplasm underneath the first polar body was squeezed out (C). The first polar body and MII chromosomes (white arrow) were confirmed by staining the squeezed-out cytoplasm (D).

3.3.3 Donor cell preparation

3.3.3.1 Preparation of domestic cat somatic cells

Domestic cat fibroblast cells were cultured in a plastic 35-mm Petri dishes (Falcon 3001) containing DMEM supplemented with 10% (v/v) FBS and 50 µg/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then synchronized with 15 µM roscovitine in culture medium for 24 h (experiment I) or with cell confluency-contact inhibition methods (experiments I, II, III). The monolayer fibroblast cells were washed twice with DMEM and then incubated in 0.25% trypsin-EDTA for 3 min at 37ºC. After trypsinisation, washing medium (DMEM supplemented with 10% FBS) was added to neutralize trypsin activity. The cells were pelleted by centrifugation at 500× g for 5 min, resuspended in the washing medium, and then maintained in this medium until SCNT manipulation. Fibroblast cells in passages 2-5 of culture were used as donor cells.

3.3.3.2 Preparation of bovine somatic cells

Cumulus cells, the source of donor nuclei, were removed from bovine oocytes by vortexing, and cultured in DMEM supplemented with 10% FBS, 1% MEM non-essential amino acid solution (Invitrogen), and 50 µg/mL of gentamicin for 7 d at 37°C in a humidified atmosphere containing 5% CO_2. Once the cumulus cells reached complete confluence, a 3-day serum starved cell monolayer was washed twice with DMEM and then incubated in 0.25% trypsin-EDTA for 3 min at 37ºC. After trypsinisation, washing medium (DMEM supplemented with 10% FBS) was added to neutralize trypsin activity. The cells were pelleted by centrifugation at 500× g for 5 min, resuspended in the washing medium, and then maintained in this medium until SCNT manipulation.

3.3.4 Nuclear transfer, fusion, activation and TSA treatment

After enucleation of each individual oocyte, the cat (experiments I and II) or bovine (experiment I) donor cell was then placed into the perivitelline space adjacent to the plasma membrane of the oocyte (Fig. 10A, B). Couplets were equilibrated for 3 min in Zimmerman cell fusion medium (Wolfe and Kraemer, 1992), transferred into a drop of

Zimmerman cell fusion medium, and then manually aligned between two electrode needles connected to a micromanipulator (MO-202D; Narishige, Tokyo, Japan). Couplets with bovine (experiment I and II) (Fig. 11A) and cat (experiment I) (Fig. 11B) ooplasm were fused and activated simultaneously with a single DC pulse of 2.3 kV/cm and 3.0 kV/cm for 30 μsec, respectively, using an electro cell fusion generator (LF101; Nepagene, Chiba, Japan). To determine the effect of different concentrations of TSA on *in vitro* developmental competence of iSCNT cat embryos (experiment II), the successfully fused couplets were cultured for 5 h in a modified synthetic oviduct fluid (mSOF) medium (Kwun et al., 2003) supplemented with 10 μg/mL of cycloheximide and TSA (Wako, Tokyo, Japan) with various concentrations [0 (experiments I, II) 25, 50 or 100 (experiment II) nM].
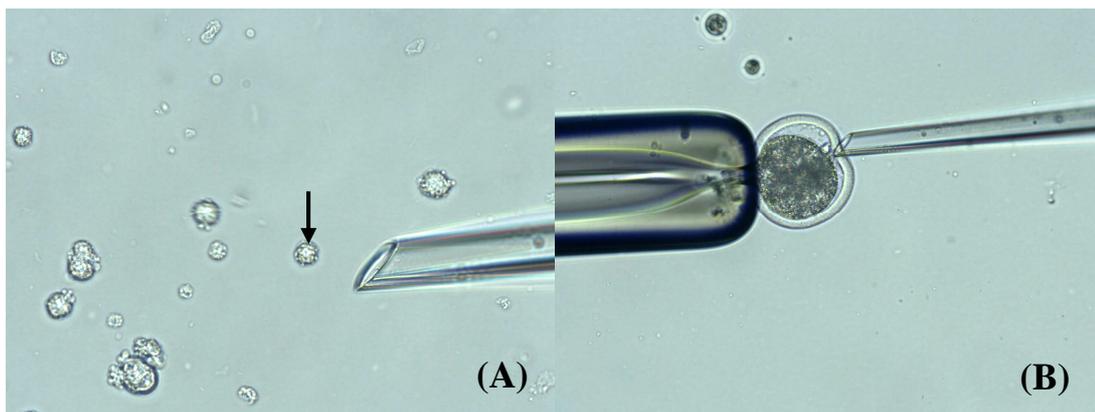
Figure 10. Nuclear transfer method; a single round-shape donor cell (arrows) was selected (A) and transferred into the perivitelline space of an enucleated oocyte (B) (X200).

3.3.5 *In vitro* embryo culture

The fused couplets were then transferred to mSOF medium with TSA at the same concentration and cultured for an additional 19 h. After 24 h of TSA treatment, embryos were washed twice with mSOF, and cultured in drops of 100 μL mSOF medium supplemented with 4 mg/mL BSA for 2 days at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2. Only cleaved embryos were further co-cultured with bovine cumulus cells in mSOF supplemented with 5% FBS at 38.5°C in a humidified 5% CO_2 atmosphere

for an additional 5 days to evaluate their ability to develop to the blastocyst stage (Fig. 12). Embryos were stained with Hoechst 33342 for counting the total cell number according to procedures previously described by Naoi et al. (2007). Embryos with more than 32 cells and with a clear blastocoele were defined as morula and blastocyst, respectively. The numbers of cleaved embryos and blastocysts were recorded.
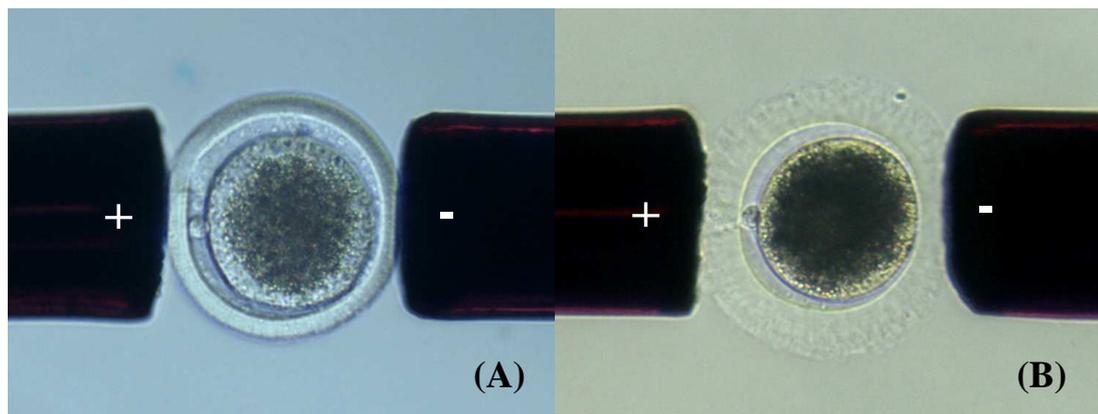
Figure 11. Fusion of NT couplets by electrical pulses; the couplet with bovine (A) and cat (B) ooplasm were manually aligned between two electrode needles connected to a micromanipulator before fused and activated simultaneously with a single DC pulse of 2.3 kV/cm and 3.0 kV/cm, respectively, for 30 μsec using an electro cell fusion generator.

3.3.6 Parthenogenesis

Parthenogenetic embryos served as embryo developmental controls. *In vitro* matured bovine oocytes were activated by a single DC pulse of 2.3 kV/cm for 30 μsec using electrode needles in a Zimmerman cell fusion medium (same methods as iSCNT embryos). Embryos were then activated in 10 μg/mL cycloheximide combined with 5 μg/mL cytochalasin B for 5 h. The activated oocytes were cultured and monitored as noted for iSCNT embryos. For TSA treatment, the parthenogenetic embryos were exposed to TSA (either 0, 25, 50, or 100 nM) for 24 h after the the onset of oocyte activation as same as iSCNT embryos.
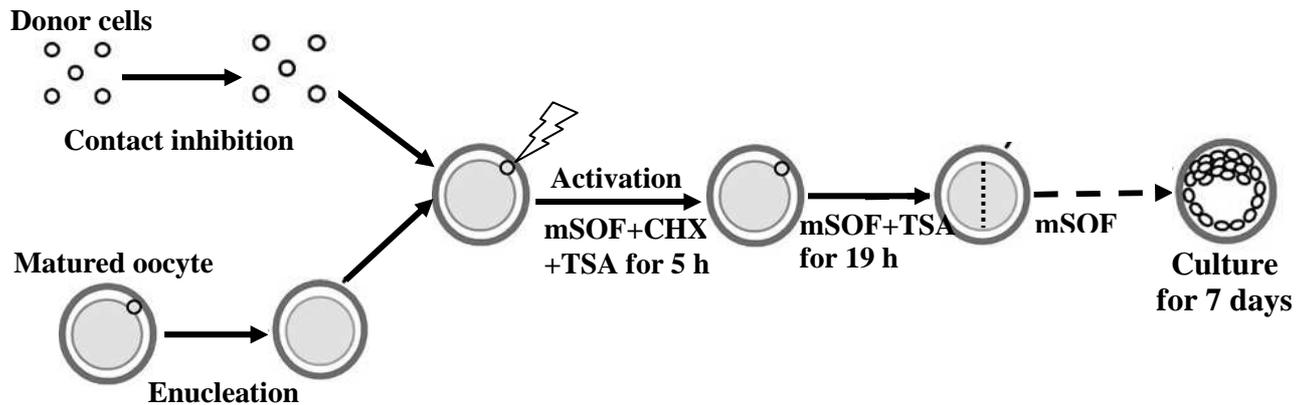
Figure 12. The scheme of experiments; to determine the effect of different concentrations of TSA on *in vitro* developmental competence of iSCNT cat embryos (experiment II), the successfully fused couplets were cultured for 5 h in mSOF medium supplemented with 10 µg/mL of cycloheximide and TSA with various concentrations (0, 25, 50 or 100 nM). The fused couplets were then transferred to mSOF medium with TSA at the same concentration and cultured for an additional 19 h. The blastocyst developmental rates were observed at d 8 of culture.

3.3.7 Statistical analysis

Data concerning the embryo developmental rates are expressed as mean ± standard error of the mean (SEM). Four to seven replications were carried out to investigate the development of cat, bovine intraspecies embryos, iSCNT cloned cat embryos and bovine parthenogenesis. The fused embryos, embryos cleaved and embryos developed to the blastocyst stage were subjected to arc sin transformation before analysis of variance (ANOVA). The transformed data were tested by ANOVA followed by Fisher's protected least significant difference (PLSD) test using the StatView program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences with a probability value *(p)* of 0.05 or less were considered statistically significant.

3.3.8 Experimental design (Table 3)

Experiment I: Effects of donor cell modification on *in vitro* **development of iSCNT cat embryos reconstructed from cat donor nuclei and bovine ooplasm in comparison with intraspeies cloned cat, bovine embryos**

The bovine oocytes were used as a recipient cytoplasm for producing the intraspecies cloned bovine and iSCNT cat embryos. The cat oocytes were used as a recipient cytoplasm for producing the intraspecies cloned cat embryos (7 replicates).

Experiment II: The effect of TSA on *in vitro* **embryo development of iSCNT cat embryos reconstructed from cat donor nuclei and bovine ooplasm**

The treatment of iSCNT cat embryos with four concentrations of TSA; 0, 25, 50 and 100 nM were performed to investigate the *in vitro* embryo development. The treatment of parthenogenetic bovine embryos with four concentrations of TSA; 0, 25, 50 and 100 nM were served as control for iSCNT cat embryos development (4 replicates).

Table 3. Procedures and conditions in each experiment

Exp.	Recipient oocyte	Donor cell	Cell treatment	Fusion Protocol	Activation protocol	Culture condition
I	Cat	Cat	CT	3.0 kV/cm for 30 μsec		D 1-3: mSOF + 0.4% BSA D 4-8: mSOF + 5.0% FBS
	Bovine	Bovine	CT	2.3 kV/cm for 30 μsec	-	D 1-3: mSOF + 0.4% BSA D 4-8: mSOF + 5.0% FBS co-cultured with bovine cumulus cells
		Cat	CT			
		Cat	Roscovitine			
II	Bovine	Cat	CT	2.3 kV/cm for 30 μsec	mSOF + 0.4% BSA + TSA (either 0, 25, 50, 100 nM) for 5 h at the onset of activation	D 1: mSOF + 0.4% BSA + TSA (either 0, 25, 50, 100 nM) for another 19 h after activation D 2-3: mSOF + 0.4% BSA D 4-8: mSOF + 5.0% FBS
	Bovine (parthenogenesis)	-	-	2.3 kV/cm for 30 μsec	mSOF + 0.4% BSA + TSA (either 0, 25, 50, 100 nM) for 5 h at the onset of activation	D 1: mSOF + 0.4% BSA + TSA (either 0, 25, 50, 100 nM) for another 19 h after activation D 2-3: mSOF + 0.4% BSA D 4-8: mSOF + 5.0% FBS

3.4 Results

3.4.1 Experiment I: Effects of donor cell modification on *in vitro* development of iSCNT cat embryos reconstructed from cat donor nuclei and bovine ooplasm in comparison with intraspeies cloned cat, bovine embryos

As shown in Table 4. The percentage of fusion of the couplets was significantly higher (P<0.05) in the roscovitine group than in contact inhibition group. Moreover, the percentage of fusion in the roscovitine group was similar to that in the homogeneous control (bovine NT) group. There were no differences in the percentages of total cleavage (the ≥ 2-cell stage) and development to the ≥ 8-cell stage among the iSCNT groups. The significant higher percentages of total cleavage (the ≥ 2-cell stage) and development to the ≥ 8-cell stage were observed in the cat NT rather than bovine NT and iSCNT cat embryos (P<0.05). There were no differences in the development to the ≥ 32-cell stage between iSCNT cat and bovine NT embryos (P>0.05). None of the iSCNT cat embryos developed to the morula or blastocyst stage. A significantly higher number of developed to the blastocyst stage was observed in the bovine NT embryos as compared to that of cat NT embryos (P<0.05) (Fig. 13).

3.4.2 Effect of TSA on *in vitro* embryo development of iSCNT cat embryos reconstructed from cat donor nuclei and bovine ooplasm

The effects of different concentrations of TSA on the *in vitro* developmental competence of iSCNT cat embryos are shown in Table 5. Treatment of early iSCNT cloned cat embryos with TSA at concentration 50 nM contributed the significant higher rate of fusion and embryo cleavage (74.1% and 84.3%, *n*=139; P<0.05) than those of untreated embryos (60.3% and 63.8%, *n*=187) and other TSA concentration treated groups (Table 1). The embryos developed to morula stage have been derived from TSA treatment at any concentration. Five from 130 fused embryos of 50 nM TSA treated group had developed to blastocyst stage (4.6%, *n*=103, P<0.05) (Fig. 13, 14); whereas no untreated embryos developed beyond the 16-cell stage. The 25 nM TSA treatment also had ability to create an iSCNT cloned cat blastocyst (0.7%, *n*=105), however, an

increasing of TSA concentration to 100 nM did not enhance the embryo development to blastocyst stage (0%, *n*=104).

The effects of different concentrations of TSA on the *in vitro* developmental competence of bovine parthenogenetic embryso are shown in Table 6. The TSA treatment at concentration 50 nM of bovine parthenogenetic embryos also yielded the significant higher rate of cleavage and embryos developed to the blastocyst stage (94.8% and 18.6%, *n*=150; P<0.05) than those of untreated embryos (90.6% and 14.4%, *n*=158; P<0.05) (Fig. 13). On the other hands, an increasing of TSA concentration to 100 nM provided the negative effects on embryo cleavage and blastocyst formation rate (81.3% and 5.8%, *n*=152) compared to those of non-treatment embryos and other TSA concentration treatment groups.

Table 4. Development *in vitro* of intraspecies cloned cat, bovine embryos and interspecies nuclear transfer embryos reconstructed from bovine ooplasm and cat somatic cells (iSCNT cat-bovine)*

Treatment	Cell treatment	No. of oocytes examined	No. (%) of fused oocytes**	No. (%) of cleaved embryos	No. (%) of embryos developed***			Total cell number****
					≥ 8-cell	≥ 32-cell	Blastocyst	
iSCNT cat	CT	165	98 (57.9 ± 7.3)^a	66 (66.8 ± 3.4)^a	14 (12.3 ± 3.9)^a	0 (0)^a	0 (0)^a	0
iSCNT cat	Roscovitine	137	105 (77.3 ± 5.1)^c	68 (66.4 ± 5.0)^a	8 (7.9 ± 2.1)^a	0 (0)^a	0 (0)^a	0
Cat NT	CT	151	102 (66.8 ± 1.5)^b	91 (92.1 ± 3.5)^c	73 (74.2 ± 3.3)^c	36 (36.5 ± 3.6)^b	20 (20.2 ± 3.3)^b	102.5 ± 6.4^a
Bovine NT	CT	150	129 (83.9 ± 2.7)^c	101 (80.0 ± 3.6)^b	78 (63.6 ± 3.8)^b	51 (39.3 ± 5.4)^b	44 (34.5 ± 3.2)^c	126.5 ± 8.0^b

*Data are expressed as the mean ± SEM. Seven replicated trials were carried out.

**Fused oocyte (%), fused oocytes/couples

***Development rate of embryos, number of embryos/number of fused oocytes.

****Total cell number, blastocysts were stained with Hoechst 33342 for counting of total cell number

[a-c] Mean values in the same columns with different superscripts are significantly different (P<0.05).

Table 5. Effects of TSA at different concentrations on the *in vitro* development of iSCNT embryos reconstructed from bovine cytoplast and cat somatic cells*

Concentrations of TSA (nM)**	No. of oocytes Examined	No. (%) of fused oocytes***	No. (%) of cleaved embryos	No. (%) of embryos developed****			Total cell number*****
				≥ 8-cell	≥ 32-cell	Blastocyst	
0	187	114 (60.3 ± 1.4)^a	73 (63.8 ± 0.8)^a	20 (17.5 ± 1.0)^a	0 (0)^a	0 (0)^a	0
25	154	105 (68.0 ± 0.9)^b	81 (77.3 ± 0.6)^b	36 (33.8 ± 2.8)^c	5 (4.7 ± 0.2)^a	1 (0.7 ± 0.7)^{ab}	53.0 ± 0
50	139	103 (74.1 ± 0.3)^c	87 (84.3 ± 4.5)^c	46 (45.4 ± 3.9)^d	12 (11.9 ± 2.9)^b	5 (4.6 ± 2.7)^b	72.8 ± 7.4
100	172	104 (60.3 ± 2.1)^a	75 (71.4 ± 2.3)^{ab}	26 (24.4 ± 2.2)^b	4 (3.2 ± 1.9)^a	0 (0)^a	0

*Data are expressed as the mean ± SEM. Four replicated trials were carried out.

**The reconstructed embryos from cat somatic cells and bovine ooplasm were treated with TSA at a specific concentration after fusion for 24 h or without any treatment (control).

*** Fused oocyte (%), fused oocytes/couples

****Development rate of embryos, number of embryos/number of fused oocytes.

[a-d] Mean values in the same columns with different superscripts are significantly different (P<0.05).

Table 6. Effects of TSA at different concentrations on the *in vitro* development of bovine parthenogenetic embryos*

Concentrations of TSA (nM)**	No. of oocytes examined	No. (%) of cleaved embryos	No. (%) of embryos developed***		
			≥ 8-cell	≥ 32-cell	Blastocyst
0	158	144 (90.6 ± 2.0)^{bc}	74 (46.7 ± 1.1)^{a}	29 (18.1 ± 0.9)^{ab}	23 (14.4 ± 0.8)^{b}
25	151	133 (88.1 ± 0.4)^{b}	70 (48.4 ± 0.3)^{a}	34 (22.4 ± 1.5)^{bc}	23 (15.3 ± 1.0)^{bc}
50	150	143 (94.8 ± 0.9)^{c}	84 (56.1 ± 1.7)^{b}	35 (23.4 ± 1.1)^{c}	28 (18.6 ± 1.0)^{c}
100	152	127 (81.3 ± 1.5)^{a}	72 (47.1 ± 0.7)^{a}	24 (14.1 ± 2.5)^{a}	11 (5.8 ± 2.2)^{a}

*Data are expressed as the mean ± SEM. Four replicated trials were carried out.

**The parthenogenesis embryos were treated with TSA at a specific concentration after fusion for 24 h or without any treatment (control).

***Development rate of embryos, number of embryos/number of fused oocytes.

[a-c] Mean values in the same columns with different superscripts are significantly different (P<0.05).
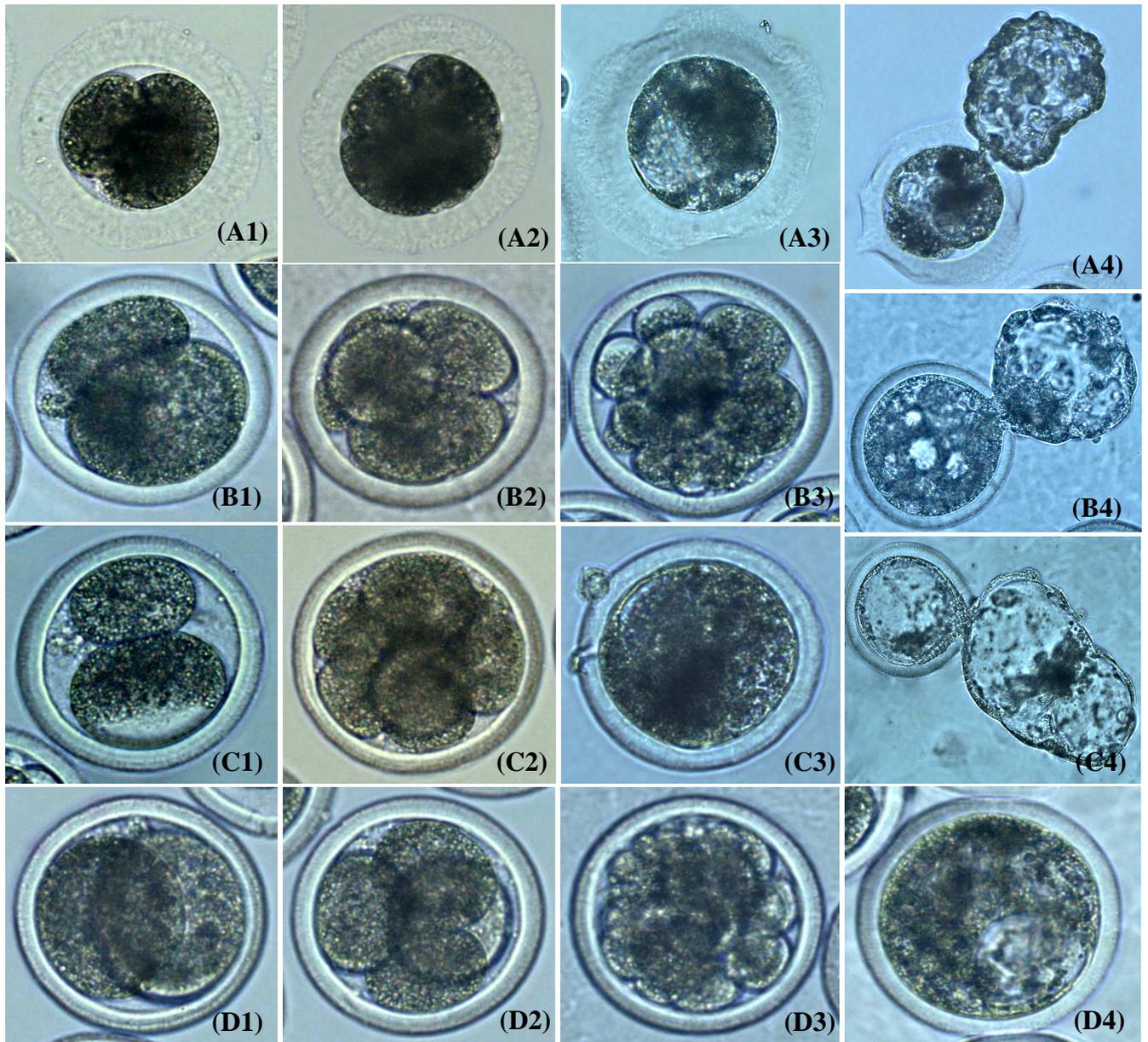
Figure 13. The development of intraspecies cloned cat, bovine embryos, iSCNT cat embryos and parthenogenetic bovine embryos; intraspecies cloned cat embryos at 2-cell (A1), 4-cell (A2), blastocyst (A3), hatching blastocyst stages (A4); intraspecies bovine cloned embryos at 2-cell (B1), 4-cell (B2), 16-cell (B3), hatching blastocyst stages (B4); 50 nM TSA treated iSCNT cat embryos at 2-cell (C1), 8-cell (C2), blastocyts (C3), hatching blastocyst stages (C4); parthenogenetic bovine embryos at 2-cell (D1), 4-cell (D2), morula (D3), and blastocyst stages (D4).
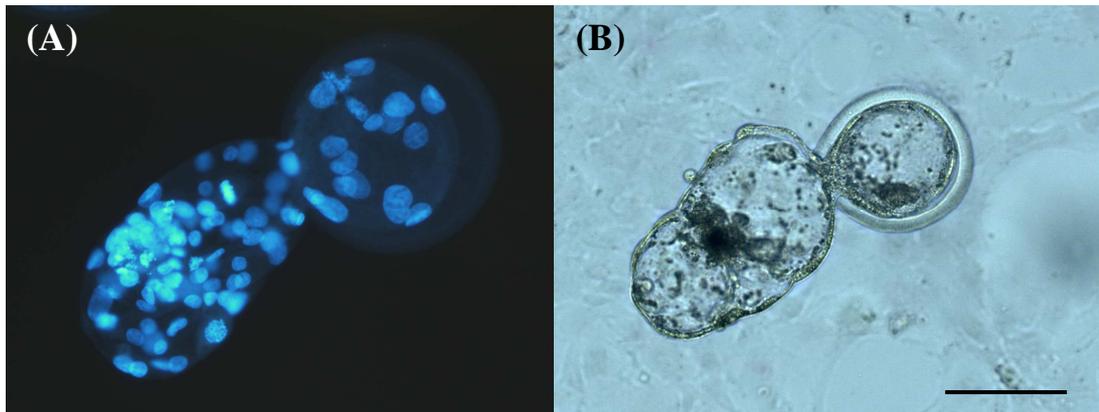
Figure 14. Development of iSCNT cloned cat to blastocyst stage after treatment with 50 nM TSA for 24 following the fusion. (A) Phase contrast and (B) Hoechst 33342 staining images of hatching iSCNT cloned cat blastocyst. (Scale bar = 100 μm)

3.5 Discussion

Effects of donor cell modification on in vitro development of iSCNT cat embryos reconstructed from cat donor cell and bovine ooplasm

Factors affecting the fusion efficiency in nuclear transfer include the cell cycle phase of donor cell, the donor cell type and the age of the recipient oocyte (Li et al., 2005). Gómez et al. (2003) reported that the method used for cell-cycle synchronization, such as roscovitine treatment, serum starvation, and contact inhibition, did not affect the fusion and cleavage rate of the couplets reconstructed from domestic cat oocytes and African wild cat donor cells. However, in this present study, the higher fusion rate of couplets reconstructed with bovine enucleated oocytes was observed in the roscovitine treatment group than that of the contact inhibition group. The mechanism of roscovitine to improving the fusion rate remains unclear. Perhaps, it might be associated with the electrosensitivity. The optimal parameters of electric pulses should be performed to achieve optimal electrofusion and remain cell viability (Trontelj et al., 2010). The Laplace equation has assumed that the voltage at the cell surface is directly proportional to the cell diameter where the smaller cells should have a greater threshold value for electropermeabilization (Neumann et al., 1989). In the present study, the slightly larger size in diameters of rocovitine treated cells were observed in comparison with contact

inhibited cells (data not shown), which might be due to the confluency of cell used. According to the Laplace equation, to reach the optimal electrofusion, a higher threshold value is required for contact inhibited cells than that of roscovitine treated cells.

The early stages of embryogenesis are normally controlled by maternally inherited products stored in the ooplasm. There are species-specific differences in the developmental period of transition from maternal to embryonic control. In the domestic cat, embryonic transcription and the timing of the transition from maternal to zygotic control of development occur by the 5- to 8-cell stage (Hoffert et al., 1997). It has been suggested that the rabbit oocytes served as a valuable recipient cytoplasm for nuclear transfer by supporting the interspecies cat-rabbit embryos develop to the blastocyst stage (Thongphakdee et al., 2006). Moreover, cat cells can also be reprogrammed in bovine and porcine cytoplasm; however, the interspecies cat embryos could not develop to the late embryonic stage (Kaedei et al., 2010). Similar to the present study, some of iSCNT cat embryos them could develop to the >8-cell stage, but none of the embryos from both treatments developed up to the morula or blastocyst stage. This indicates that the modification of donor cell by cell cycle synchronization did not overcome developmental arrest in interspecies cloned embryos. It is possible that this embryo developmental block might be related to the lack of transition from the maternal to embryonic control or mitochondrial incompatibility between the recipient oocytes and donor cells, which has been reported by (Thongphakdee et al., 2008) that the genotype of mitochondrial DNA (mtDNA) in the interspecies cloned cat-bovine embryos has shown the heteroplasmy with a reduction in copy number of mtDNA resulting to the embryo developmental block at the 8-cell stage.

In the next experiments, however, the cell confluency-contact inhibition method was used for donor cell preparation for nuclear transfer due to the ease and economical in preparation.

Effect of TSA on in vitro embryo development of iSCNT cat embryos reconstructed from cat donor nuclei and bovine ooplasm

The iSCNT technique gives great promise for conservation of wild or endangered animal species. However, the incomplete nuclear reprogramming, the low blastocyst rate and the abnormal epigenetic reprogramming are still the major problems (Shi et al., 2008; Wu et al., 2010). Since the epigenetic modification of early intra-species SCNT cloned embryos with histone deacetylase (HDAC) inhibitor TSA improved their *in vitro* embryo development, e.g. pigs (Li et al., 2008; Zhang et al., 2007), mice (Kishigami et al., 2006), and cattle (Sawai et al., 2012), several studies on iSCNT, e.g. the guar-bovine (Srirattana et al., 2012), the black-footed cat-domestic cat (Gómez et al., 2011), the human-rabbit (Shi et al., 2008) and the whale-bovine (Bhuiyan et al., 2010) have speculated to improve the success rate by using TSA. As well as our present study, to determine the effects of the epigenetic modifier on the *in vitro* developmental competence of the iSCNT cloned cat embryos, the embryos were exposed to TSA at a specific concentration (0, 25, 50 or 100 nM) after fusion of the enucleated bovine oocytes and cat donor cells for a total 24 h. Results revealed that the treatment of 50 nM TSA contributed the significant higher rates of fusion, cleavage, the development to eight-cell or morula stages, and also blastocyst formation compared to non-TSA treated group. Although, we founded that TSA has an efficiency to improve the development *in vitro* of iSCNT cat embryos, other studies on iSCNT did not observe the positive effects of TSA on embryo development. Srirattana et al. (2012) has reported that treatment with 50 nM TSA for 10 h after fusion had no effects on the rates of fusion, cleavage, or the *in vitro* development of iSCNT cloned gaur-bovine embryos. Moreover, one iSCNT guar calf was born from a recipient receiving blastocysts from the non-TSA treated group. Similar to the study by Shi et al. (2008), which suggested that TSA treatment at concentration 100 nM for 6 h during activation does not improve the blastocyst developmental rate of iSCNT cloned human-rabbit embryos.

Bovine oocytes have the ability to support development of sei whale nuclei up to the compacted morula stage in case of non-TSA treatment, but the embryos could not

develop beyond 8-cell stage if treated with 50 nM TSA for 14 h starting from post-activation treatment (Bhuiyan et al., 2010). The difference of results between our- and other studies is likely to be associated with the TSA application (concentration, timing and the onset of treatment), species-specific effect as well as phylogenetically distant between oocyte and somatic cell donor. The selection of optimized TSA application in different species might be an important key to improve the success rate in animal cloning (Wang et al., 2011) , especially for the iSCNT, which the genetic distance between donor cell and recipient cytoplast is involved. Nevertheless, an increasing the TSA concentration to 100 nM in the present study exhibited the negative effects on the development of iSCNT cloned cat embryos. This is possibly due to a long-time exposure of high TSA concentration used. Treatment of TSA has been reported to significantly reduce the success rates of animal cloning when the concentration is too high or exposure is too long and the developmental defects after implantation might also be caused by an overdose of TSA (Zhao et al., 2009).

In conclusion, treatment of 50 nM TSA for 24 h after fusion of the couplets between bovine recipient cytoplasts and cat donor cells improves the embryos developmental competence to the blastocyst stage. To determine the effect of TSA treatment whether it could modify the acetylation levels on lysine residues in the early development of iSCNT cloned cat embryos to be similar to normal IVF embryos is needed.

CHAPTER IV

DIFFERENTIAL ACETYLATION AND DI-METHYLATION OF HISTONE H3 LYSINE
DURING EARRLY DEVELOPMENT OF INTERSPECIES CLONED CAT EMBRYOS

4.1 Abstract

The present study was aimed to determine the differential acetylation on histone H3 lysine 9 (K9), 18 (K18), 23 (K23) and di-methylation on histone H3 lysine 9 (K9) in the cat donor cells and iSCNT cat embryos at the early stage between with and without 50 nM TSA treatment compared to bovine IVF embryos. Experiment I was conducted to assess the acetylation levels on histone H3K9, H3K18, and H3K23 in 50 nM TSA-treated and non-treated cat donor cell. The results show that the acetylation levels on H3K9, H3K18 and H3K23 of TSA-treated cat cells were significant higher than those of non-TSA treated cells ($P<0.05$). Experiment II and III were conducted to determine the acetylation patterns on histone H3K9, H3K18, H3K23 (experiment II) and di-methylation on H3K9 (experiment III) of early-stage iSCNT cat embryos treated with 50 nM TSA and un-treated embryos (control) in comparison with bovine IVF embryos. The results revealed that the acetylation levels of AcH3K9, AcH3K18 and AcH3K23 in TSA-treated embryos and bovine IVF embryos were higher than that of control embryos at all examined stages (2 h PF, PN, 2-cell, 4-cell and 8-cell). Exceptionally, in 6 h PN stage, the levels of AcH3K9 and AcH3K23 in embryos treated with 50 nM of TSA and bovine IVF embryos were significant lower than that of control embryos. At PN stage, the significantly higher intensity levels of Me2H3K9 were found in embryos treated with TSA and bovine IVF than that of control embryos. This suggest that the treatment of 50 nM TSA for 24 h after fusion in iSCNT cat embryos contribute the beneficial effects on the modification of acetylation levels of lysine residues (K9, K18 and K23) on histone H3 and di-methylation levels on histone H3K9 during the early embryogenesis.

4.2 Introduction

Interspecies somatic cell nuclear transfer (iSCNT) has been continuously developed for the purpose of endangered animal conservation as well as for analyzing the interactions between the donor nucleus and the recipient cytoplasm (Yamochi et al., 2012). However, the incomplete nuclear reprogramming, the low blastocyst rate, and the abnormal epigenetic reprogramming are still the major problems for this technique (Shi et al., 2008; Wu et al., 2010). Several researches is being investigated into understanding the mechanism caused an aberrant epigenetic formation (DNA methylation or histone modifications), which can leads to de-regulation of a number of vital genes in cloned embryos (Maalouf et al., 2008). Therefore, abnormal patterns of histone acetylation could seriously affect the development of cloned embryos (Shi et al., 2008). To alter the patterns of histone acetylation in cloned embryos to be similar to those of normal embryos, the treatment of early stage embryos with Trichostatin A (TSA), histone deacetylase (HDAC) inhibitor, has been suggested (Lee et al., 2010).

Histone acetylation and de-acetylation, the regulators of gene activity in eukaryotic cells, have important roles for cell growth, cell cycle control, development, differentiation and survival during the embryo development (Gray and Dangond, 2006). It is known that the de-acetylation of core histones by HDAC leads to transcriptional repression (Pazin and Kadonaga, 1997) by catalyzing the removal of acetyl groups for the epsilon-amino groups of distinct lysine residues in the amino-terminal tail of core histones (Finnin et al., 1999; Tribus et al., 2005). The HDAC inhibitors, TSA, trapoxin, and n-butyrate, accumulate highly acetylated histone species in cells and cause a variety of phenotypic changes (Nakajima et al., 1998). Acetylation on H3K9 plays an important role on gene transcripts activation (Sawai et al., 2012), whereas the H3K18 acetylation appears to be general mark of active transcription (Pham et al., 2007). H3K23 is located within the epitope of histone H3, where the acetylation on this residue could regulate the recognition of the chromodomain of Polycomb proteins (Robin et al., 2007). Histone lysine methylation has different effects depending on the residue that is methylated, for example, the methylation on H3K9 is generally correlated with

transcriptional repression (Robin et al., 2007). Histone lysine methyaltion is mediated by histone methyltransferase (HMTs), in which G9a (EuHMTase-2) is the major methylase responsible for di-methylation of H3K9 in euchromatic regions (Tachibana et al., 2001). It is noteworthy that the certain combinations of acetyl and methyl modifications of lysines in histone tails might have antagonistic or synergistic biological effects (Rice and Allis, 2001).

Domestic cat nuclei could be reprogrammed when transferred to bovine oocyte; however, the embryos were not able to develop beyond 8-cell stage (Thongphakdee et al., 2008). This developmental block might be caused by an aberrant of epigenetic patterns before embryonic genome activation (EGA), the most important event at the early development for embryo viability (Meirelles et al., 2004). The EGA occurs at 5-cell to 8- cell in domestic cat (Hoffert et al., 1997) and 8-cell to 16-cell stage in cow (Camous et al., 1986). The results in part II shown that the treatment of iSCNT cat-cow embryos with TSA at concentration 50 nM for 24 h after fusion could support the embryo development until the blastocyst stage, whereas none of the un-treated iSCNT cat embryos developed beyond 8-16 cell stage. Therefore, the assessment of the covalent patterns of acetylation and methylation on histone H3 might be valuable to learn about its role on early iSCNT cloned cat embryos development. In the present study, we demonstrate the covalent patterns of acetylation on histone H3 lysine 9, 18 and 23 (AcH3K9, AcH3K18 and AcH3K23) and di-methylation on H3K9 in iSCNT cat embryos treated with 50 nM TSA compared to un-treated embryos (control).

4.3 Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise. Media were prepared weekly, filtered (0.2 µm, # 16534 Sartorius, Minisart) and kept in sterile tubes weekly. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air for overnight before use.

4.3.1 The iSCNT cloned cat production

The production of iSCNT cloned cat embryos was processed according to the methods in Chapter III. The procedures are described in brief as follows;

Oocyte preparation

Bovine COCs were matured in maturation medium that consisted of TCM 199 with Earle's salts supplemented with 0.05 g taurine, 0.02 IU/mL of follicle stimulating hormone, 5% FBS, 40 µg/mL of EGF and 50 µg/mL of gentamicin. About 10 COCs were incubated for 22 h in the drop of maturation medium (100 µl) covered with mineral oil in a polystyrene culture dish at 38.5°C in a humidified atmosphere containing 5% CO_2.

Preparation of domestic cat somatic cells

Domestic cat fibroblast cells were cultured in plastic 35-mm Petri dishes containing DMEM supplemented with 10% (v/v) FBS and 50 µg/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO_2. A confluency fibroblast cells was trypsinized with 0.25% trypsin for culture in the next passage and storage in liquid nitrogen. Fibroblast cells in passages 2–5 of culture were used as donor cells.

The SCNT, activation, in vitro culture of embryos, and TSA treatment

After maturation culture, bovine COCs were denuded mechanically in a TCM199 medium supplemented with 0.1% (w/v) hyaluronidase. Oocytes with the first polar body extrusion were collected and then incubated for 10 min in 3 µg/mL of Hoechst 33342 diluted in manipulation medium supplemented with 5% FBS and 5 µg/mL of cytochalasin B. Oocytes were then washed, transferred to a drop of manipulation medium. The zona pellucida above the first polar body was cut with a glass needle. Then a small volume of cytoplasm underneath the first polar body was squeezed out. Complete enucleation was confirmed by staining the squeezed-out cytoplasm. After enucleation of each oocyte, the donor cell was then placed into the perivitelline space adjacent to the plasma membrane of the oocyte. Couplets with bovine ooplasm were fused and activated simultaneously with a single DC pulse of 2.3 kV/cm for 30 µs using an electro cell fusion generator.

Fused couplets were cultured for 5 h in mSOF medium supplemented with 10 µg/mL of cycloheximide and 50 nM of TSA and then transferred to mSOF medium with 50 nM TSA and cultured for an additional 19 h. After 24 h of TSA treatment, embryos were cultured in drops of 100 µL mSOF medium supplemented with 4 mg/mL BSA for 2 days at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2. The embryos cultured without TSA treatment were served as control. To determine the covalent patterns of acetylation on H3K9, H3K18, H3K23 and Me2H3K9, the embryos were collected at a2 h, 6 h post-fusion, pronuclear, 2-cell, 4-cell and 8-cell stages.

4.3.2 Fluorescent immunodetection of acetylation on H3K9, H3K18 and H3K23 in TSA-treated and non-treated domestic cat fibroblast cells

All following steps were carried out at room temperature (RT) and all solutions were prepared in 1% BSA/PBS, unless otherwise stated. The sterile coverglasses with TSA-treated and non-treated domestic cat cells were fixed with 1% paraformaldehyde for 5 min and then permeabilized with PBS containing 0.1% Triton-x 100 for 5 min prior to the fluorescence immunodetection. The following procedure of fluorescence immunodetection was performed in 96-well plates (MS-0096s; Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After washing the cells twice with PBS containing 0.3% polyvinyl alcohol (PVA; sigma) (PBS/PVA), cells were incubated with 10% goat serum (Nichirei, Tokyo, Japan) in PBS for 1 h to block non-specific binding. Cells were incubated with primary antibody (rabbit polyclonal acetyl-histone H3K9 antibody (AcH3K9); rabbit polyclonal acetyl-histone H3K18 antibody (AcH3K18); rabbit polyclonal acetyl-histone H3K23 antibody (AcH3K23); 5 µg/ml or rabbit polyclonal Histone H3 antibody; 5 µg/mL; Cell Signaling, Danvers, MA, USA) in a moisture chamber for 1 h. For the negative control, normal rabbit IgG (Dako, Kyoto, Japan) was used after adjusting to the working concentration as same as primary antibody. Cells were then washed twice in PBS/PVA and subsequently incubated in 4 µg/mL Alexa 594-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) for 1 h in a moisture chamber under dark condition, followed by washed twice with PBS/PVA. Cells were counterstained with 5 µg/mL 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) in distilled water for 10 min

in a moisture chamber under dark condition and washed twice with PBS/PVA before mounted with anti-fading reagent in glycerol/PBS (Invitrogen) on the glass slides and sealed. Images were visualized and obtained with a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) equipped with a Nikon DS-Ri1 digital camera (Nikon), and images in jpeg format were acquired using NIS-Element D 3.1 (Nikon) imaging software package in Dell Optilex 960 PC (Dell, Inc., Austin, TX, USA) work station.

4.3.3 Fluorescent immunodetection of acetylation on H3K9, H3K18, H3K23 and di-methylation on H3K9 in iSCNT cloned cat embryos

The iSCNT cloned cat embryos at 2 h-, 6 h post-fusion (PF), pronuclear (PN), 2-cell, 4-cell and 8-cell stages from 50 nM TSA treatment and control groups were subjected to perform the AcH3K9, AcH3K18 and AcH3K23 fluorescent immunodetection. In case of the Me2H3K9 assessment, the iSCNT cloned cat embryos at 2 h-, 6 h PF and PN stages were used. The PN stage embryos were collected after 20 h PF, the 2-cell stage embryos were collected after 24 h PF (end-point of TSA treatment), whereas the 4-cell and 8-cell stage embryos were collected at day 3 of the culture. To compare with naturally fertilized embryos, *in vitro* fertilized (IVF) bovine embryos were used. IVF was carried out according to the method described by (Taniguchi et al., 2007). The PN and 2-cell stage embryos were collected at 20 h and 24 h post-insemination (PI), respectively, and the 4- and 8-cell stage embryos were collected at 48 h PI for fluorescent immunodetection.

All following steps were carried out at room temperature (RT) and all solutions were prepared in 1%BSA/PBS, unless otherwise stated. Embryos were fixed in 3.7% paraformaldehyde overnight at 4ºC, permeabilized with 0.1% TritonX-100 (Sigma-Aldrich)/PBS for 40 min and then stored in 1% (w/v) BSA/PBS overnight at 4°C. Permeabilized embryos were incubated with 10% goat serum (Nichirei, Tokyo, Japan)/PBS for 1 h to block non-specific binding before incubated with primary antibody (5 µg/ml of rabbit polyclonal acetyl-histone H3K9, H3K18, H3K23 antibody, 5 µg/ml of rabbit polyclonal di-methyl-histone H3K9 or 5 µg/ml of rabbit polyclonal histone H3 antibody; Cell Signaling Technology Inc., Danvers, MA, USA) in a moisture chamber

overnight. Normal rabbit IgG (Dako, Kyoto, Japan) was used as the negative control. Embryos were subsequently incubated in 4 µg/ml of Alexa 594-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) for 1 h in a moisture chamber before counterstained with 5 µg/ml of DAPI (Invitrogen). Images were obtained with a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) equipped with a Nikon DS-Ri1 digital camera (Nikon). Then images in jpeg format were acquired using the NIS-Element D 3.1 (Nikon) imaging software package running on a work station (Dell Optiplex 960 PC; Dell Inc., Austin, TX, USA).
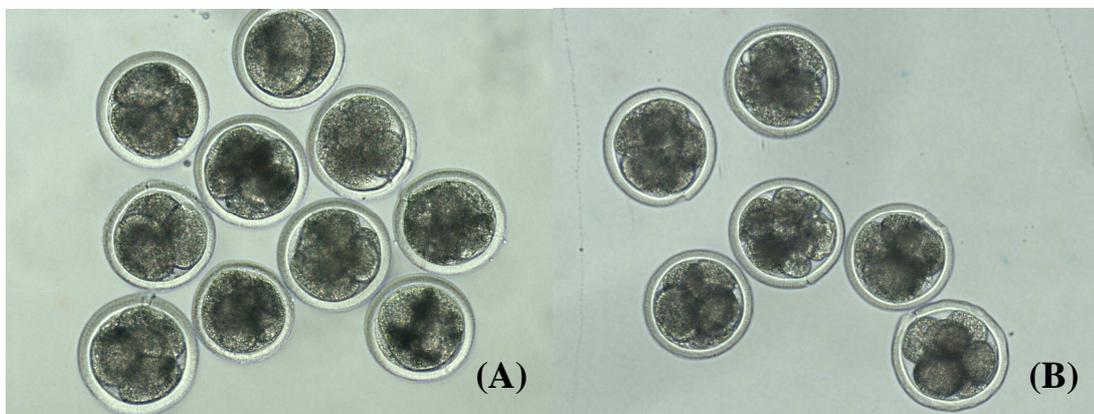
Figure 15. The 50 nM TSA-treated iSCNT cat embryos at 2-cell, 4-cell (A) and 8-cell stage (B) were collected for fluorescent immunodetection of acetylation on H3K9, H3K18 and H3K23.

4.3.4 Semi-quantification of fluorescence intensities in the embryos

The fluorescence images of each nucleus within an embryo were taken under the following conditions: Alexa Fluor 594 dye, DAPI, and in bright fields. The signal intensities of fluorescence from AcH3 histone H3 and DAPI-nucleic acid staining were measured automatically using imaging software under the area of nucleus by manually outlining a limited area of each nucleus within an embryo, except overlapping or folded nuclei. Fluorescence intensities of embryonic cytoplasm and background were quantified using the same method. The mean intensity in each examined nucleus was

recorded. The relative intensity levels of acetyl-histone H3 (AcH3) and di-methyl-histone H3 (Me2H3) in each nucleus were calculated using the following formula.

$$\text{Relative intensity in one nucleus} = \frac{\text{AcH3/Me2H3 (Mean intensity of nucleus- mean intensity of cytoplasm)}}{\text{DAPI (Mean intensity of nucleus- mean intensity of cytoplasm)}}$$

Relative intensity levels of histone H3 in each nucleus were also calculated using the same formula. Subsequently, average values of relative intensity levels of AcH3, Me2H3 and histone H3 in each embryo were calculated. These average values of each embryo were used for additional calculations to ascertain the average value of relative intensity levels of AcH3, Me2H3 and histone H3 in each treatment. The data compensation, in each treatment, during the experiments was performed using the average value of relative intensity levels of AcH3, Me2H3 and histone H3 in control samples without TSA treatment.

4.3.5 Statistical analysis

The relative intensity levels of acetylation on histone H3K9, H3K18 and H3K23 in donor cell between TSA treatment and non-treatment were compared by Student's *t*-test using the StatView program. The relative intensity levels of acetylation on histone H3K9, H3K18, H3K23 and di-methylation on H3K9 in embryos between TSA treatment, non-treatment and bovine IVF were analyzed by one-way ANOVA. Differences with a probability value *(p)* of 0.05 or less were considered statistically significant.

4.3.6 Experimental designs

Experiment I: Characterization of acetylation on H3K9, H3K18 and H3K23 in cat donor cells with or without treatment of 50 nM TSA for 24 h

To determine the effect of TSA on AcH3K9, AcH3K18 and AcH3K23 of donor cells, the domestic cat fibroblast cells were seeded on the sterile coverglasses in plastic 35-mm Petri dishes containing culture medium with or without TSA (final concentration, 0 or 50 nM) for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 prior to fluorescence immunodetection (6 replicates).

Experiment II: Characterization of acetylation on histone H3K9, H3K18 and H3K23 in iSCNT cat embryos with or without treatment of 50 nM TSA incomparison with bovine IVF embryos

To determine the pattern of acetylation on histone H3K9, H3K18 and H3K23 in iSCNT cat embryos between with and without treatment of 50 nM TSA in comparison with bovine IVF embryos, The iSCNT cloned cat embryos at 2 h-, 6 h post-fusion (PF), pronuclear (PN), 2-cell, 4-cell and 8-cell stages from 50 nM TSA treatment and control groups were subjected to perform the AcH3K9, AcH3K18 and AcH3K23 fluorescent immunodetection. The PN stage embryos were collected after 20 h PF, the 2-cell stage embryos were collected after 24 h PF (end-point of TSA treatment), whereas the 4-cell and 8-cell stage embryos were collected at day 3 of the culture. The pattern of acetylation on histone H3K9, H3K18 and H3K23 in bovine IVF embryos was served as naturally fertilized embryos (4 replicated).

Experiment III: Characterization of di-methylation on histone H3K9 in iSCNT cat embryos with or without treatment of 50 nM TSA incomparison with bovine IVF embryos

To determine the pattern of di-methylation on histone H3K9 in iSCNT cat embryos between with and without treatment of 50 nM TSA in comparison to bovine IVF embryos, the iSCNT cloned cat embryos at 2 h PF, 6 h PF and PN stages were used. The pattern of di-methylation on histone H3K9 in bovine IVF embryos was served as naturally fertilized embryos (4 replicated).

4.4 Results

4.4.1 Experiment I: Characterization of acetylation on histone H3K9, H3K18 and H3K23 in cat donor cells with or without treatment of 50 nM TSA for 24 h

The levels of acetylation on histone H3K9, H3K18 and H3K23 were evaluated after treatment with or without 50 nM TSA on cat donor cells for 24 h (Fig. 16, 17). All of cat cell nucleus showed positive immuno-reactivity in AcH3K9, AcH3K18, AcH3K23 and

histone H3 independent on the treatment (Fig. 17). There were no any differences of the acetylation levels on histone H3K9, H3K18 and H3K23 between nucleuses within the treatment. The acetylation levels on histone H3K9, H3K18 and H3K23 of TSA-treated cat cells were significant higher than those of non-TSA treated cells (P<0.05) (Fig. 16). However, the levels of histone H3 were not significant differences between TSA treated- and non-TSA treated cells as well as negative control (P>0.05). No significant differences on the background intensity were found between those experimental groups (P>0.05).
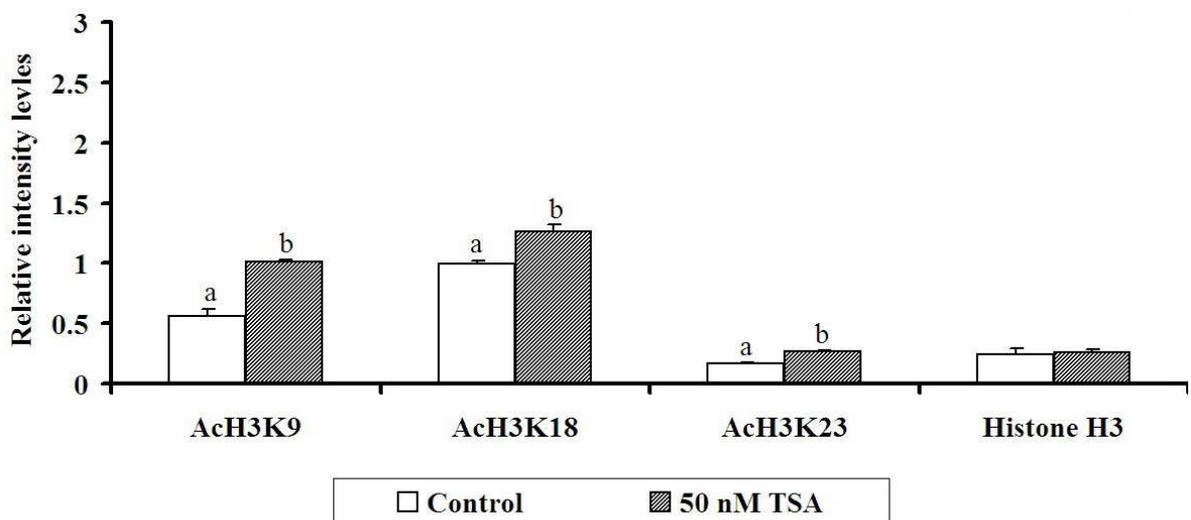
Figure 16. Relative intensity levels of acetylation on histone H3K9, H3 K18 and H3K23 of cat donor cells treated with or without 50 nM TSA for 24 h. Each bar represents the mean ± SEM. Different superscripts within same stage differ significantly (P<0.05).

4.4.2 Characterization of acetylation on H3K9, H3K18 and H3K23 in iSCNT cat embryos with or without treatment of 50 nM TSA for 24 h post-fusion in comparison with bovine IVF embryos

All nuclei of the iSCNT cloned cat embryos and bovine IVF at any examined stages showed positive immuno-reactivity in acetyl-histone H3 and di-methyl-histone H3, irrespective of the TSA treatment (Fig. 18, 19, 20, 21). The levels of histone H3 in the embryos were similar in all embryonic stages between the three groups (Fig. 22E).

There were no differences of histone H3 levels in the embryos was found among the embryonic stages, irrespective of the TSA treatment (Fig. 22E).

The levels of AcH3K9, AcH3K18 and AcH3K23 in the iSCNT cat embryos with or without (control) the treatments of 50 nM TSA and bovine IVF embryos were evaluated (Fig. 18, 19, 20, 21, 22). The fluorescence intensities in AcH3K9 of control embryos were significant lower than those of embryos treated with 50 nM of TSA at all stages of examined embryos ($P<0.05$) (Fig. 18, 22A). Exceptionally, in 6 h post-fusion stage, the levels of AcH3K9 in embryos treated with 50 nM of TSA (0.99 ± 0.05) were significant lower than those of control embryos (2.82 ± 0.17). Nuclear intensities of AcH3K9 in TSA-treated embryos at the PN, 4-cell and 8-cell stages were similar to those in bovine IVF embryos at the same stage, whereas the intensity of TSA-treated embryos at 2 h and 6 h PF were significantly lower ($P<0.05$) than that of 2 h and 6 h PI IVF embryos, respectively.

In embryos treated with 50 nM of TSA and bovine IVF, the signal intensities of AcH3K18 were significantly stronger than control embryos at all examined stages ($P<0.05$) (Fig. 19, 22B). Exceptionally, in all groups, their AcH3K18 signal levels were similar at 6 PF/PI stages. In contrast to the AcH3K9 levels, the embryos treated with 50 nM of TSA and bovine IVF showed the increasing of AcH3K18 levels from 6 h PF/PI to PN stages.

At 6 h PF/PI stages, control iSCNT embryos expressed the stronger AcH3K23 levels than that of bovine IVF and iSCNT embryos treated with 50 nM TSA ($P<0.05$) (Fig. 20, 22C). On the other hands, the significantly higher signal levels of AcH3K23 were represented in the embryos treated with 50 nM of TSA and bovine IVF rather than those of control embryos at PN and 8-cell stages ($P<0.05$). There were no significant differences on AcH3K23 signal intensity between those three groups at 2 h PF/PI, 2-cell and 4-cell stages ($P>0.05$).

4.4.3 Experiment III: *Characterization of di-methylation on H3K9 in iSCNT cat embryos with or without treatment of 50 nM TSA for 24 h post-fusion in comparison with bovine IVF embryos*

The levels of Me2H3K9 in the iSCNT cat embryos with or without (control) the treatments of 50 nM TSA and bovine IVF embryos were demonstrated (Fig. 21, 22D). Embryos treated with 50 nM TSA showed the significantly higher intensity levels of Me2H3K9 than that of control embryos at 2 h PF and PN stages (P<0.05). At PN stage, the signal intensity of Me2H3K9 of embryos treated with 50 nM TSA were resembled to bovine IVF embryos. However, no significant differences of Me2H3K9 levels were observed between those three groups (P>0.05).
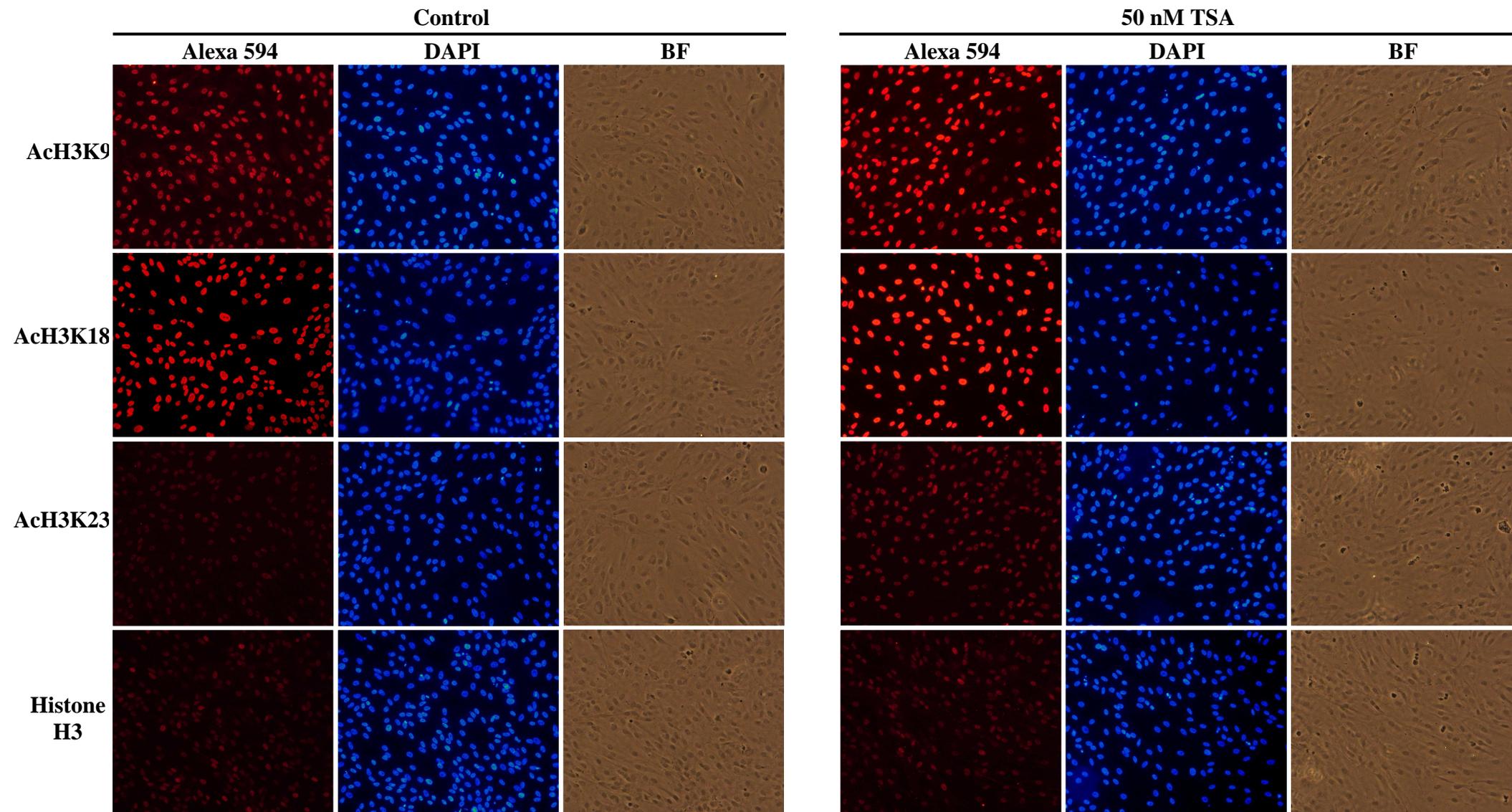
Figure 17. Immunolocalization of acetylation on histone H3K9, H3K18, H3K23 and histone H3 of cat donor cells treated without (left, control) or with (right) 50 nM TSA for 24 h. Each sample was counterstained with DAPI to visualize DNA.
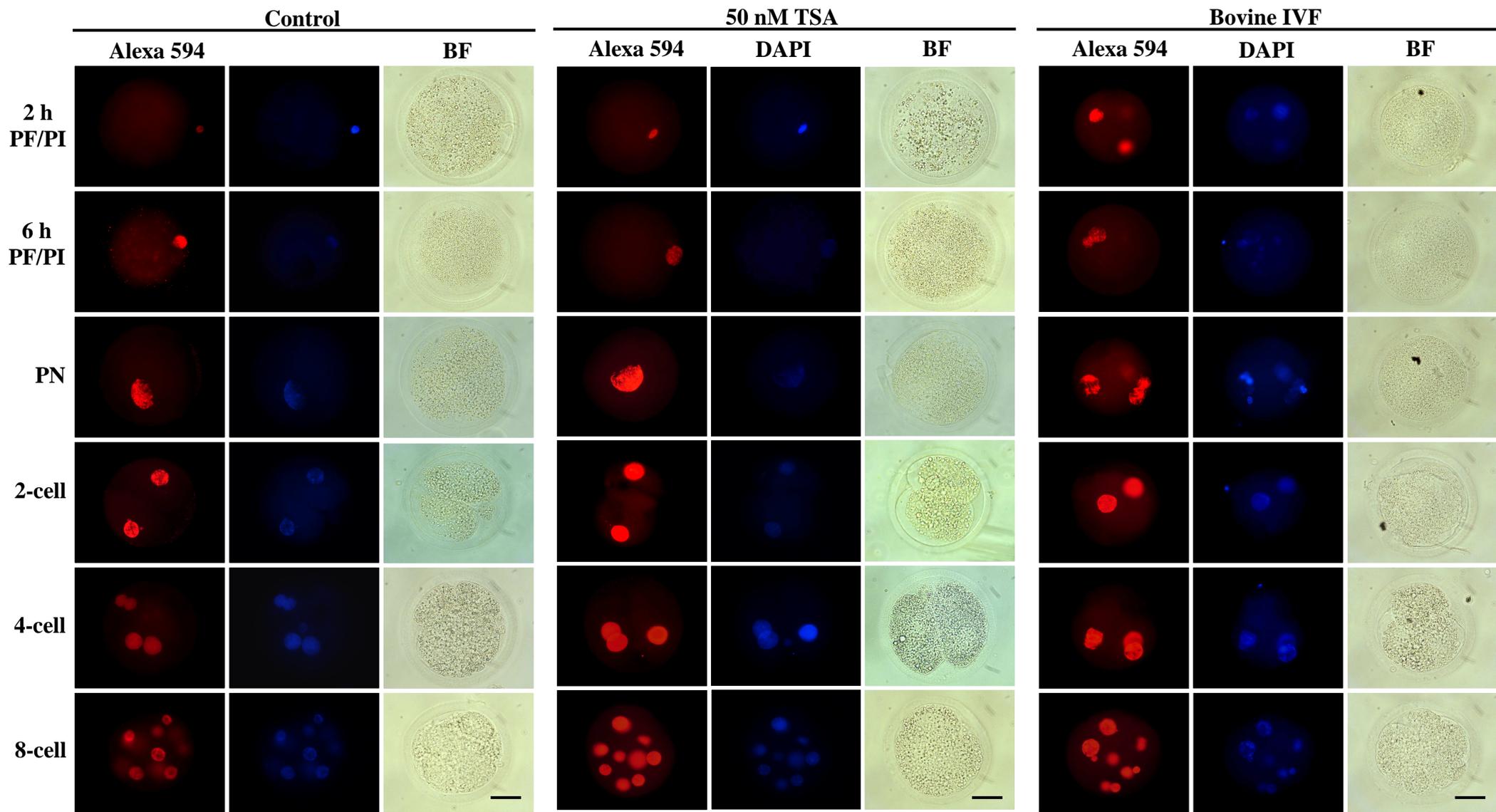
Figure 18. Immunolocalization of acetylation on histone H3K9 of iSCNT cat embryos treated without (left, control) or with (middle) 50 nM TSA in comparison with bovine IVF embryos (right) at 2 h post-fusion/post-insemination (PF/PI), 6 h PF/PI, pronuclear (PN), 2-cell, 4-cell and 8-cell stage. Each sample was counterstained with DAPI to visualize DNA. Scale bar = 50 μm.
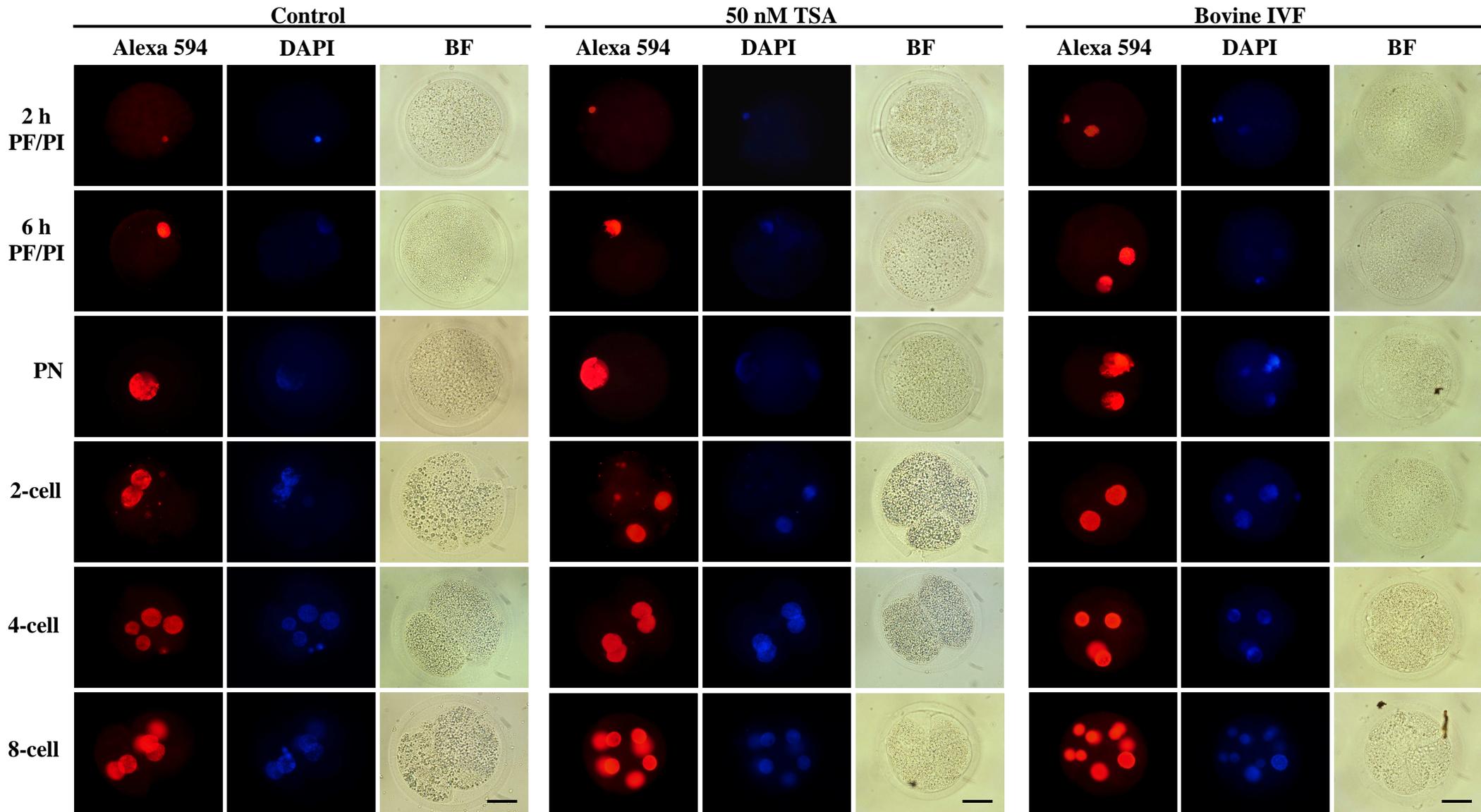
Figure 19. Immunolocalization of acetylation on histone H3K18 of iSCNT cat embryos treated without (left, control) or with (middle) 50 nM TSA in comparison with bovine IVF embryos (right) at 2 h post-fusion/post-insemination (PF/PI), 6 h PF/PI, pronuclear (PN), 2-cell, 4-cell and 8-cell stage. Each sample was counterstained with DAPI to visualize DNA. Scale bar = 50 μm.
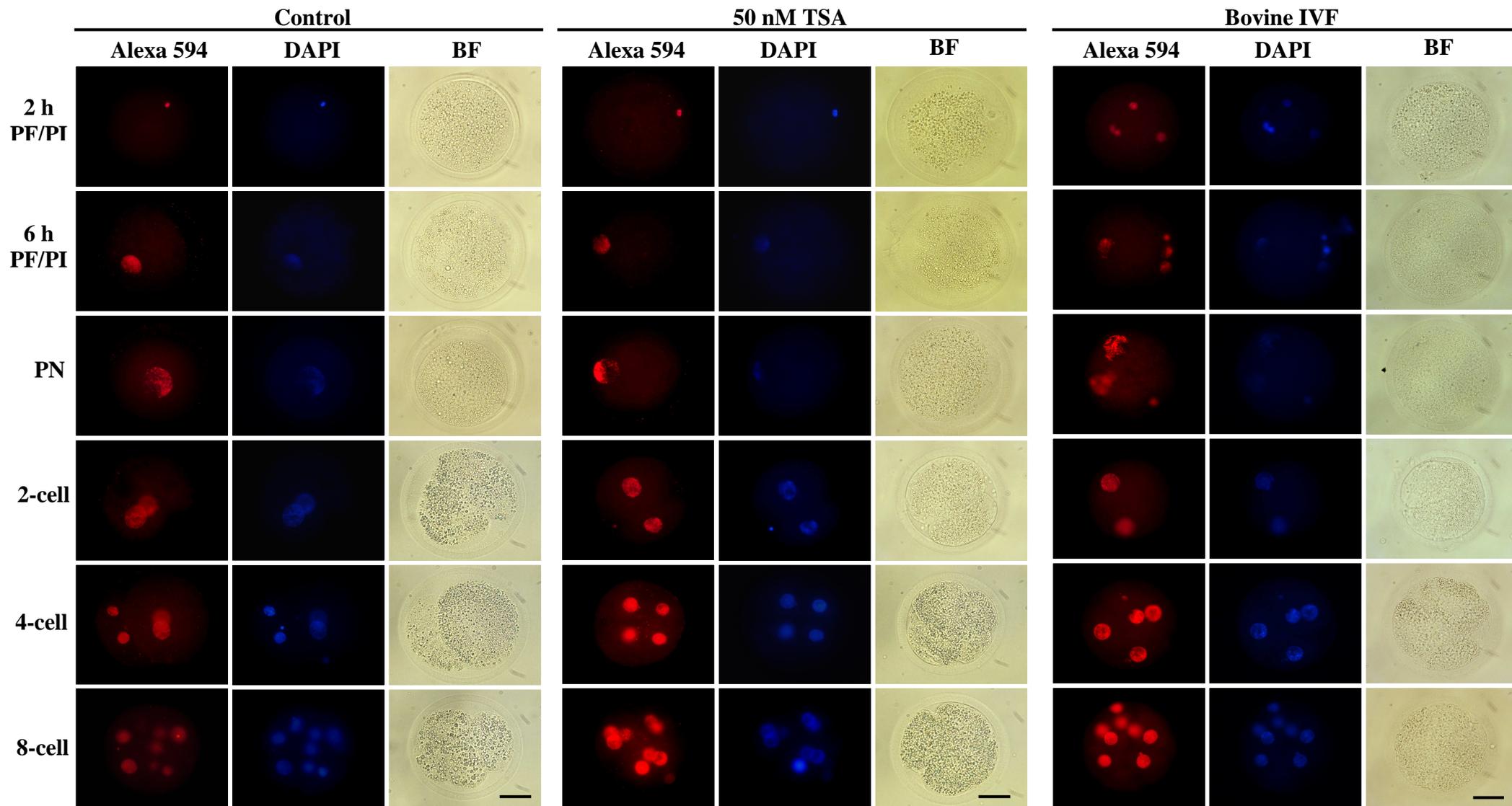
Figure 20. Immunolocalization of acetylation on histone H3K23 of iSCNT cat embryos treated without (left, control) or with (middle) 50 nM TSA in comparison with bovine IVF embryos (right) at 2 h post-fusion/post-insemination (PF/PI), 6 h PF/PI, pronuclear (PN), 2-cell, 4-cell and 8-cell stage. Each sample was counterstained with DAPI to visualize DNA. Scale bar = 50 μm.
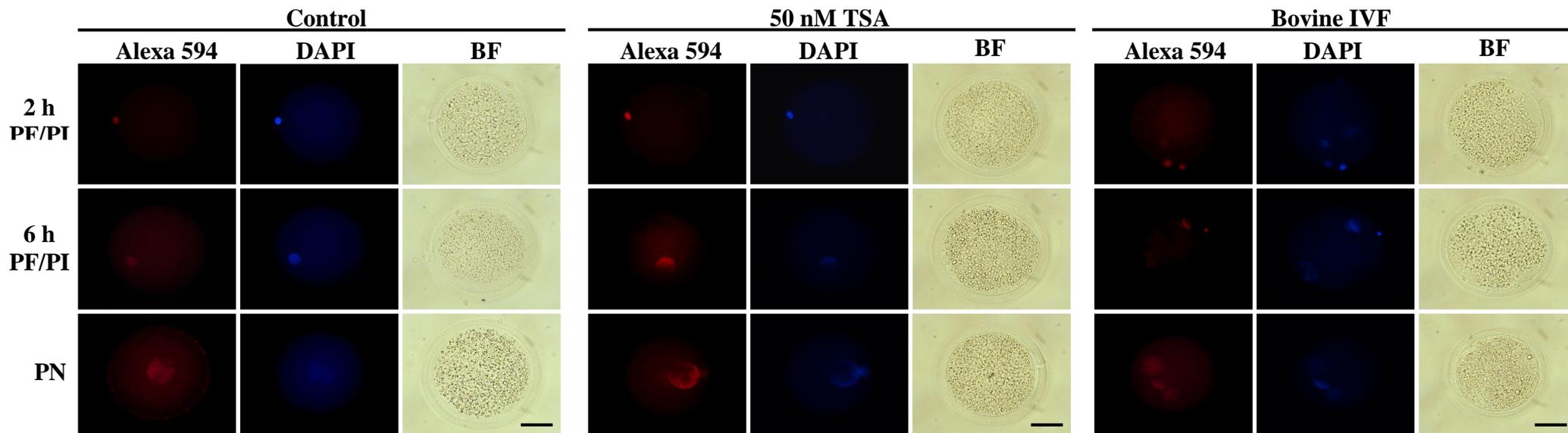
Figure 21. Immunolocalization of di-methylation on histone H3K9 of iSCNT cat embryos treated without (left, control) or with (middle) 50 nM TSA in comparison with bovine IVF embryos (right) at 2 h post-fusion/post-insemination (PF/PI), 6 h PF/PI, and pronuclear (PN) stages. Each sample was counterstained with DAPI to visualize DNA. Scale bar = 50 μm.

(A)

(B)

(C)
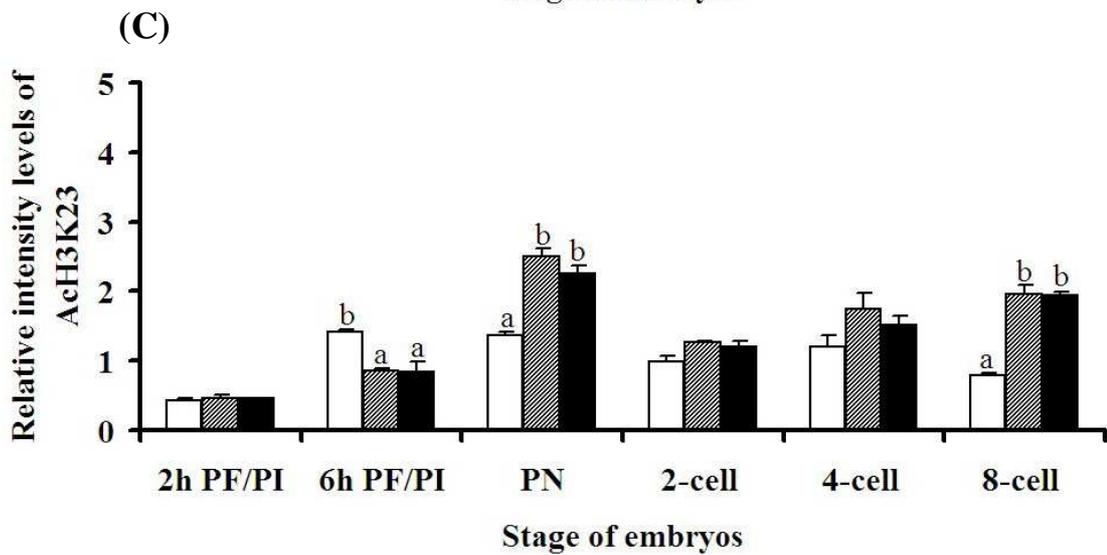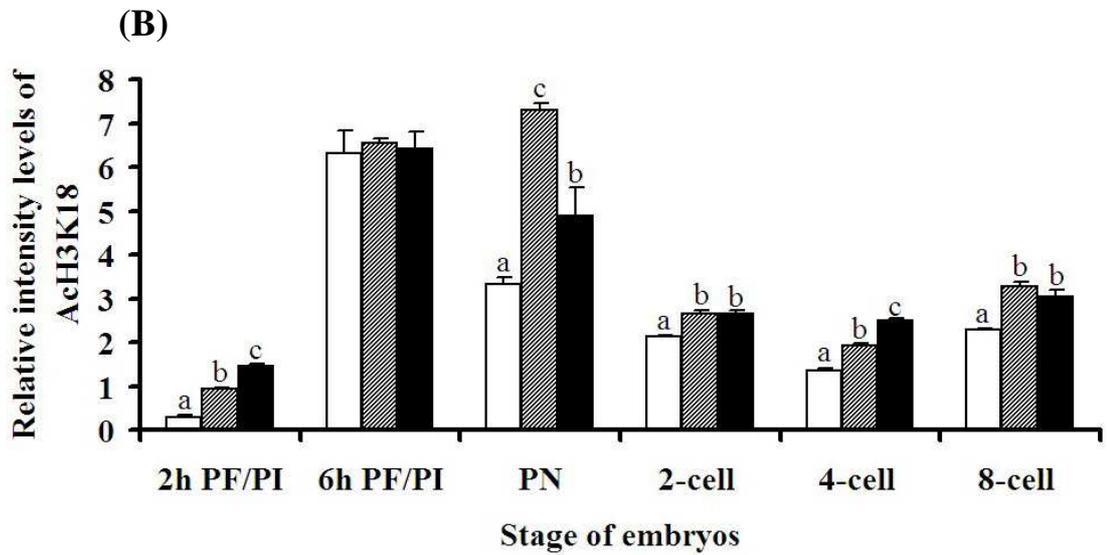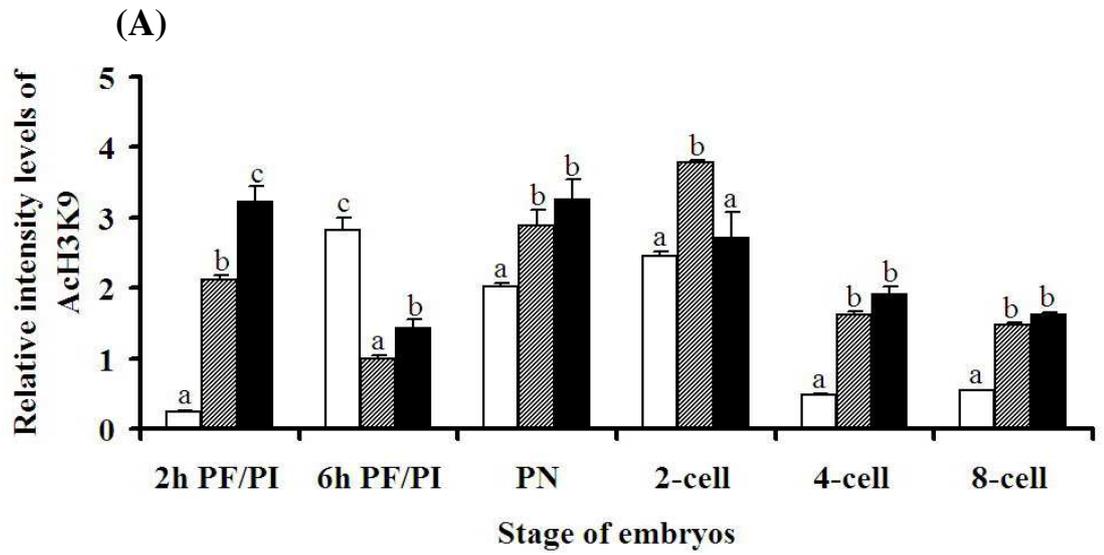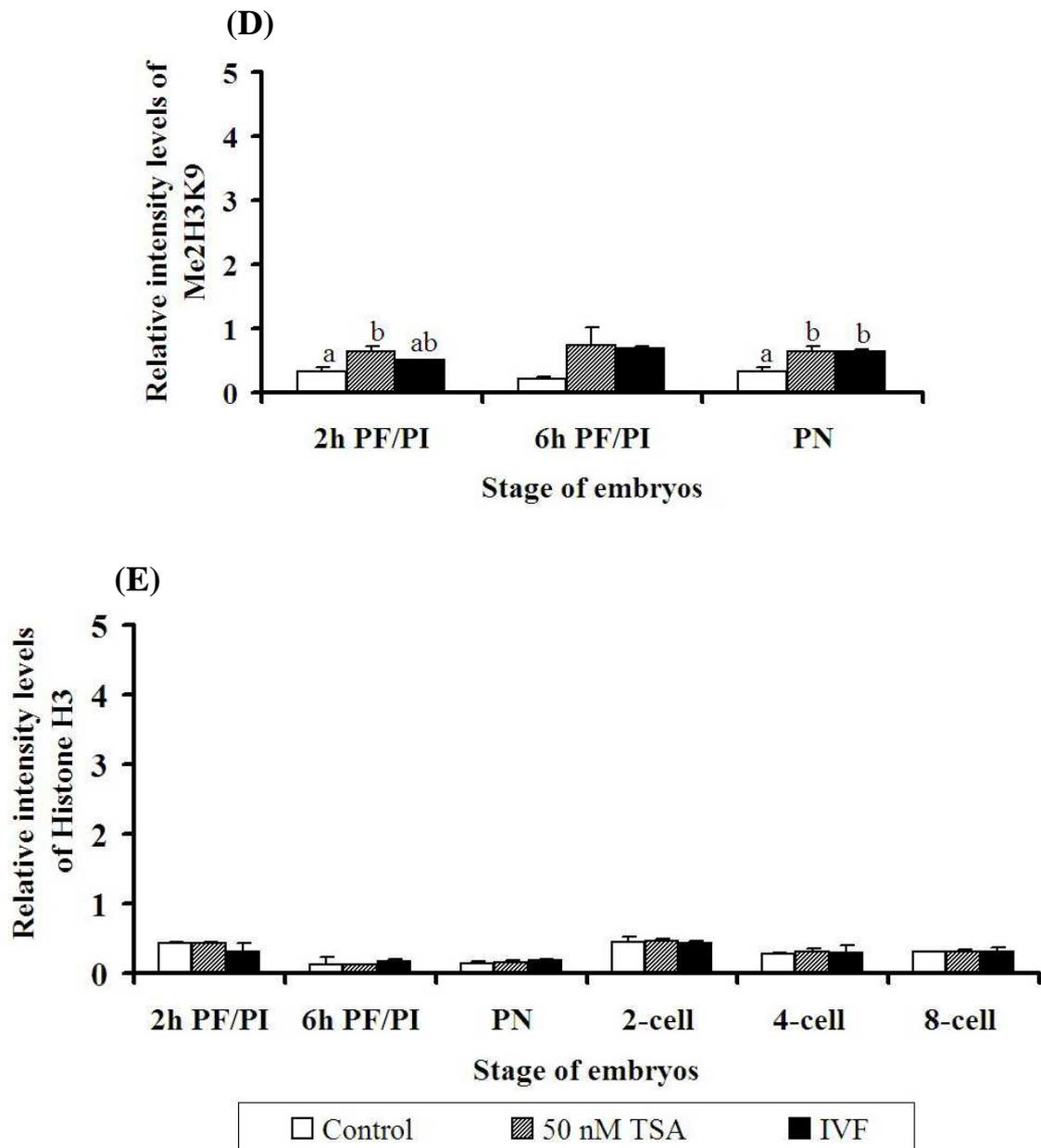
Figure 22. Relative intensity levels of acetylation on H3K9 (AcH3K9) (A), H3K18 (AcH3K18) (B), H3K23 (AcH3K23) (C) and di-methylation on H3K9 (Me2H3K9) (D) and histone H3 (E) in the 2 h PF/PI, 6 h PF/PI, PN, 2-cell, 4-cell, and 8-cell stages of iSCNT cat embryos treated without or with 50 nM TSA in comparison to *in vitro* fertilized bovine embryos. Seven to ten iSCNT cat embryos in each staining were used to estimate the levels of AcH3K9, AcH3K18, AcH3K23, Me2H3K9 and histone H3. Each bar represents the mean ± SEM. [a-c] show significant differences (P<0.05).

4.5 Discussion

Trichostatin A (TSA) has been suggested to modify the covalent patterns of histone acetylation in cloned embryos. In the previous study (chapter II), we found that the treatment of iSCNT cat-cow embryos with TSA at concentration 50 nM for 24 h after fusion could support the embryo development until the blastocyst stage, whereas none of the un-treated iSCNT cat embryos developed beyond 8-16 cell stage. Histone acetylation and de-acetylation, the regulators of gene activity in eukaryotic cells, have important roles for cell growth, cell cycle control, development, differentiation and survival during the embryo development. However, it is noteworthy that the certain combinations of acetyl and methyl modifications of lysines in histone tails might have antagonistic or synergistic biological effects. Therefore, the assessment of the differential patterns of acetylation and methylation on histone H3 might be valuable to learn about its role on early iSCNT cloned cat embryos development.

It has been generally known that the deacetylation of core histones by HDAC leads to transcriptional repression (Pazin and Kadonaga, 1997) by catalyzing the removal of acetyl groups for the epsilon-amino groups of distinct lysine residues in the amino-terminal tail of core histones (Finnin et al., 1999; Tribus et al., 2005). The HDAC inhibitors, TSA, trapoxin, and n-butyrate, accumulate highly acetylated histone species in cells and cause a variety of phenotypic changes (Nakajima et al., 1998). The acetylation on histone H3 initially occurs at lysine 14, 23, 18 and 9, respectively (Turner, 1991; Wu et al., 2010). Therefore, H3K9 is thought to be a corresponds to a hyperacetylation state (Wu et al., 2010), and highly acetylated core histones are associated with transcriptionally active genes (Eriksson et al., 2012). However, it is noteworthy that more active gene transcription resulted from the abnormal high levels of histone acetylation often occur in cloned embryos than *in vitro* fertilization (IVF) embryos (Wu et al., 2010).

In the nuclear reprogramming process of cloned embryos, the somatic cell memory is erased by histone deacetylation at some specific chromatin locations to create a relative naive set of chromatin onto which the zygotic program would be built

(Rybouchkin et al., 2006). The occurred deacetylation allowed the transferred somatic genome to exhibit an acetylation levels in a similar pattern to the oocyte chromosomes, which mimic the situation of normal MII oocytes and fertilized embryos (Wang et al., 2007). This process is thought to be occurred during oocyte activation (Rybouchkin et al., 2006). To re-establish embryonic epigenetic characteristics and gene expression in NT embryos, re-acetylation of histone is needed at the pronuclear formation to 2-cell stage (Shi et al., 2008). The patterns of histone deacetylation-reacetylation were evidently differences between the intra- and inter-species SCNT embryos. It has been reported by Shi et al. (2008), which demonstrated that histone deacetylation of iSCNT cloned human-rabbit embryos did not undergo until activation, though the acetylated core histones were highly accumulated. They suggested that HDACs in the rabbit oocytes could not deacetylate the human fibroblast cell chromatin in a limited time resulted to the incomplete of nuclear reprogramming and genomes remodeling, which are the important processes to achieve full-term development of embryos (Solter, 2000). As well as iSCNT cloned black-footed cat-domestic cat embryos, the embryo-treated with 100 nM TSA for 20 h resembled the acetylation patterns on H3K9 in IVF counterpart embryos, however, the embryo developmental competence was not enhanced and genomic reprogramming of blastocysts also showed the differences of reprogramming pattern observed in cat IVF embryos (Gómez et al., 2011). Nevertheless, it should be noted that the investigation of acetylation patterns of this previous study was ended at 20 h PF; therefore it remains questionable whether an aberrant histone acetylation took place before EGA in iSCNT cloned black-footed cat embryos.

Hyperacetylation of histone H3 is considered as the active mark associating with ongoing transcription. For example, histone H3 acetylation at position K9 is related to accessible chromatin structure for transcription. In the present study, we investigated the effect of 50 nM TSA on differential patterns of AcH3K9, AcH3K18, AcH3K23 and Me2H3K9 in early stages of iSCNT cat embryos. The results show that the acetylation levels on H3K9 and H3K23 of TSA-treated embryos and bovine IVF embryos were significant higher than that of control embryos at all examined stages except for the 6 h

PF, which the AcH3K9 and AcH3K23 levels of treated embryos was de-acetylated. This transient de-acetylation is possibly to associated with a rapidly lost of the maturation-promoting factor (MPF) and the mitogen-activated protein kinase (MAPK) activities in recipient cytoplast (Maalouf et al., 2008) as well as to allow the transferred somatic genome to exhibit an acetylation levels in a similar pattern to the oocyte chromosomes, which mimic the situation of normal MII oocytes and fertilized embryos (Wang et al., 2007). Our results are in agreement with (Gómez et al., 2011), who reported that the acetylation level on H3K9 of cat IVF embryos was observed to be low at 6 h PI before sharply increased at 20 h PI. Nonetheless, the de-acetylation phenomenon at 6 h PF/PI was not found in AcH3K18 of the TSA-treated group as same as control and bovine IVF embryos.

To re-establish embryonic epigenetic characteristics and gene expression in cloned embryos, re-acetylation of histone is needed at the stage of pronuclear formation to 2-cell stage (Shi et al., 2008). It has been reported by Shi et al. (2008) which demonstrated that histone de-acetylation of iSCNT cloned human-rabbit embryos did not undergo until activation, though the acetylated core histones were highly accumulated. They suggested that HDACs in the rabbit oocytes could not deacetylate the human fibroblast cell chromatin in a limited time resulted to the incomplete of nuclear reprogramming and genomes remodeling, which are important for achieve full-term development of embryos (Solter, 2000). Our results show that the high levels of acetylation on H3K9 and H3K18 were observed from PN to 2-cell stages in TSA-treated embryos, which resemble to IVF embryos. Moreover, in the chapter III, we observed that 4.6% of 50 nM TSA treated embryos could reach to the blastocyst stages. Therefore, we believe that the cat chromatin was possibly de-acetylated by HDACs in the bovine oocytes before the re-acetylation of histone lysine residues (e.g. H3K9, H3K18) occurred during the PN to 2-cell stages of TSA-treated embryos within a limited time of nuclear reprogramming, leads to the embryo could develop to blastocyst stage.

It has been reported that de-acetylation events occurring during oocyte activation are independent from reactivation of the genes responsible for the ability of donor cells

to develop to blastocysts after SCNT (Rybouchkin et al., 2006). Moreover, we observed that the treatment of 50 nM TSA increased the rates of cleavage and blastocyst formation compared to the TSA non-treatment group. These observations indicate that the effect of TSA is most likely to be associated with the events of re-acetylation in the stages of pronuclear formation to 2-cell. In these stages, high acetylation of H3K9 is necessary to establish embryonic epigenetic characteristics and gene expression in cloned embryos (Stein et al., 1997; Worrad et al., 1995). However, the underlying mechanism of TSA to increase the acetylation level of histone lysine residue in the early stage of iSCNT cat embryos has remained unclear. Reportedly, TSA strongly induces acetylation of the genome by blocking the HDAC enzyme (Lee et al., 2010), which changes the chromatin structure, enhances DNA demethylation, and increases the transcriptional activity of the donor cell genome (D'Alessio et al., 2007). Maalouf et al. (2009) also suggested that the induction of histone acetylation by TSA improves opening of the chromatin, sustaining mobility and relocalizing of constitutive heterochromatin as well as other genomic sequences. Consequently, we suppose that TSA treatment improves nuclear remodeling of iSCNT cat embryos via modified histone acetylation, which is important for early embryo development and subsequent stages.

In the chapter III, we observed that the iSCNT embryos without TSA treatment were unable to develop beyond the 16-cell stage. The embryonic genome activation (EGA) at the early embryonic stage is the most important event for early embryo development (Meirelles et al., 2004). The developmental failure to the blastocyst stage in iSCNT *rhesus* monkey-cow embryos probably resulted from the down-regulation of EGA, in which the impaired nucleologenesis and aberrant nucleolar formation were involved (Song et al., 2009). The EGA occurs at the 5-cell to 8-cell stages in domestic cat (Hoffert et al., 1997) and the 8-cell to 16-cell stages in cow (Camous et al., 1986). Therefore, the developmental arrest at 8-cell to 16-cell stages of iSCNT cat embryos without TSA treatment might be related to insufficient reprogramming of donor nuclei and/or epigenetic status before EGA. However, the homologous intensity patterns of histone acetylation from the morula to blastocyst stages between IVF and SCNT embryos have

been reported (Wu et al., 2010). In this study, we observed that the acetylation levels on histone H3K9, H3K18 and H3K23 in TSA-treated 4-cell and 8-cell iSCNT cat embryos more closely resemble bovine IVF embryos. In contrast, intensities of AcH3K9 in non-TSA treated iSCNT cat embryos at 4-cell and 8-cell stages were apparently lower than those of IVF embryos, indicating that an aberrant histone acetylation before embryonic genomic activation induced the developmental arrest at 8-cell to 16-cell stages (Wu et al., 2010). Moreover, the treatment of TSA has been suggested to support a more accurate regulation of developmental genes at the early development (Maalouf et al., 2009). These results indicate that the normal reprogramming of epigenetic markers including histone acetylation before EGA is the key to the success of iSCNT embryonic development.

The gradually depletion of AcH3K9 levels in bovine IVF embryos from 2-cell to 8-cell stage might have resulted from the dynamic change of GCN5, an ubiquitous histone acetyltransferase (HAT) (Schiltz et al., 1999; Wu et al., 2010). The high levels of GCN5 transcripts occur in bovine germinal vesicle (GV), metaphase II (MII) oocytes and 2-cell stage embryos before markedly decrease at the 8-cell stage (McGraw et al., 2003). Likewise, our results revealed that histone H3K9 in TSA-treated embryos and bovine IVF embryos were gradually deacetylated from 2-cell to 8-cell stage. It is possible that the treatment of 50 nM TSA for 24 h after fusion in the iSCNT cat embryos was imitate to the normal embryo development, which the hyperacetylation is acquired during the PN (20 h post-fusion) to 2-cell stage (24 h post-fusion). Consequently, the end point of TSA treatment at 24 h may inhibit the hyperacetylation that might be occurred after the embryos developed beyond 2-cell leads to the consistent with the dynamic change of GCN5. On the other hands, the significant hyperacetylation of H3K18 was observed at 4-cell and 8-cell stages of swamp buffalo IVF embryos (Suteevun et al., 2006). Similarly, in this study, we found that the hyperacetylation on H3K18 occurred in 8-cell stage bovine IVF embryos and iSCNT embryos treated with TSA.

The methylation on H3K9 generally correlates with transcriptional repression (Robin et al., 2007). It has been suggested that factors present in the recipient cytoplast

are responsible for inducing de-methylation of the transferred nucleus and the process is not species-specific (Gómez et al., 2011). Surprisingly, we observed that the Me2H3K9 levels through the early embryo development (2h PF to PN stages) of control embryos apparently lower than that of TSA-treated iSCNT embryos and bovine IVF embryos. Perhaps, this phenomenon might be associated with acetylated histone on lysine 9 and 23 that the de-acetylation at 6 h PF/PI must be required. This suggest that, under TSA treatment, bovine cytoplasm was enable to alter the de-methylation in chromatin of cat donor cell in a sufficient level that the de-acetylation could be occurred at 6 h PF.

In conclusion, the treatment of 50 nM TSA for 24 h after fusion in iSCNT cat embryos provide the beneficial effects on the modification of acetylation levels of lysine residues (K9, K18 and K23) and di-methylation level of lysine 9 on histone H3 in the early embryogenesis, although the acetylation patterns may different among the residues. We suggest that the acetylation patterns of H3K9, H3K18, H3K23 and Me2H3K9 induced by TSA in the present study were sufficient to resemble the naturally fertilized embryos leads to the improvement of the *in vitro* development of iSCNT cat embryos. However, it should be noted that these beneficial effects may not be derived from different concentrations and durations of TSA treatment.

CHAPTER V

IN VIVO DEVELOPMENT OF TSA-TREATED INTERSPECIES
CAT EMBRYOS RECONSTRUCTED FROM CAT DONOR NUCLEI
AND BOVINE RECIPIENT CYTOPLASM

5.1 Abstract

To achieve the ultimate goal of the use of iSCNT, the *in vivo* developmental capacity of TSA-treated iSCNT cat embryos was investigated in this study. Five recipients were administered with 150 IU of PMSG (s/c). At 72 and 120 h later, ovulation was induced by administering 100 and 400 IU of hCG (s/c), respectively. The estrous induced-cats were mated with vasectomized male cat for 4-5 days prior to ET. Forty eight hours after second hCG administration, laparotomy was performed and the ovarian response was evaluated by the number of CH. The TSA-treated iSCNT cat embryos at the 2- to 4 cell stage were transferred into the oviducts with the fimbria. A tom cat catheter containing embryos attached with a tuberculin syringe was performed in four recipients by inserting into the oviducts until the tip reached the ampulla, whereas, a glass pipette was used in one recipients due to the narrowing of the oviducts. The total of 224 embryos at 2- to 4-cell stages was successfully transferred into five recipients. The pregnancy was assessed at day 30 and day 45 after the embryo transfer by using real-time, B-mode ultrasonography. However, none of the recipients receiving TSA-treated iSCNT cat became pregnant.

5.2 Introduction

To achieve the ultimate goal of the use of SCNT, the *in vivo* developmental capacity of cloned embryos is investigated and the number of live offspring becomes the index of SCNT efficiency. Since *in vitro* production of cloned cat embryos is obstructed by the low success rate caused by embryo developmental block at the morula stage and fragmentation, the transfer of cloned cat embryos is usually performed at an early stage (1 to 2 cell stages) (Thongphakdee, 2007). The acceptance of

pregnancy involving a number of placenta and fetuses that can produce sufficient progesterone to maintain pregnancy requires many reconstructed embryos (more than 30) to be transferred (Gómez et al., 2004). Yin et al. (2005) has successfully produced the cloned kitten by transferring 65 cloned embryos at 2-4 cell stage into the recipient. The kitten was vigorous and at birth and appeared to be completely normal, but died three days pots-parturition from starvation as it refused to suck colostrums (Yin et al., 2005).

Due to the birth of Indian desert cat (Pope et al., 1993) and an early pregnancy of interspecies panda-rabbit cloned embryos (Chen et al., 2002), it is demonstrated that the domestic cat is not only a model for development of *in vitro* assisted reproductive technologies, but it cal also serve as a successful recipient of embryos from closely related small non-domestic cats (Gómez et al., 2003). The previous report has been shown that the 1.4% of inter-generic cloned leopard cat fetus could develop in the domestic cat uterus for up to 45 d after embryo transfer; however, all of the reconstructed embryos failed to develop to term (65 days) (Yin et al., 2006). Similarly to the study of Gómez et al. (2003), the interspecies cloned African wild cat embryos were transferred to the uteri of 11 synchronized domestic cat recipients on day 6 or 7 after oocyte aspiration. Unfortunately, no pregnancies were observed by ultrasonography assessment on day 21 postovulation.

Since the epigenetic modification of early intra-species cloned embryos with histone deacetylase (HDAC) inhibitor TSA has been reported to improve their *in vitro* embryo development, e.g. pigs (Li et al., 2008; Zhang et al., 2007), mice (Kishigami et al., 2006), and cattle (Sawai et al., 2012), several studies on iSCNT, e.g. the guar-bovine (Srirattana et al., 2012), the black-footed cat-domestic cat (Gómez et al., 2011), the human-rabbit (Shi et al., 2008) and the Sei whale-bovine (Bhuiyan et al., 2010) have speculated to improve the success rate by using TSA. Although, in the present study, we could successfully produced the TSA-treated iSCNT cat blastocyst (chapter III) with modification of acetylation and di-methylation levels of lysine residues (K9, K18,K23) on histone H3 in the early embryogenesis (chapter IV). However, the ultimate goal of the *in*

vitro procedure is to transfer produced embryos with subsequent pregnancies. Therefore, full-term development capacity of TSA-treated cloned cat embryos was assessed by transferring them into a domestic cat oviduct.

5.3 Materials and methods

All chemicals were purchased from Sigma–Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise. Media were prepared weekly, filtered (0.2 µm, # 16534 Sartorius, Minisart) and kept in sterile tubes weekly. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air for overnight before use.

5.3.1 The iSCNT cloned cat production

The production of iSCNT cloned cat embryos was processed according to the methods in Chapter III and IV. The procedures are described in brief as follows;

Oocyte preparation

Bovine COCs were matured in maturation medium that consisted of TCM 199 with Earle's salts supplemented with 0.05 g taurine, 0.02 IU/mL of follicle stimulating hormone, 5% FBS, 40 µg/mL of EGF and 50 µg/mL of gentamicin. About 10 COCs were incubated for 22 h in the drop of maturation medium (100 µL) covered with mineral oil in a polystyrene culture dish at 38.5°C in a humidified atmosphere containing 5% CO_2.

Preparation of domestic cat somatic cells

Domestic cat fibroblast cells were cultured in plastic 35-mm Petri dishes containing DMEM supplemented with 10% (v/v) FBS and 50 µg/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO_2. A confluency fibroblast cells was trypsinized with 0.25% trypsin for culture in the next passage and storage in liquid nitrogen. Fibroblast cells in passages 2–5 of culture were used as donor cells.

The SCNT, activation, in vitro culture of embryos, and TSA treatment

After maturation culture, bovine COCs were denuded mechanically in a TCM199 medium supplemented with 0.1% (w/v) hyaluronidase. Oocytes with the first polar body extrusion were collected and then incubated for 10 min in 3 µg/mL of Hoechst 33342 diluted in manipulation medium supplemented with 5% FBS and 5 µg/mL of cytochalasin B. Oocytes were then washed, transferred to a drop of manipulation medium. The zona pellucida above the first polar body was cut with a glass needle. Then a small volume of cytoplasm underneath the first polar body was squeezed out. Complete enucleation was confirmed by staining the squeezed-out cytoplasm. After enucleation of each oocyte, the donor cell was then placed into the perivitelline space adjacent to the plasma membrane of the oocyte. Couplets with bovine ooplasm were fused and activated simultaneously with a single DC pulse of 2.3 kV/cm for 30 µs using an electro cell fusion generator.

In vitro embryo culture

The successfully fused couplets were cultured for 5 h in mSOF supplemented with 10 µg/mL of cycloheximide and 50 nM of TSA and then transferred to mSOF medium with 50 nM TSA and cultured for an additional 19 h. Thereafter, embryos were washed twice with mSOF, and cultured in drops of 100 µL mSOF medium supplemented with 4 mg/mL BSA at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 prior to ET.

5.3.2 Estrus induction, embryo transfer and pregnancy assessment

Animal care and use

The mixed breed domestic cats aged 1-7 years, weighing 3-6 kg were used as embryo recipients. The cats were individual-housed in environmental conditions. The domestic tom cat was housed in an individual cage under the same conditions as the females. The cats were fed once daily (Science Diet, Hill Pet Nutrition, USA). Fresh water was given ad libitum. All animal procedures were approved by the Ethical Committee for

experimental animals of the Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan.

Estrus and ovulation inductions

The estrus and ovulation inductions were performed according to Yin et al. (2006). Cats were administered with 150 IU of pregnant mare's serum gonadotropin (PMSG; Kyoritsu Seiyaku, Tokyo, Japan) subcutaneously (s/c). The response to PMSG treatment was checked by observing changes in estrus behavior, characterized by the continuous rubbing of the head and neck against convenient objects, constant vocalizing, lordosis posturing and rolling. PMSG-unresponsive cats were to be excluded. At 72 and 120 h later, ovulation was induced by administering 100 and 400 IU of human chrorionic gonadotrophin (hCG; Kyoritsu Seiyaku) (s/c), respectively. Serum estradiol concentrations were assessed for 3 times in one recipient. The estrous induced-cats were mated with vasectomized male cat for 4-5 days prior to ET.

Embryo transfer technique

The PMSG-hCG treated cats were deprived of food and water for 9-12 h before the operation. Forty eight hours after second hCG administration, the recipients were anaesthetized with a combination of 3 mg/kg xylazine (Seton®, Laboratorios Calier, S.A., Spain) and 10 mg/kg ketamine (Calypsol®, Gdeon Richther Ltd, Budapest, 68 Hungry), intramuscularly. Laparotomy was performed and the ovarian response was evaluated by the number of corpus hemorragicum (CH). The TSA-treated iSCNT cat embryos (Fig. 24) at the 2 to 4 cell stage were transferred into the oviducts with the fimbria being held using tissue forceps. A tom cat catheter (Sovereign Tomcat 8890-703013; Sherwood Medical, St, Louis, MO, USA) containing embryos attached with a tuberculin syringe was inserted into the oviducts until the tip reached the ampula. A volume of 0.2 ml air was flushed to deposit the embryos into the ampulla (Fig.25). In cases where transfer by catheter was not possible due to the narrow oviduct, a glass pipette was used. The oviduct was punctured in the ampulla region using a 26 g needle. The tip of a glass pipette was heated and pulled over. A pipette containing embryos was then inserted

through the hole made by the needle and the embryos were introduced into the ampulla. Then oviducts and ovaries were gently placed back inside the abdominal cavity. After embryo deposition, the caudal midline incision was sutured.

Pregnancy assessment

The pregnancy was assessed at day 30 and day 45 after the embryo transfer by using real-time, B-mode ultrasonography.

5.3.3 Statistical analysis

The number of CH and transferred embryos was presented as mean ± SD

5.4 Results

The response to PMSG-hCG treatment

Serum estradiol concentrations in all recipients were gradually higher from the onset to the end of PMSG-hCG treatment (Table 3). Three recipients (D8054, D5073S, D7041) were well response to the PMSG-hCG treatment, demonstrated by the 4-6 CH on the ovaries (Fig. 23); although, some follicles were not ovulated. The cat S40IFC slightly responded to PMSG-hCG, showing only 2 CH. The S38KFC showed a hyper-ovulated response to PMSG-hCG administration, having 33 CHs.

Table 7. The profile of recipients used in this study

Cat ID	Age at ET	Serum estradiol concentration*(pg)		
		1	2	3
D8054	3 years 7 months	44.6	139.0	169.0
S38KFC	1 years 3 months	88.8	191.0	233.0
D5073S	6 years 9 months	44.4	54.9	62.6
S40IFC	3 years 2 months	46.4	62.3	95.8
D7041	5 years 7 months	36.0	-	61.2

* Serum was collected at the time of PMSG injection (1), first (2) and second (3) hCG injection.

Figure 23. Evaluation of the ovarian responses of PMSG-hCG treated recipients; after laparotomy, the uterus (u) and ovaries (o) were observed in terms of ovarian response regarding the number of fresh CH (arrows)

The TSA-treated iSCNT cat embryo transfer

The glass pipette transfer was used in one recipient (D8054) due to the small oviducts. Meanwhile, the urinary catheters were easily inserted into the oviduct for transferring the embryos in another four recipients (Fig. 25). Total of 224 embryos at 2- to 4-cell stages (Fig. 24) was successfully transferred into five recipients. However, none of the recipients receiving iSCNT cat embryos became pregnant.
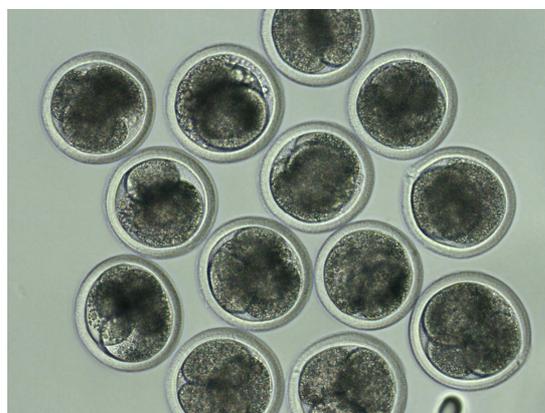
Figure 24. Transferred 2- to 4- cell stage TSA treated-iSCNT cat embryos at 48 h post-fusion
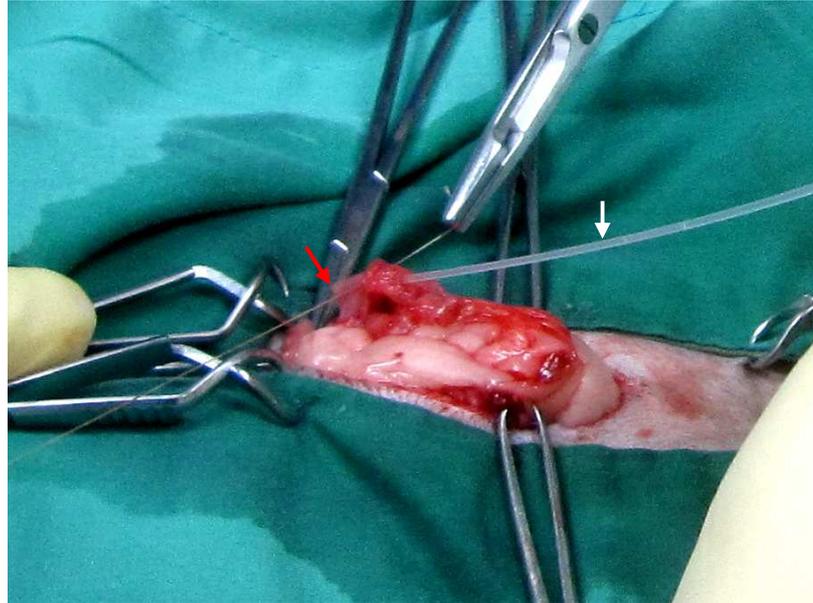
Figure 25. Embryo transfer by tom cat catheter (white arrow); embryos were loaded in a urinary catheter with approximately 3 µl culture medium (mSOF supplemented with BSA). The catheter was inserted into the oviduct via the fimbria until the tip reached the ampulla (red arrow).

Table 8. The ability to establish pregnancy in PMSG-hCG treated recipients receiving iSCNT cat embryos

Cat ID	Ovulation* R/L (n)	Follicle** R/L (n)	Transferred embryos*** R/L (n)	Pregnancy/Implantation	Note
D8054	3/3 (6)	0/1 (1)	15/15 (30)	-/-	L, R: glass pipette transfer
S38KFC	15/18 (33)	0/0 (0)	53/25 (78)	-/-	
D5073S	2/3 (5)	4/1 (5)	0/25 (25)	-/-	P4 injection weekly until pregnancy assessment****
S40IFC	0/2 (2)	2/0 (2)	0/45 (45)	-/-	P4 injection weekly until pregnancy assessment****
D7041	2/2 (4)	0/0 (0)	0/46 (46)	-/-	P4 injection weekly until pregnancy assessment****

* Number of corpus heamorragica (CH) on the right (R) and left (L) ovaries

** Number of non-ovulated follicles on the right (R) and left (L) ovaries; which were then aspirated by needle

*** Number of transferred embryos in right (R) and left (L) oviducts

**** Progesterone injection was performed in the recipients due to the few number of observed CH

5.5 Discussion

Although, the TSA-treated iSCNT cloned cat embryos developed to the blastocyst stage *in vitro* in a previous study (chapter III), we were not able to produce a pregnancy after embryo transfer. This might be due to many factors relating to both the embryo and the recipient. In chapter III, we demonstrated that the successfully production of iSCNT cat embryos were obtained by treating the reconstructed embryos with TSA at concentration 50 nM fro 24 h after fusion; however, the blastocyst developmental rate was still very low (4.6%) compared to cat NT (20.2%). The first IVF kittens in Thailand were born in year 2007 by Thongphakdee et al. (2007) with the observed *in vitro* blastocyst rate at 61.4%. It is suggested that the developmental competence and quality of embryos are one of the crucial factor affecting pregnancy establishment (Thongphakdee, 2007). Nevertheless, the normal cloned kitten have been produced using fetal donor cells with the 4% of *in vitro* developmental rate to the blastocyst stage (Yin et al., 2005).

The induction of ovulation and embryo transfer also plays the important role on the successful embryo transfer. In this study, the recipients responded to 150 IU PMSG and 100-400 IU hCG administration by presenting the corpora haemorrhagica (mean 10.0 ± 5.8). However, some follicles (mean 1.6 ± 0.9) in three recipients were not ovulated at the time of ET. It might be due to the failure of copulation between male cat and recipients. One recipient slightly responded to PMSG-hCG, whereas another one showed a hyper-ovulated response to PMSG-hCG administration. It has been reported that most of PMSG-hCG treated cats ovulated in average numbers, hyper- and non-response cats could be found (Thongphakdee, 2007). This indicated that administration of 150 IU eCG and 100-400 IU hCG was effective for the induction of estrus and ovulation for ET. The glass pipette transfer was used in one recipient due to the narrowing of oviducts. The obstacle of this method was the trauma and hemorrhagic of the oviductal tissues, which could impair the transferred embryo quality.

The intricate mechanism required to sustain pregnancy still largely unknown, particularly, for interspecies embryos. During the nuclear transfer procedure, small

numbers of donor cell mitochondria that have not undergone ubiquitination are introduced into reconstructed embryos, led to the heterogenous mitochondrial population (Lee et al., 2010; Sutovsky et al., 2004). The increasing of mitochondrial heteroplasmy, an incompatibility of the mitochondrial and nuclear genomes could negatively affect the mitochondria functioning, was found when iSCNT was performed (St John et al., 2004). The mitochondrial incompatibility between the recipient bovine oocytes and cat donor cells has been reported by Thongphakdee et al. (2008) that the genotype of mitochondrial DNA (mtDNA) in the interspecies cloned cat-bovine embryos has shown the heteroplasmy with a reduction in copy number of mtDNA resulting to the embryo developmental block at the 8-cell stage. Unfortunately, heteroplasmy of the TSA-treated iSCNT cat embryos in this present study were not evaluated. Although, the mitochondrial heteroplasmy were detected in pre-implantation iSCNT panda-rabbit embryos, only panda donor cell mitochondria were found at the early stage of pregnancy (Chen et al., 2002). It is possible that the incompatibility between the donor cell and recipient cytoplasm were associated with the developmental failures in interspecies cloned embryos (Lee et al., 2010). Furthermore, the development of cloned embryos after implantation might be suppressed by an altered mitochondrial respiration.

In addition, the pregnancy maintenance requires reciprocal signaling between the fetus, including the extraembryonic membranes, and the endometrium (Lee et al., 2010). Lymphocytic invasion of the placenta was considered as a factor caused the failure of pregnancy. This phenomenon was reported in several cross-species pregnancy, such as horse to donkey (Anderson, 1988) and *Mus caroli* to *Mus musculus* (Lee et al., 2010).

In conclusion, we demonstrated that pregnancy in recipients receiving iSCNT cat embryos could not be established in this study. Several factors are thought to be involved in this failure including the developmental competence and quality of embryos, the induction of ovulation in recipients and the embryo transfer procedure, and the physiology incompatibility between fetus and uterus. Further research is required to evaluate the mitochondrial heteroplasmy pattern of TSA-treated iSCNT cat embryos.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

Interspecies somatic cell nuclear transfer (iSCNT): potentiality, reality and prospective direction

Interspecies somatic cell nuclear transfer (iSCNT) involves transfer of donor cell nuclei from one species to the enucleated oocytes of another species. This technology was established for many reasons including production of embryos from species which oocytes are difficult to obtain or where their collection is under restricted control (Thongphakdee et al., 2008) as well as endangered species conservation. Live offspring from a few endangered mammalian species have been produced by iSCNT. Mouflon and Argali cloned embryos have been derived by transferring somatic cells into enucleated domestic sheep oocytes (Janssen et al., 2004; Lanza et al., 2000). The reconstructed gaur, banteng and yak embryos were obtained after transfer of cells into enucleated domestic cow oocytes (Li et al., 2007; Loi et al., 2001; Sansinena et al., 2005). For the endangered felids, the domestic cat oocytes were widely used as recipients to supported the development of cloned embryos such as black-footed cat and African wild cat, which could successfully establish the pregnancy and live offspring were born (Gómez et al., 2006)

As mentioned above, it seems that iSCNT can be well established for wildlife conservation. However, in fact, this technique still has many obstacles that cause the failure of iSCNT embryo production. It has been known that the ability of iSCNT embryos developed to the blastocyst stage decreases as the taxonomic distance between the donor and recipient species increases. In many areas, the conservation of endangered animal is required; but the oocytes from closely related species are difficult to obtain. Therefore, it is impossible to avoid the use of recipient oocytes from the animal with far genetic distances. This is why the "universal recipient cytoplasm" including bovine and rabbit oocytes has been used for iSCNT in several studies. Bovine oocytes support replication of transferred nuclei in various species with significantly greater evolutionary

divergence, including rats (Dominko et al., 1999), pigs (Dominko et al., 1999), monkeys (Dominko et al., 1999; Simerly et al., 2004), chickens (Kim et al., 2004), whales (Ikumi et al., 2004) and humans (Chang et al., 2003); however, no pregnancy had carried to full-term in the recipients receiving iSCNT pig and monkey embryos.

According to our knowledge, there are very few reports concerning the ability of bovine oocytes to reprogram the nucleus of felid species. Owing to the similarities between the domestic cat and wild cats, the domestic cat is widely used as a model organism for development of assisted reproductive technologies (ARTs) in all felids The iSCNT cat embryo reconstructed from cat somatic cell and bovine ooplasm has been created by Thongphakdee et al. (2008), however, no any iSCNT cloned cat embryos had developed beyond the 8-cell stage. Similarly, our present study (chapter III) demonstrated that the developmental block of iSCNT cat embryos occurred at 8- to 16-cell stages. We suggested that this developmental failure is highly related to the insufficiency reprogramming of the donor cell nuclei after transplantation into enucleated oocytes from different species.

To improving the efficiency of iSCNT cat embryo production, the modification of donor cells prior to use and modification of culture procedures following embryo reconstruction were considered in this present study.

Cell cycle synchronization plays a crucial role on the success of the cloned embryo development. The G0/G1 stage of the cell cycle is considered as a suitable stage contributing the high development rate of reconstructed embryo when MII oocyte is used as the recipient cytoplasm (Boquest et al., 1999; Campbell et al., 1996; Gómez et al., 2003; Liu et al., 2004). Moreover, a first live cloned sheep has been produced from presumptive G0 phase mammary cells (Wilmut et al., 1997). These results suggested that the chromatin at the G0/G1 phase is amenable to nuclear reprogramming for the cloned embryo. In chapter II of this study, we successfully synchronizeed the cat donor cells to G0/G1 stage using the serum starvation, confluency-contact inhibition and roscovitine treatment. Due to the ease and economical in preparation, the confluency-contact inhibition method was selected for synchronizing

the cat cell in the production of iSCNT cat embryos. In addition, the apoptotic cells were in an acceptable level in comparison with the serum starvation method.

When the confluency-contact inhibited cat cells or roscovitine treated-cells were transferred into the enucleated bovine oocytes, the development of iSCNT cat embryos was stopped at the 8- to 16-cell stage. This demonstrated that the modification of donor cell alone could not improve the developmental competence of iSCNT cat embryos. Therefore, modification of culture procedures following embryo reconstruction is subsequently required.

Since the epigenetic modification of early intraspecies SCNT cloned embryos with HDAC inhibitor TSA improved their *in vitro* embryo development, e.g. pigs (Li et al., 2008; Zhang et al., 2007), mice (Kishigami et al., 2006), and cattle (Sawai et al., 2012), several studies on iSCNT, e.g. the guar-bovine (Srirattana et al., 2012), the black-footed cat-domestic cat (Gómez et al., 2011), the human-rabbit (Shi et al., 2008) and the sei whale-bovine (Bhuiyan et al., 2010) have speculated to improve the success rate by using TSA. As well as our present study in chapter III, results revealed that the treatment of 50 nM TSA for 24 h after fusion contributed the significant higher rates of fusion, cleavage, the development to 8-cell or morula stages, and also blastocyst formation compared to non-TSA treated group. Although, we founded that TSA has an efficiency to improve the development *in vitro* of iSCNT cat embryos. We have further confirmed in chapter IV whether the effects of TSA treatment at concentration 50 nM on iSCNT cat embryo development associated with the modification of acetylation levels on histone H3K9, H3K18 and H3K23 and di-methylation level on histone H3K9.

Histone acetylation and deacetylation, the regulators of gene activity in eukaryotic cells, have important roles for cell growth, cell cycle control, development, differentiation and survival during the embryo development. It has been generally known that the deacetylation of core histones by HDAC leads to transcriptional repression (Pazin and Kadonaga, 1997) by catalyzing the removal of acetyl groups for the epsilon-amino groups of distinct lysine residues in the amino-terminal tail of core histones (Finnin et al., 1999; Tribus et al., 2005). The HDAC inhibitors, TSA, accumulate highly acetylated

histone species in cells and cause a variety of phenotypic changes In chapter IV, the treatment of 50 nM TSA for 24 h after fusion in iSCNT cat embryos have been proved to provide the beneficial effects on the modification of acetylation levels of lysine residues (K9, K18 and K23) on histone H3 and di-methylation on H3K9 in the early embryogenesis to be resembled to those of bovine IVF embryos. The acetylation levels on H3K9, H3K18 and H3K23 and di-methylation H3K9 of TSA-treated embryos were significant higher than those of control embryos at all examined stages, exceptionally for some certain stages. We have suggested that the acetylation patterns of H3K9, H3K18 and H3K23 induced by TSA in this study were sufficient to improve the *in vitro* development of iSCNT cat embryos to blastocyst stage.

To achieve the ultimate goal of the use of iSCNT, the *in vivo* developmental capacity of 50 nM TSA-treated iSCNT cat embryos was investigated in chapter V. The total of 224 embryos at 2- to 4-cell stages was transferred into five recipients. However, none of the recipients receiving TSA-treated iSCNT cat became pregnant. Several factors are thought to be involved in this pregnancy failure including the developmental competence and quality of embryos, the induction of ovulation in recipients and the embryo transfer procedure, and the physiology incompatibility between fetus and uterus.

The implantation rate has been found to be associated with the cleavage rate and degree of fragmentation. Moreover, in case of IVF, the embryos transferred at the 4-cell stage showed the twice implantation rate than 2-cell embryos. In year 2005, the cloned kitten was obtained from the transferred 2- to 4-cell stage reconstructed cat embryos (Yin et al., 2005). In spite of these ealier studies, the iSCNT cat embryos at 2- to 4-cell stage were used for ET in this study (chapter V). Unfortunately, we were not able to produce a pregnancy in 5 recipients. The failure of pregnancy in iSCNT cat was possibly related to the number of transferred embryos and passage of the donor cell line. The cloned kitten produced by Yin et al., (2005) was born from a recipient receiving a total 65 embryos, which reconstructed from the first passage of skin fibroblast cell line. However, the iSCNT cat embryos were derived from the donor cells at 2nd to 5th passages. The passage of cell lines has shown the importance on chromosomal stability

in cloned embryos. The chromosomal abnormalities were observed to increases when the passage of the donor cell increases. In African wild cat, the cell line at passage 9th showed the chromosomal abnormalities 59%, whereas 43% was obsrerved in 1st passage. When the 4th passaged-cells were transferred in to the enucleated cat oocytes, 50% of chromosomal abnormalities were presented in African wild cat cloned embryos (Gómez et al., 2006).

Since up to now there is no report of success in the production of non-bovid specie offspring derived by iSCNT reconstructed from bovine ooplasm, the genetic distance between the host oocyte and the donor nucleus is believed to be one of the factors deciding the developmental ability of the embryo/offspring (Ikumi et al., 2004; Thongphakdee, 2007). Although, the addition of TSA in the cultured medium of iSCNT cat embryos improved the *in vitro* developmental competence to the blastocyst stage (chapter III) by modifying the acetylation on histone H3 (chapterIV), but the pregnancy of iSCNT cat could not be established after embryo transfer (chapter V). This indicated that TSA treatment provides the beneficial effect on *in vitro* iSCNT cat development at the early embryogenesis, which the epigenetic reprogramming have played an important role on embryo survival. On the other hands, TSA could not overcome the obstacle of mitochondria heteroplasmy influencing suvivability *in utero* of iSCNT embryos.

Conclusion and further investigation

In conclusion, our study has shown that the domestic cat donor cells was possibly to reprogrammed in bovine oocytes, which the reconstructed iSCNT cat embryos could successfully developed to the blastocyst stages when TSA was supplemented in the culture medium. Although the production of TSA-treated iSCNT cat offspring has not been achieved, many factors affected the *in utero* developmental block from this study have been discussed. Further studies on iSCNT could be required in an attempt to overcome the *in utero* developmental block and the mitochondrial incompatibilies in iSCNT embryos.

REFERENCES

Akshey, Y.S., Malakar, D., De, A.K., Jena, M.K., Garg, S., Dutta, R., Pawar, S.K. and Mukesh, M. 2010. Hand-made cloned goat *(Capra hircus)* embryos-a comparison of different donor cells and culture systems. Cell Reprogram. 12(5): 581-588.

Anderson, G.B. 1988. Interspecific pregnancy: barriers and prospects. Biol Reprod. 38(1): 1-15.

Arat, S., Rzucidlo, S.J. and Stice, S.L. 2003. Gene expression and in vitro development of inter-species nuclear transfer embryos. Mol Reprod Dev. 66(4): 334-342.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. and Echelard, Y. 1999. Production of goats by somatic cell nuclear transfer. Nat Biotechnol. 17(5): 456-461.

Bannister, A.J. and Kouzarides, T. 2005. Reversing histone methylation. Nature. 436(7054): 1103-1106.

Bertos, N.R., Wang, A.H. and Yang, X.J. 2001. Class II histone deacetylases: structure, function, and regulation. Biochem Cell Biol. 79(3): 243-252.

Bestor, T.H. 2000. The DNA methyltransferases of mammals. Hum Mol Genet. 9(16): 2395-2402.

Bhuiyan, M.M., Suzuki, Y., Watanabe, H., Lee, E., Hirayama, H., Matsuoka, K., Fujise, Y., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2010. Production of Sei whale *(Balaenoptera borealis)* cloned embryos by inter- and intra-species somatic cell nuclear transfer. J Reprod Dev. 56(1): 131-139.

Bjerling, P., Silverstein, R.A., Thon, G., Caudy, A., Grewal, S. and Ekwall, K. 2002. Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and *in vivo* specificity. Mol Cell Biol. 22(7): 2170-2181.

Boquest, A.C., Day, B.N. and Prather, R.S. 1999. Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells. Biol Reprod. 60(4): 1013-1019.

Briggs, R. and King, T. 1952. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc Natl Acad Sci U S A. 38(5): 455-463.

Caamano, J.N., Rodriguez, A., Salas, A., Munoz, M., Diez, C., Prather, R.S. and Gómez, E. 2008. Flow cytometric cell cycle analysis of cultured brown bear fibroblast cells. Cell Biol Int. 32(7): 855-859.

Camous, S., Kopecny, V. and Flechon, J.E. 1986. Autoradiographic detection of the earliest stage of [3H]-uridine incorporation into the cow embryo. Biol Cell. 58(3): 195-200.

Campbell, K.H., Ritchie, W.A. and Wilmut, I. 1993. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod. 49(5): 933-942.

Campbell, K.H., Loi, P., Otaegui, P.J. and Wilmut, I. 1996. Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod. 1(1): 40-46.

Campbell, K.H. 1999. Nuclear equivalence, nuclear transfer, and the cell cycle. Cloning. 1(1): 3-15.

Campbell, K.H. and Alberio, R. 2003. Reprogramming the genome: role of the cell cycle. Reprod Suppl. 61: 477-494.

Campbell, K.H., Fisher, P., Chen, W.C., Choi, I., Kelly, R.D., Lee, J.H. and Xhu, J. 2007. Somatic cell nuclear transfer: Past, present and future perspectives. Theriogenology. 68: 214-231.

Chang, K.H., Lim, J.M., Kang, S.K., Lee, B.C., Moon, S.Y. and Hwang, W.S. 2003. Blastocyst formation, karyotype, and mitochondrial DNA of interspecies embryos derived from nuclear transfer of human cord fibroblasts into enucleated bovine oocytes. Fertil Steril. 80(6): 1380-1387.

Chen, D.Y., Wen, D.C., Zhang, Y.P., Sun, Q.Y., Han, Z.M., Liu, Z.H., Shi, P., Li, J.S., Xiangyu, J.G., Lian, L., Kou, Z.H., Wu, Y.Q., Chen, Y.C., Wang, P.Y. and Zhang, H.M. 2002. Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. Biol Reprod. 67(2): 637-642.

Chen, T., Zhang, Y.L., Jiang, Y., Liu, J.H., Schatten, H., Chen, D.Y. and Sun, Q.Y. 2006. Interspecies nuclear transfer reveals that demethylation of specific repetitive sequences is determined by recipient ooplasm but not by donor intrinsic property in cloned embryos. Mol Reprod Dev. 73(3): 313-317.

Chesne, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. and Renard, J.P. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol. 20(4): 366-369.

Cho, S.R., Ock, S.A., Yoo, J.G., Mohana Kumar, B., Choe, S.Y. and Rho, G.J. 2005. Effects of confluent, roscovitine treatment and serum starvation on the cell-cycle synchronization of bovine foetal fibroblasts. Reprod Domest Anim. 40(2): 171-176.

Choresca, C.H., Jr., Koo, O.J., Oh, H.J., Hong, S.G., Gómez, D.K., Kim, J.H., Lee, B.C. and Park, S.C. 2009. Different culture conditions used for arresting the G0/G1 phase of the cell cycle in goldfish *(Carassius auratus)* caudal fin-derived fibroblasts. Cell Biol Int. 33(1): 65-70.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A. and Robl, J.M. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science. 280(5367): 1256-1258.

Collas, P., Pinto-Correia, C., Ponce de Leon, F.A. and Robl, J.M. 1992. Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. Biol Reprod. 46(3): 501-511.

Coller, H.A. 2007. What's taking so long? S-phase entry from quiescence versus proliferation. Nat Rev Mol Cell Biol. 8(8): 667-670.

D'Alessio, A.C., Weaver, I.C. and Szyf, M. 2007. Acetylation-induced transcription is required for active DNA demethylation in methylation-silenced genes. Mol Cell Biol. 27(21): 7462-7474.

Ding, X., Wang, Y., Zhang, D., Guo, Z. and Zhang, Y. 2008. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology. 70(4): 622-630.

Dominko, T., Mitalipova, M., Haley, B., Beyhan, Z., Memili, E., McKusick, B. and First, N.L. 1999. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biol Reprod. 60(6): 1496-1502.

Eggan, K. and Jaenisch, R. 2002. Nuclear reprogramming: biological and technological constraints. In: Principles of cloning. J. Cibelli, R.P. Lanza, K.H.S. Campbell and M.D. West (ed.). New York: Elsevier Science. 85-108.

Enright, B.P., Jeong, B.S., Yang, X. and Tian, X.C. 2003. Epigenetic characteristics of bovine donor cells for nuclear transfer: levels of histone acetylation. Biol Reprod. 69(5): 1525-1530.

Enright, B.P., Sung, L.Y., Chang, C.C., Yang, X. and Tian, X.C. 2005. Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2'-deoxycytidine. Biol Reprod. 72(4): 944-948.

Eriksson, I., Joosten, M., Roberg, K. and Ollinger, K. 2012. The histone deacetylase inhibitor trichostatin A reduces lysosomal pH and enhances cisplatin-induced apoptosis. Exp Cell Res. 319(1): 12-20.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R. and Pavletich, N.P. 1999. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature. 401(6749): 188-193.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. 2003. Pregnancy: a cloned horse born to its dam twin. Nature. 424(6949): 635.

Gao, S., McGarry, M., Priddle, H., Ferrier, T., Gasparrini, B., Fletcher, J., Harkness, L., De Sousa, P., McWhir, J. and Wilmut, I. 2003. Effects of donor oocytes and culture conditions on development of cloned mice embryos. Mol Reprod Dev. 66(2): 126-133.

Gibbons, J., Arat, S., Rzucidlo, J., Miyoshi, K., Waltenburg, R., Respess, D., Venable, A. and Stice, S. 2002. Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. Biol Reprod. 66(4): 895-900.

Gómez, M.C., Jenkins, J.A., Giraldo, A., Harris, R.F., King, A., Dresser, B.L. and Pope, C.E. 2003. Nuclear transfer of synchronized African wild cat somatic cells into enucleated domestic cat oocytes. Biol Reprod. 69(3): 1032-1041.

Gómez, M.C., Pope, C.E., Cole, A. and Dresser, B.L. 2004. Production of rusty spotted cat embryos by inter-generic nuclear transfer and another pregnancy in a domestic cat after transfer of African wild cat embryos produced by inter-species nuclear transfer. In: Proceedings of the 15th International Congress on Animal Reproduction. Porto Seguro, Brazil. 562.

Gómez, M.C., Pope, C.E. and Dresser, B.L. 2006. Nuclear transfer in cats and its application. Theriogenology. 66(1): 72-81.

Gómez, M.C., Pope, C.E., Biancardi, M.N., Dumas, C., Galiguis, J., Morris, A.C., Wang, G. and Dresser, B.L. 2011. Trichostatin A modified histone covalent pattern and enhanced expression of pluripotent genes in interspecies black-footed cat cloned embryos but did not improve *in vitro* and *in vivo* viability. Cell Reprogram. 13(4): 315-329.

Gray, S.G. and Dangond, F. 2006. Rationale for the use of histone deacetylase inhibitors as a dual therapeutic modality in multiple sclerosis. Epigenetics. 1(2): 67-75.

Han, Z.M., Chen, D.Y., Li, J.S., Sun, Q.Y., Wang, P.Y., Du, J. and Zhang, H.M. 2003. Flow cytometric cell-cycle analysis of cultured fibroblasts from the giant panda, *Ailuropoda melanoleuca L*. Cell Biol Int. 27(4): 349-353.

Hashem, M.A., Bhandari, D.P., Kang, S.K., Lee, B.C. and Suk, H.W. 2006. Cell cycle analysis of *in vitro* cultured goral *(Naemorhedus caudatus)* adult skin fibroblasts. Cell Biol Int. 30(9): 698-703.

Hoffert, K.A., Anderson, G.B., Wildt, D.E. and Roth, T.L. 1997. Transition from maternal to embryonic control of development in IVM/IVF domestic cat embryos. Mol Reprod Dev. 48(2): 208-215.

Hwang, W., Kim, K., Jin, Y., Kim, Y., Chung, H. and Yoon, T. 2001. Interspecies somatic cell nuclear transfer for the production of endangered Korean Tiger *(Panthera Tigiris Altaica)*. Theriogenology. 55: 271.

Ikumi, S., Sawai, K., Takeuchi, Y., Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Interspecies somatic cell nuclear transfer for *in vitro* production of Antarctic minke whale *(Balaenoptera bonaerensis)* embryos. Cloning Stem Cells. 6(3): 284-293.

IUCN 2009. The Users' Guide to the IUCN Red List web site. Version 1.0 (March 2009)

Iwamoto, H.S., Saeki, K., Kishigami, S., Kasamatsu, A., Tatemizo, A. and Abe, Y. 2007. Effects of trichostatin A on development of bovine somatic cell nuclear transfer embryos. Reprod Fertil Dev. 19(1): 142-142.

Jang, G., Kim, M.K., Oh, H.J., Hossein, M.S., Fibrianto, Y.H., Hong, S.G., Park, J.E., Kim, J.J., Kim, H.J., Kang, S.K., Kim, D.Y. and Lee, B.C. 2007. Birth of viable female dogs produced by somatic cell nuclear transfer. Theriogenology. 67(5): 941-947.

Janssen, D.L., Edward, M.L., Koster, J.A., Lanza, R.P. and Ryder, O.A. 2004. Postnatal management of cryptordich banteng calves cloned by nuclear transfer utilizing frozen fibroblast cultures and enucleated cow ova. Reprod Fertil Dev. 16(2): 224-224.

Kaedei, Y., Fujiwara, A., Tanihara, F., Namula, Z., Viet, V.L. and Otoi, T. 2010. *In vitro* development of cat interspecies nuclear transfer using pig's and cow's cytoplasm. Bull Vet Inst Pulawy. 54: 405-408.

Keefer, C.L. 2008. Lessons learned from nuclear transfer (cloning). Theriogenology. 69(1): 48-54.

Khammanit, R., Chantakru, S., Kitiyanant, Y. and Saikhun, J. 2008. Effect of serum starvation and chemical inhibitors on cell cycle synchronization of canine dermal fibroblasts. Theriogenology. 70(1): 27-34.

Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Hwang, W.S., Hossein, M.S., Kim, J.J., Shin, N.S., Kang, S.K. and Lee, B.C. 2007. Endangered wolves cloned from adult somatic cells. Cloning Stem Cells. 9(1): 130-137.

Kim, T.M., Park, T.S., Shin, S.S., Han, J.Y., Moon, S.Y. and Lim, J.M. 2004. An interclass nuclear transfer between fowl and mammal: *in vitro* development of chicken-to-

cattle interclass embryos and the detection of chicken genetic complements. Fertil Steril. 82(4): 957-959.

Kishigami, S., Mizutani, E., Ohta, H., Hikichi, T., Thuan, N.V., Wakayama, S., Bui, H.T. and Wakayama, T. 2006. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun. 340(1): 183-189.

Kishigami, S., Bui, H.T., Wakayama, S., Tokunaga, K., Van Thuan, N., Hikichi, T., Mizutani, E., Ohta, H., Suetsugu, R., Sata, T. and Wakayama, T. 2007. Successful mouse cloning of an outbred strain by trichostatin A treatment after somatic nuclear transfer. J Reprod Dev. 53(1): 165-170.

Kues, W.A., Anger, M., Carnwath, J.W., Paul, D., Motlik, J. and Niemann, H. 2000. Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. Biol Reprod. 62(2): 412-419.

Kwun, J., Chang, K., Lim, J., Lee, E., Lee, B., Kang, S. and Hwang, W. 2003. Effects of exogenous hexoses on bovine *in vitro* fertilized and cloned embryo development: Improved blastocyst formation after glucose replacement with fructose in a serum-free culture medium. Mol Reprod Dev. 65(2): 167-174.

Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D. and Damiani, P. 2000. Cloning of an endangered species *(Bos gaurus)* using interspecies nuclear transfer. Cloning. 2(2): 79-90.

Lee, B., Wirtu, G.G., Damiani, P., Pope, E., Dresser, B.L., Hwang, W. and Bavister, B.D. 2003. Blastocyst development after intergeneric nuclear transfer of mountain bongo antelope somatic cells into bovine oocytes. Cloning Stem Cells. 5(1): 25-33.

Lee, B.C., Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Hossein, M.S., Kim, J.J., Kang, S.K., Schatten, G. and Hwang, W.S. 2005. Dogs cloned from adult somatic cells. Nature. 436(7051): 641.

Lee, H.S., Yu, X.F., Bang, J.I., Cho, S.J., Deb, G.K., Kim, B.W. and Kong, I.K. 2010. Enhanced histone acetylation in somatic cells induced by a histone deacetylase

inhibitor improved inter-generic cloned leopard cat blastocysts. Theriogenology. 74(8): 1439-1449.

Lee, J.H., Marfil, M., Panarce, M., Medina, M. and Campbell, K.H.S. 2006. Establishment of pregnancy by ovine nuclear transfer embryos reconstructed using caffeine-treated *in vitro* matured oocytes as cytoplast recipients. Reprod Fertil Dev. 18(2): 135-136.

Li, E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet. 3(9): 662-673.

Li, J., Svarcova, O., Villemoes, K., Kragh, P.M., Schmidt, M., Bogh, I.B., Zhang, Y., Du, Y., Lin, L., Purup, S., Xue, Q., Bolund, L., Yang, H., Maddox-Hyttel, P. and Vajta, G. 2008. High *in vitro* development after somatic cell nuclear transfer and trichostatin A treatment of reconstructed porcine embryos. Theriogenology. 70(5): 800-808.

Li, Y., Dai, Y., Du, W., Zhao, C., Wang, H., Wang, L., Li, R., Liu, Y., Wan, R. and Li, N. 2006[a]. Cloned endangered species takin *(Budorcas taxicolor)* by inter-species nuclear transfer and comparison of the blastocyst development with yak (Bos grunniens) and bovine. Mol Reprod Dev. 73(2): 189-195.

Li, Y., Dai, Y., Du, W., Zhao, C., Wang, L., Wang, H., Liu, Y., Li, R. and Li, N. 2007. *In vitro* development of yak *(Bos grunniens)* embryos generated by interspecies nuclear transfer. Anim Reprod Sci. 101(1-2): 45-59.

Li, Z., Sun, X., Chen, J., Leno, G.H. and Engelhardt, J.F. 2005. Factors affecting the electrofusion of mouse and ferret oocytes with ferret somatic cells. Mol Reprod Dev. 72(1): 40-47.

Li, Z., Sun, X., Chen, J., Liu, X., Wisely, S.M., Zhou, Q., Renard, J.P., Leno, G.H. and Engelhardt, J.F. 2006[b]. Cloned ferrets produced by somatic cell nuclear transfer. Dev Biol. 293(2): 439-448.

Liu, C.T., Yu, K.C. and Ju, J.C. 2004. Cell cycle stage analysis of rabbit foetal fibroblasts and cumulus cells. Reprod Domest Anim. 39(6): 385-390.

Loi, P., Ptak, G., Barboni, B., Fulka, J., Jr., Cappai, P. and Clinton, M. 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol. 19(10): 962-964.

Lorthongpanich, C., Laowtammathron, C., Muenthaisong, S., Vetchayan, T., Ketudat-Cairns, M. and Likitdecharote, B. 2004. *In vitro* development of enucleated domestic cat oocytes reconstructed with skin fibroblasts of domestic and leopard cats. Reprod Fertil Dev. 16(2): 149-150.

Lu, F., Shi, D., Wei, J., Yang, S. and Wei, Y. 2005. Development of embryos reconstructed by interspecies nuclear transfer of adult fibroblasts between buffalo *(Bubalus bubalis)* and cattle *(Bos indicus)*. Theriogenology. 64(6): 1309-1319.

Maalouf, W.E., Alberio, R. and Campbell, K.H. 2008. Differential acetylation of histone H4 lysine during development of *in vitro* fertilized, cloned and parthenogenetically activated bovine embryos. Epigenetics. 3(4): 199-209.

Maalouf, W.E., Liu, Z., Brochard, V., Renard, J.P., Debey, P., Beaujean, N. and Zink, D. 2009. Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin remodeling as well as developmental potential to term. BMC Dev Biol. 9(11): doi: 10.1186/1471-213X-9-11.

Massip, A. and Leibo, S. 2002. Factors influencing cryopreservation of domestic animal embryos. In: Assessment of Mammalian Embryo Quality: Invasive and Non-invasive Techniques. A. Van Soom and M. Boerjan (ed.). Dordrecht, The Netherlands: Kluwer's Academic. 121-138.

McGraw, S., Robert, C., Massicotte, L. and Sirard, M.A. 2003. Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. Biol Reprod. 68(2): 383-389.

Meirelles, F.V., Caetano, A.R., Watanabe, Y.F., Ripamonte, P., Carambula, S.F., Merighe, G.K. and Garcia, S.M. 2004. Genome activation and developmental block in bovine embryos. Anim Reprod Sci. 82-83: 13-20.

Melican, D., Butler, R., Hawkins, N., Chen, L.H., Hayden, E., Destrempes, M., Williams, J., Lewis, T., Behboodi, E., Ziomek, C., Meade, H., Echelard, Y. and Gavin, W.

2005. Effect of serum concentration, method of trypsinization and fusion/activation utilizing transfected fetal cells to generate transgenic dairy goats by somatic cell nuclear transfer. Theriogenology. 63(6): 1549-1563.

Mitalipov, S.M., White, K.L., Farrar, V.R., Morrey, J. and Reed, W.A. 1999. Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-trisphosphate. Biol Reprod. 60(4): 821-827.

Moreira, P.N., Fernandez-Gonzalez, R., Ramirez, M.A., Perez-Crespo, M., Rizos, D., Pintado, B. and Gutierrez-Adan, A. 2006. Differential effects of culture and nuclear transfer on relative transcript levels of genes with key roles during preimplantation. Zygote. 14(1): 81-87.

Mori, M., Otoi, T. and Suzuki, T. 2002. Correlation between the cell number and diameter in bovine embryos produced in vitro. Reprod Domest Anim. 37(3): 181-184.

Murakami, M., Otoi, T., Wongsrikeao, P., Agung, B., Sambuu, R. and Suzuki, T. 2005. Development of interspecies cloned embryos in yak and dog. Cloning Stem Cells. 7(2): 77-81.

Nakajima, H., Kim, Y.B., Terano, H., Yoshida, M. and Horinouchi, S. 1998. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res. 241(1): 126-133.

Naoi, H., Otoi, T., Shimamura, T., Karja, N.W., Agung, B., Shimizu, R., Taniguchi, M. and Nagai, T. 2007. Developmental competence of cat oocytes from ovaries stored at various temperature for 24 h. J Reprod Dev. 53(2): 271-277.

Neumann, E., Sowers, A.E. and Jordan, C.A. 1989. Electroporation and electrofusion. In: Cell biology. (ed.). New York and London: Plenum Press. 436.

Niemann, H. and Wrenzycki, C. 2000. Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: implications for subsequent development. Theriogenology. 53(1): 21-34.

Niemann, H., Wrenzycki, C., Lucas-Hahn, A., Brambrink, T., Kues, W.A. and Carnwath, J.W. 2002. Gene expression patterns in bovine *in vitro*-produced and nuclear

transfer-derived embryos and their implications for early development. Cloning Stem Cells. 4(1): 29-38.

Pazin, M.J. and Kadonaga, J.T. 1997. What's up and down with histone deacetylation and transcription? Cell. 89(3): 325-328.

Pham, H., Ferrari, R., Cokus, S.J., Kurdistani, S.K. and Pellegrini, M. 2007. Modeling the regulatory network of histone acetylation in *Saccharomyces cerevisiae*. Mol Syst Biol. 3: 153.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. 407(6800): 86-90.

Pope, C.E., Keller, G.L. and Dresser, B.L. 1993. *In vitro* fertilization in domestic and non-domestic cats including sequences of early nuclear events, development *in vitro*, cryopreservation and successful intra- and interspecies embryo transfer. J Reprod Fertil Suppl. 47: 189-201.

Pope, C.E., Johnson, C.A., McRae, M.A., Keller, G.L. and Dresser, B.L. 1998. Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. Anim Reprod Sci. 53(1-4): 221-236.

Rice, J.C. and Allis, C.D. 2001. Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol. 13(3): 263-273.

Robin, P., Fritsch, L., Philipot, O., Svinarchuk, F. and Ait-Si-Ali, S. 2007. Post-translational modifications of histones H3 and H4 associated with the histone methyltransferases Suv39h1 and G9a. Genome Biol. 8(12): 270.

Rybouchkin, A., Kato, Y. and Tsunoda, Y. 2006. Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol Reprod. 74(6): 1083-1089.

Sansinena, M.J., Hylan, D., Hebert, K., Denniston, R.S. and Godke, R.A. 2005. Banteng (Bos javanicus) embryos and pregnancies produced by interspecies nuclear transfer. Theriogenology. 63(4): 1081-1091.

Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W. and Dean, W. 2003. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr Biol. 13(13): 1116-1121.

Sarma, K. and Reinberg, D. 2005. Histone variants meet their match. Nat Rev Mol Cell Biol. 6(2): 139-149.

Sawai, K., Fujii, T., Hirayama, H., Hashizume, T. and Minamihashi, A. 2012. Epigenetic status and full-term development of bovine cloned embryos treated with trichostatin A. J Reprod Dev. 58(3): 302-309.

Schiltz, R.L., Mizzen, C.A., Vassilev, A., Cook, R.G., Allis, C.D. and Nakatani, Y. 1999. Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. J Biol Chem. 274(3): 1189-1192.

Shi, H., Maier, S., Nimmrich, I., Yan, P.S., Caldwell, C.W., Olek, A. and Huang, T.H. 2003. Oligonucleotide-based microarray for DNA methylation analysis: principles and applications. J Cell Biochem. 88(1): 138-143.

Shi, L.H., Miao, Y.L., Ouyang, Y.C., Huang, J.C., Lei, Z.L., Yang, J.W., Han, Z.M., Song, X.F., Sun, Q.Y. and Chen, D.Y. 2008. Trichostatin A (TSA) improves the development of rabbit-rabbit intraspecies cloned embryos, but not rabbit-human interspecies cloned embryos. Dev Dyn. 237(3): 640-648.

Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L. and Westhusin, M. 2002. A cat cloned by nuclear transplantation. Nature. 415(6874): 859.

Simerly, C., Navara, C., Hyun, S.H., Lee, B.C., Kang, S.K., Capuano, S., Gosman, G., Dominko, T., Chong, K.Y., Compton, D., Hwang, W.S. and Schatten, G. 2004. Embryogenesis and blastocyst development after somatic cell nuclear transfer in nonhuman primates: overcoming defects caused by meiotic spindle extraction. Dev Biol. 276(2): 237-252.

Solter, D. 2000. Mammalian cloning: advances and limitations. Nat Rev Genet. 1(3): 199-207.

Song, B.S., Lee, S.H., Kim, S.U., Kim, J.S., Park, J.S., Kim, C.H., Chang, K.T., Han, Y.M., Lee, K.K., Lee, D.S. and Koo, D.B. 2009. Nucleologenesis and embryonic genome activation are defective in interspecies cloned embryos between bovine ooplasm and rhesus monkey somatic cells. BMC Dev Biol. 9(44): doi: 10.1186/1471-213X-9-44.

Song, J., Hua, S., Song, K. and Zhang, Y. 2007. Culture, characteristics and chromosome complement of Siberian tiger fibroblasts for nuclear transfer. In Vitro Cell Dev Biol Anim. 43(7): 203-209.

Srirattana, K., Imsoonthornruksa, S., Laowtammathron, C., Sangmalee, A., Tunwattana, W., Thongprapai, T., Chaimongkol, C., Ketudat-Cairns, M. and Parnpai, R. 2012. Full-term development of gaur-bovine interspecies somatic cell nuclear transfer embryos: effect of trichostatin A treatment. Cell Reprogram. 14(3): 248-257.

St John, J.C., Lloyd, R.E., Bowles, E.J., Thomas, E.C. and El Shourbagy, S. 2004. The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. Reproduction. 127(6): 631-641.

Stein, P., Worrad, D.M., Belyaev, N.D., Turner, B.M. and Schultz, R.M. 1997. Stage-dependent redistributions of acetylated histones in nuclei of the early preimplantation mouse embryo. Mol Reprod Dev. 47(4): 421-429.

Stice, S.L., Gibbons, J., Rzucidlo, J.R. and Baile, C.A. 2000. Improvements in nuclear transfer procedures will increase commercial utilization of animal cloning. Asian. Australas. J. Anim. Sci. 13: 856-860.

Stumpf, T.T., Terlouw, S.L., Funahashi, H. and Prather, R.S. 1992. Premature chromosomal condensation of nuclei transferred into *in vitro* matured porcine oocytes. J. Biol. Reprod. 48: 175.

Sun, Y.H., Chen, S.P., Wang, Y.P., Hu, W. and Zhu, Z.Y. 2005. Cytoplasmic impact on cross-genus cloned fish derived from transgenic common carp *(Cyprinus carpio)* nuclei and goldfish *(Carassius auratus)* enucleated eggs. Biol Reprod. 72(3): 510-515.

Suteevun, T. 2005. Epigenetics of cloned swamp buffalo embryos, cloned cattle fetuses and calves. In: PhD Thesis. Suranaree University of Technology, Thailand.

Suteevun, T., Parnpai, R., Smith, S.L., Chang, C.C., Muenthaisong, S. and Tian, X.C. 2006. Epigenetic characteristics of cloned and *in vitro*-fertilized swamp buffalo *(Bubalus bubalis)* embryos. J Anim Sci. 84(8): 2065-2071.

Sutovsky, P., Van Leyen, K., McCauley, T., Day, B.N. and Sutovsky, M. 2004. Degradation of paternal mitochondria after fertilization: implications for heteroplasmy, assisted reproductive technologies and mtDNA inheritance. Reprod Biomed Online. 8(1): 24-33.

Tachibana, M., Sugimoto, K., Fukushima, T. and Shinkai, Y. 2001. Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. J Biol Chem. 276(27): 25309-25317.

Taniguchi, M., Ikeda, A., Arikawa, E., Wongsrikeao, P., Agung, B., Naoi, H., Nagai, T. and Otoi, T. 2007. Effect of cryoprotectant composition on *in vitro* viability of in vitro fertilized and cloned bovine embryos following vitrification and in-straw dilution. J Reprod Dev. 53(4): 963-969.

Thongphakdee, A., Numchaisrika, P., Omsongkram, S., Chatdarong, K., Kamolnorranath, S., Dumnui, S. and Techakumphu, M. 2006. *In vitro* development of marbled cat embryos derived from interspecies somatic cell nuclear transfer. Reprod Domest Anim. 41(3): 219-226.

Thongphakdee, A. 2007. *In vitro* and *in vivo* development of marbled cat and flat-headed cat embryos derived by somatic cell nuclear transfer. In: PhD Thesis. Chulalongkorn University, Thailand.

Thongphakdee, A., Numchaisrika, P., Rungsiwiwat, R., Kamolnorranath, S., Chatdarong, K. and Techakumphu, M. 2007. Inter-generic marbled cat and flat-headed cat cloned blastocyst generated from domestic cat and rabbit oocytes. Reprod Fertil Dev. 19(163.

Thongphakdee, A., Kobayashi, S., Imai, K., Inaba, Y., Tasai, M., Tagami, T., Nirasawa, K., Nagai, T., Saito, N., Techakumphu, M. and Takeda, K. 2008. Interspecies nuclear transfer embryos reconstructed from cat somatic cells and bovine ooplasm. J Reprod Dev. 54(2): 142-147.

Thongphakdee, A., Siriaroonrat, B., Manee-in, S., Klincumhom, N., Kamolnorranath, S., Chatdarong, K. and Techakumphu, M. 2010. Intergeneric somatic cell nucleus transfer in marbled cat and flat-headed cat. Theriogenology. 73(1): 120-128.

Tribus, M., Galehr, J., Trojer, P., Brosch, G., Loidl, P., Marx, F., Haas, H. and Graessle, S. 2005. HdaA, a major class 2 histone deacetylase of Aspergillus nidulans, affects growth under conditions of oxidative stress. Eukaryot Cell. 4(10): 1736-1745.

Trontelj, K., Usaj, M. and Miklavcic, D. 2010. Cell electrofusion visualized with fluorescence microscopy. J Vis Exp. 41):

Turner, B.M. 1991. Histone acetylation and control of gene expression. J Cell Sci. 99(13-20.

van der Heijden, G.W., Derijck, A.A., Ramos, L., Giele, M., van der Vlag, J. and de Boer, P. 2006. Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin. Dev Biol. 298(2): 458-469.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature. 394(6691): 369-374.

Wang, F., Kou, Z., Zhang, Y. and Gao, S. 2007. Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. Biol Reprod. 77(6): 1007-1016.

Wang, Y., Zeng, S. and Zhu, S. 2002. Effects of the donor blastomere cell cycle stages on the development of rabbit nuclear transplantation embryos. Acta. Zoology. Sinica. 48(3): 368-374.

Wang, Y.S., Xiong, X.R., An, Z.X., Wang, L.J., Liu, J., Quan, F.S., Hua, S. and Zhang, Y. 2011. Production of cloned calves by combination treatment of both donor cells

and early cloned embryos with 5-aza-2/-deoxycytidine and trichostatin A. Theriogenology. 75: 819-825.

Waurich, R., Ringleb, J., Braun, B.C. and Jewgenow, K. 2010. Embryonic gene activation in *in vitro* produced embryos of the domestic cat *(Felis catus)*. Reproduction. 140(4): 531-540.

Wen, D.C., Yang, C.X., Cheng, Y., Li, J.S., Liu, Z.H., Sun, Q.Y., Zhang, J.X., Lei, L., Wu, Y.Q., Kou, Z.H. and Chen, D.Y. 2003. Comparison of developmental capacity for intra- and interspecies cloned cat *(Felis catus)* embryos. Mol Reprod Dev. 66(1): 38-45.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature. 385(6619): 810-813.

Wolfe, B.A. and Kraemer, D.C. 1992. Methods in bovine nuclear transfer. Theriogenology. 37: 5-15.

Worrad, D.M., Turner, B.M. and Schultz, R.M. 1995. Temporally restricted spatial localization of acetylated isoforms of histone H4 and RNA polymerase II in the 2-cell mouse embryo. Development. 121(9): 2949-2959.

Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Lemme, E., Korsawe, K. and Niemann, H. 2004. Gene expression patterns in *in vitro*-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome? Anim Reprod Sci. 82-83: 593-603.

Wu, X., Li, Y., Xue, L., Wang, L., Yue, Y., Li, K., Bou, S., Li, G.P. and Yu, H. 2010. Multiple histone site epigenetic modifications in nuclear transfer and *in vitro* fertilized bovine embryos. Zygote. 19(1): 31-45.

Yamochi, T., Kida, Y., Oh, N., Ohta, S., Amano, T., Anzai, M., Kato, H., Kishigami, S., Mitani, T., Matsumoto, K., Saeki, K., Takenoshita, M., Iritani, A. and Hosoi, Y. 2012. Development of interspecies cloned embryos reconstructed with rabbit *(Oryctolagus cuniculus)* oocytes and cynomolgus monkey *(Macaca fascicularis)* fibroblast cell nuclei. Zygote. 5(1-9): Epub ahead of print.

Yin, X.J., Lee, H.S., Lee, Y.H., Seo, Y.I., Jeon, S.J., Choi, E.G., Cho, S.J., Cho, S.G., Min, W., Kang, S.K., Hwang, W.S. and Kong, I.K. 2005. Cats cloned from fetal and adult somatic cells by nuclear transfer. Reproduction. 129(2): 245-249.

Yin, X.J., Lee, Y., Lee, H., Kim, N., Kim, L., Shin, H. and Kong, I. 2006[a]. *In vitro* production and initiation of pregnancies in inter-genus nuclear transfer embryos derived from leopard cat *(Prionailurus bengalensis)* nuclei fused with domestic cat *(Felis silverstris catus)* enucleated oocytes. Theriogenology. 66(2): 275-282.

Yin, X.J., Lee, Y.H., Jin, J.Y., Kim, N.H. and Kong, I.K. 2006[b]. Nuclear and microtubule remodeling and in vitro development of nuclear transferred cat oocytes with skin fibroblasts of the domestic cat *(Felis silvestris catus)* and leopard cat *(Prionailurus bengalensis)*. Anim Reprod Sci. 95(3-4): 307-315.

Yin, X.J., Lee, H.S., Yu, X.F., Kim, L.H., Shin, H.D., Cho, S.J., Choi, E.G. and Kong, I.K. 2008. Production of second-generation cloned cats by somatic cell nuclear transfer. Theriogenology. 69(8): 1001-1006.

Zhang, Y., Li, J., Villemoes, K., Pedersen, A.M., Purup, S. and Vajta, G. 2007. An epigenetic modifier results in improved *in vitro* blastocyst production after somatic cell nuclear transfer. Cloning Stem Cells. 9(3): 357-363.

Zhao, J., Ross, J.W., Hao, Y., Spate, L.D., Walters, E.M., Samuel, M.S., Rieke, A., Murphy, C.N. and Prather, R.S. 2009. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod. 81(3): 525-530.

Zhou, H. and Guo, Z. 2006. Heterogenous nuclear transfer embryo reconstructed by bovine oocytes and their subsequent development. In vitro Cell Dev Biol Anim. 42(1-2): 16-19.

Zhou, Q., Renard, J.P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A. and Cozzi, J. 2003. Generation of fertile cloned rats by regulating oocyte activation. Science. 302(5648): 1179.

Appendix

List of publications and proceedings

Publications

1. Wittayarat, M., Thongphakdee, A., Saikhun, K., Chatdarong, K., Otoi, T. and Techakumphu M. 2013. Cell cycle synchronization of skin fibroblast cells in four species of family Felidae. Reprod Domest Anim. 48(2). 305-10.

2. Wittayarat, M., Fujiwara, A., Kaedei, Y., Chatdarong, K., Techakumphu, M., Sato, Y., Tanihara, F., Taniguchi, M. and Otoi, T. 2013. Cell cycle analysis and interspecies nuclear transfer of cat cells treated with chemical inhibitors. Acta Vet. Hung. DOI: 10.1556/AVet.2013.050.

3. Wittayarat, M., Sato, Y., Do, L. T. K., Morita, Y., Chatdarong, K., Techakumphu, M., Taniguchi, M. and Otoi, T. 2013. Histone deacetylase inhibitor improves the development and acetylation levels of cat-cow interspecies cloned embryos. Cell. Reprogram. (In press).

Proceedings

1. Wittayarat, M., Thongphakdee, A., Chatdarong, K., Saikhun, K. and Techakumphu, M. 2009. Cell Cycle Analysis of Cultured Skin Fibroblasts from the Leopard (*Panthera pardus*). Proceeding of the 8th Chulalongkorn University Veterinary Annual Conference, 3 April 2009, Faculty of Vet. Science, Chulalongkorn University, Bangkok, Thailand.

2. Wittayarat, M., Thongphakdee, A., Chatdarong, K. Saikhun, K., Otoi, T. and Techakumphu, M. 2010. Cell cycle analysis of cultured skin fibroblasts from the Asian golden cat *(Pardofelis temminckii),* Marbled cat *(Pardofelis marmorata),* Leopard *(Panthera pardus)* and Siamese cat *(Felis Catus)*. Proceeding of the 9th Chulalongkorn University Veterinary Annual Conference, 1 April 2010, Faculty of Vet. Science, Chulalongkorn University. 40 (1). 139.

3. Wittayarat, M., Thongphakdee, A., Chatdarong, K. Saikhun, K., Otoi, T. and Techakumphu, M. 2010. Cell Cycle Analysis of Cultured Skin Fibroblasts from the Asian golden cat *(Pardofelis temminckii),* Marbled cat *(Pardofelis*

marmorata), Leopard *(Panthera pardus)* and Siamese cat *(Felis Catus).* Proceeding of the 1st Wildlife ARTs Workshop; Eld's Deer model, 31 March 2010, Khao Kheow Open Zoo, Chonburi, Thailand.

4. <u>Wittayarat, M.,</u> Namula, Z., Luu, V. V., Do, L. T. K., Sato, Y., Taniguchi, M., Otoi T. 2013. Effect of trichostatin A on *in vitro* embryo development of interspecies nuclear transfer embryos reconstructed from cat donor nuclei and bovine cytoplasm. Proceeding of the 39th Annual Conference of the IETS, 19-22 January, 2013, Hannover, Germany.

BIOGRAPHY

Miss Manita Wittayarat was born on November 16th 1983 in Chai Nat province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 1st class honour from Faculty of Veterinary Science, Chulalongkorn University, in 2008. In 2008, she received a scholarship from the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program: The Office of the Higher Education Commission to perform a PhD program of Theriogenology at the Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her focus research is about interspecies somatic cell nuclear transfer in felid species and their epigenetic patterns after modulation of cultured procedure.