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ปีการศึกษา 2550

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

IN VITRO AND *IN VIVO* DEVELOPMENT OF MARBLED CAT AND FLAT-HEADED CAT EMBRYOS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER

Miss Ampika Thongphakdee

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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
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Thesis Title	*IN VITRO* AND *IN VIVO* DEVELOPMENT OF MARBLED CAT AND FLAT-HEADED CAT EMBRYOS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER
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อัมพิกา ทองภักดี : การพัฒนาภายนอกและภายในร่างกายของตัวอ่อนแมวลายหินอ่อนและแมวป่าหัวแบนจากการย้ายฝากนิวเคลียสด้วยเซลล์โซมาติก (*IN VITRO* AND *IN VIVO* DEVELOPMENT OF MARBLED CAT AND FLAT-HEADED CAT EMBRYOS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER) อ.ที่ปรึกษา : ศ.น.สพ. มงคล เตชะกำพุ อ.ที่ปรึกษาร่วม: รศ.สพ.ญ. เกวลี ฉัตรดรงค์ น.สพ. ในริโอะ ไซโตะ , 110 หน้า

การทดลองที่ 1 ศึกษาหาวิธีการที่เหมาะสมของระยะเวลาการเลี้ยงโอโอไซต์ การเชื่อมเซลล์ต้นแบบกับโอโอไซต์ และการกระตุ้นตัวอ่อนในสัตว์ตระกูลแมว โอโอไซต์ได้จากรังไข่แมวบ้านที่ได้รับการทำหมัน เซลล์ต้นแบบได้จากแมวลายหินอ่อนที่ตายในกรงเลี้ยง โคลนตัวอ่อนที่ได้จากโอโอไซต์ที่เลี้ยงนาน 24 ชม.มีอัตราการพัฒนามากกว่าตัวอ่อนที่ผลิตจากโอโอไซต์ที่เลี้ยงนาน 36 และ 42 ชม. (ระยะโมรูลา 9.2, 4.3 และ 0% ตามลำดับ, P<0.05) ประสิทธิภาพเชื่อมติดของเซลล์แมวลายหินอ่อนและโอโอไซต์ด้วยการเชื่อมด้วยกระแสไฟฟ้า 2.1 และ 2.4 กิโลโวลต์/ซม. นาน 80 ไมโครเซกเคิล 2 ครั้ง และ 2.4 กิโลโวลต์/ซม. นาน 50 ไมโครเซกเคิล 3 ครั้ง มีอัตรา 46, 48.5 และ 67.7% ตามลำดับ (P>0.05) การพัฒนาของตัวอ่อนโคลนแมวลายหินอ่อนและแมวบ้านมีอัตราไม่แตกต่างกัน (ระยะโมรูลา 8 และ 8.3%, P>0.05) การเติมไซโตคาลาซิน บี ในน้ำยากระตุ้นช่วยให้โอโอไซต์มีอัตราการพัฒนาสู่ระยะบลาสโตซีสมากกว่าการไม่เติมไซโตคาลาซิน บี (8.3 และ 35%, P>0.05) สรุปว่าโอโอไซต์ที่เลี้ยงเป็นเวลานาน 24 ชม เหมาะสมในการเป็นโอโอไซต์ตัวรับในการผลิตโคลนตัวอ่อนแมวลายหินอ่อน การเชื่อมเซลล์ด้วยกระแสไฟฟ้า 2.4 กิโลโวลต์/ซม. นาน 50 ไมโครเซกเคิล 3 ครั้ง ให้อัตราการเชื่อมติดที่มีประสิทธิภาพ มีความเป็นไปได้ที่เซลล์ไฟโบรบลาสแมวลายหินอ่อนถูกพัฒนาไปเป็นตัวอ่อนและเจริญสู่ระยะโมรูลาด้วยโอโอไซต์แมวบ้าน การกระตุ้นโอโอไซต์ด้วยการเลี้ยงในน้ำยากระตุ้นที่เติมไซโตคาลาซิน บี ช่วยพัฒนาอัตราการเจริญสู่ระยะบลาสโตซีส

การทดลองที่ 2 ศึกษาการพัฒนาของโคลนตัวอ่อนแมวลายหินอ่อนและแมวป่าหัวแบนซึ่งผลิตจากโอโอไซต์แมวบ้านและกระต่าย จากการผลิตตัวอ่อนด้วยโอโอไซต์แมวบ้าน ตัวอ่อนแมวป่าหัวแบนมีอัตราการเจริญสู่ระยะโมรูลา (53%) มากกว่าตัวอ่อนแมวลายหินอ่อน (23%) และแมวบ้าน (11%) แต่มีอัตราการเจริญของระยะบลาสโตซีสไม่แตกต่างกัน (8.3, 5 และ 8.5% ตามลำดับ, P>0.05) จากการผลิตตัวอ่อนด้วยโอโอไซต์กระต่ายอัตราการเจริญของระยะบลาสโตซีสของแมวป่าหัวแบน (8.5%) แมวบ้าน (7.57%) ต่ำกว่าในกระต่าย (14.3%, P<0.05) แต่ไม่ต่างจากแมวลายหินอ่อน (11.5%, P>0.05) มีความแตกต่างของอัตราการเชื่อมติดของเซลล์ และอัตราการพัฒนาของตัวอ่อนซึ่งผลิตจากเซลล์แมวป่าหัวแบน 3 ตัว แต่อัตราการพัฒนาของตัวอ่อนแมวป่าหัวแบนที่ผลิตจากเซลล์จากเพศผู้และเพศเมียไม่มีความแตกต่างกัน (P>0.05) สรุปว่าสามารถผลิตตัวอ่อนแมวลายหินอ่อน แมวป่าหัวแบนจากโอโอไซต์แมวบ้านและกระต่ายได้โดยวิธีการย้ายฝากนิวเคลียส อัตราการพัฒนาของโคลนตัวอ่อนของแมวป่าหัวแบนแตกต่างกันขึ้นอยู่กับเซลล์ของสัตว์แต่ละตัว แต่ไม่ขึ้นกับเพศของเซลล์ต้นแบบ

การทดลองที่ 3 ศึกษาการพัฒนาภายนอกร่างกายของตัวอ่อนแมวบ้านที่ได้จากการปฏิสนธิภายนอกร่างกาย (ไอวีเอฟ) และการย้ายฝากสู่แมวตัวรับ และการตั้งท้องของตัวรับภายหลังการย้ายฝากโคลนตัวอ่อนแมวป่าหัวแบน ตัวอ่อนไอวีเอฟแบ่งตัวที่ 18-27 ชม. มีอัตราการเจริญของระยะบลาสโตซีส และจำนวนเซลล์สูงกว่าตัวอ่อนที่แบ่งตัวที่ >27- 42 ชม. (61.4 กับ 18.6 %, 106 ± 43 กับ 60 ± 27 เซลล์) ย้ายฝากตัวอ่อนระยะคลีเวจสู่แมวตัวรับ 6 ตัว (25 ± 9) แมวตัวรับทั้งหมดตั้งท้อง และได้ลูกแมวทั้งหมด 5 ตัวจากตัวรับ 3 ตัว ย้ายฝากโคลนตัวอ่อนแมวป่าหัวแบน (5 ตัว, 41.4 ± 13 ตัวอ่อน) โคลนตัวอ่อนแมวป่าหัวแบน และแมวบ้าน (5 ตัว, 29.8 ± 20.8 ตัวอ่อน) และ โคลนตัวอ่อนแมวป่าหัวแบนร่วมกับตัวอ่อนไอวีเอฟ (4 ตัว, 55 ± 15 ตัวอ่อน) ตัวรับที่ได้รับการย้ายฝากโคลนตัวอ่อนแมวป่าหัวแบนร่วมกับตัวอ่อนไอวีเอฟตั้งท้อง 1 ตัว และให้กำเนิดลูกแมวไอวีเอฟ สรุปว่าแมวตัวรับสามารถตั้งท้องได้ภายหลังการย้ายฝากตัวอ่อนไอวีเอฟ และตัวอ่อนที่แบ่งตัวที่ 18-27 ชม.ภายหลังการเลี้ยงร่วมกับตัวอสุจิเหมาะสมนำมาย้ายฝากตัวอ่อนเนื่องจากมีอัตราการพัฒนาและคุณภาพดีกว่าตัวอ่อนที่แบ่งตัวในภายหลัง การที่ไม่สามารถผลิตลูกโคลนแมวป่าหัวแบนและแมวบ้านได้เนื่องจากข้อจำกัดของอัตราการพัฒนาและคุณภาพของตัวอ่อน

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ปีการศึกษา 2550

ลายมือชื่อนิสิต อัมพิกา ทองภักดี
ลายมือชื่ออาจารย์ที่ปรึกษา [signature]
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KEY WORDS : MARBLED CAT/ FLAT-HEADED CAT/ EMBRYO DEVELOPMENT/ SOMATIC CELL NUCLEAR TRANSFER

AMPIKA THONGPHAKDEE: *IN VITRO* AND *IN VIVO* DEVELOPMENTS OF MARBLED CAT AND FLAT-HEADED CAT EMBRYOS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT 3[e] CYCLE THESIS COADVISOR: ASSOC. PROF. KAYWALEE CHATDARONG, D.V.M., MSc, Ph.D., NORIO SAITO, D.V.M. 110 pp.

EXP. I was conducted to find appropriate *in vitro* maturation (IVM), fusion and activation protocols for somatic cell nuclear transfer (SCNT) in felid species. The oocytes were obtained from ovaries of ovariohysterectomized domestic cats (DC). The donor cells were obtained from a marbled cat (MC) died in captivity. The developmental rates of MC-DC (donor cell-recipient oocyte) cloned embryos derived from oocytes cultured for 24 h were greater than those cultured for 36 and 42 h (morula stage; 9.2, 4.3 and 0%, respectively, $P<0.05$). The fusion efficiency of the MC-DC couplets using the 2 pulses of 2.1 and 2.4 kV/cm for 80 μs and 3 pulses of 2.4 kV/cm for 50 μs was 46, 48.5 and 67.7% ($P > 0.05$). The development of cloned embryos derived from MC and DC fibroblast cells was not different (morula rate; 8 vs. 8.3%, $P>0.05$). Addition of the cytochalasin B in the activation medium provided a grater blastocyst rate compared to that without the cytochalasin B (8.3 vs. 35%, $P<0.05$). In conclusion, DC oocyte matured at 24 h is suggested to be used as recipient ooplasm for cloned MC embryos. The MC-DC couplets are fused efficiently by the introducing of 3 pulses of 2.4 kV/cm for 50 μs. The MC fibroblast cells demonstrate the possibility to be reprogrammed in the DC oocytes, resulting in the development to the morula stage. Exposure of mature DC oocytes to cycloheximide and cytochalasin B after electrical stimulation improves the rate of blastocyst formation.

EXP. II was conducted to investigate 1) the development of MC and FC cloned embryos reconstructed from DC and rabbit (RB) oocytes and 2) the effect of individual cell line and gender on FC-DC cloned embryo production. Using DC oocytes, the FC-DC couplets yielded a greater percentage of morula (53%) than those of MC- (23%) and DC-DC couplets (11%, $P<0.05$). However, the number of couplets achieving blastocyst expansion did not differ (MC; 5, FC; 8.3 and DC; 8.5%, $P>0.05$). Using RB oocytes, The blastocyst rate of FC- (8.5%) and DC-RB embryos (7.7%) was lower than those of RB-RB (14.3%, $P<0.05$) but not MC-RB embryos (11.5%, $P>0.05$). The variations of fusion and developmental rate through morula of FC-DC couplets were observed among three cell lines. The development of FC-DC embryos derived from female and male donor cells was not different ($P>0.05$). These results indicated that the MC and FC embryos could be produced successfully by SCNT of either DC or RB oocytes. Individual cell line but not gender of donor cells influenced the development of cloned FC embryos.

EXP. III was conducted to investigate 1) *in vitro* development of DC IVF embryos and their transfer to recipients and 2) pregnancy establishment of recipients receiving FC-DC cloned embryos. The IVM oocytes were co-cultured with sperm for 18 h. The blastocyst rate and cell number of blastocysts derived from cleaved embryos collected at 18-27 h were greater than those cleaved during >27-42 h (61.4 vs. 18.6% and 106 ± 43 vs. 60 ± 27 cells, $P<0.05$). Total 150 of cleaved IVF embryos were transferred to 6 recipients (mean 25 ± 9). All recipients became pregnant resulting in five kittens delivered from three recipients. The recipients receiving FC-DC cloned (n=5, 41.4 ± 13 embryos), FC- and DC-DC cloned (n=5, 29.8 ± 20.8 embryos) and FC-DC cloned and DC IVF embryos (n=4, 55 ± 15 embryos) were observed the pregnancy establishment. One of the recipients receiving FC-DC cloned and DC IVF embryos became pregnant and delivered IVF kittens. In conclusion, the pregnancy could be established after DC IVF embryo transfers, and embryos cleaved early at 18-27 h pi would rather be used for transfer than those cleaved later due to the greater development and quality. The FC- and DC-DC cloned offspring were not able to be produced which may be caused by the low development and quality of the embryos.

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Academic year 2007 Co-advisor's signature...K. Chatdarong

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LIST OF ABBREVIATIONS

AI	artificial insemination
ARTs	assisted reproductive technologies
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CH	corpus heamorragicum
CO_2	carbon dioxide
DC	domestic cat
DCP	domestic cat parthenote
DMAP	dimethylaminopurine
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
eCG	equine chrorionic gonadotrophin
ET	embryo transfer
FBS	fetal bovine serum
FSH	follicle stimulating hormone
FC	flat-headed cat
h	hour
hCG	human chrorionic gonadotrophin
hepes	N-(2-hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid)
ICSI	intra cytoplasmic sperm injection
ICM	inner cell mass
ig-NT	inter-generic nuclear transfer
i.m.	intramuscular
IVM	*in vitro* maturation
IVF	*in vitro* fertilization
kV/cm	kilo volt per centimetre
L	line

LOS	large offspring syndrome
M199	Medium 199
MAPK	mitogen activated protein kinases
MC	marbled cat
$MgSO_4$	magnesium sulphate
MPF	maturation promoting factor
mt-DNA	mitochrondrial deoxyribonucleic acid
MII	methaphase of meiosis II
min	minute
ml	milliliter
mM	millimole
mOsm	milliosmole
NT	nuclear transfer
Oct-4	POU transcription factor Octamer-4 transcript
pi	post insemination
PBS	phosphate buffered saline
RB	rabbit
rpm	revolutions per min
s/c	subcutaneous
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
SOFaa	synthetic oviductal fluid plus amino acids
μg/ml	microgram per milliliter
μl	microlitre
μm	micrometer
μs	microsecond

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In a world of rapidly growing numbers of humans it is becoming more difficult to save wild spaces, animals and plants. Today, most of the 36 species of the Felidae family except for the domestic cat *(Felis silvertris catus)* are classified as threatened, vulnerable or endangered. Illegal hunting, the trade expansion in their parts and pollution are still serious causes of declining numbers in their population. There are 9 species of non-domestic felids in Thailand; the tiger *(Panthera tigris*), the leopard or panther *(Pathera pardus*), the clouded leopard *(Neofelis nebulosa*), the leopard cat *(Felis bengalensis*), the fishing cat *(Felis viverrina),* the asian golden cat *(Felis temmincki*), the jungle cat *(Felis chaus*), the marbled cat *(Pardofelis marmorata*) and the flat-headed cat *(Prionailurus planiceps*) (Lekagul and Mcneely, 1997). The last two species, marbled cat (Fig. 1) and flat-headed cat (Fig.2) are small wild cats of Southeast Asia, classified as a different genus from the domestic cat. They are considered to be critically endangered in CITES appendix I. Today, in Thailand, very few flat-headed cats exist in captivity and few marbled cats in the wild have been discovered (Grassman and Michale, 2002).

The application of conservation strategies, i.e. habitat management and protection, translocation, captive breeding and reproductive technology is necessary for maintaining of their genetic diversity (Holt and Pickard, 1999). *In situ* conservation, natural breeding is the first choice for sustaining the endangered wildlife population and its genetic diversity in zoos. However, natural breeding in captivity can unexpectedly fail due to factors such as male aggression, behavioral incompatibility and infertility. Consequently, captive breeding management and assisted reproductive technologies (ARTs) have been recognized as alternative tools for expanding the captive wildlife population.

Figure 1. The marbled cat is only slightly bigger than the domestic cat. The body markings are faded like those seen in marble and the bushy tail is very long extending to a length longer than the head and the body combined (www.bigcatrescue.org/marbled_cat.htm).

Figure 2. The flat-headed cat (A,B) looks like the domestic cat but it has a typical body shade, a flattened skull, long snout, small ears set well down on the sides of the skull, stumpy legs and a comparatively short tail.

To date, basic to advanced ARTs, play an important role in the felid captive breeding program (Pope, 2000). Basic reproductive biotechnologies including reproductive physiology and hormonal analyses have been propagated to monitor oestrous cycles and detect pregnancy. Advanced reproductive procedures such as germ cell (Axner et al., 2004) and embryo cryopreservation (Gómez et al., 2003b), artificial insemination (AI) (Chatdarong et al., 2007; Zambelli and Count, 2005), *in vitro* fertilization (IVF) (Comizzoli et al., 2003; Kanda et al., 1998; Karja et al., 2002; Murakami et al., 2002), intracytoplasmic sperm injection (ICSI) (Bogliolo et al., 2001), embryo transfer (ET) (Goodrowe et al., 1988) and nuclear transfer (NT) (Gómez et al., 2003a; Shin et al., 2002) have been studied in the last decade. However, use of these advanced techniques has limited because of the low success rates. Nevertheless, the development of advanced reproductive biotechnologies is needed for future research into endangered felid species.

In Thailand, genome resource banking and ARTs, i.e. AI, IVF and NT have been developed at the Zoological Park Organization (ZPO) in collaboration with local universities including Chulalongkorn University's faculty of Veterinary Science. CryoBanking of gametes, embryos and somatic cells have been established with an aim of maintaining the genetic diversity by producing genetically desired offspring. Currently, the ZPO has successfully established a semen bank of 8 endangered felid species. Semen analysis and crypreservation has been conducted and is readily available for research and routine AI and IVF activities. For the cell bank, tissues of endangered felids collected during annual physical examination or from fresh carcasses are routinely cultured and maintained as cell lines. The fibroblast cells of several endangered species including the marbled cat and the flat-headed cat have been successfully preserved in the cell bank for further study including NT.

In 1997, the birth of Dolly (Wilmut et al., 1997) produced by an unnatural process called the "somatic cell nuclear transfer technique" (SCNT) or production of identical offspring from somatic cells (Fig. 3), gave rise to large scale studies on reproductive cloning, which aims to expand the population of valuable trait animals (Domiko et al., 1999; Miyoshi et al., 2002). The SCNT is also regarded as having the potential for conserving endangered species of wildlife (Holt et al., 2004).

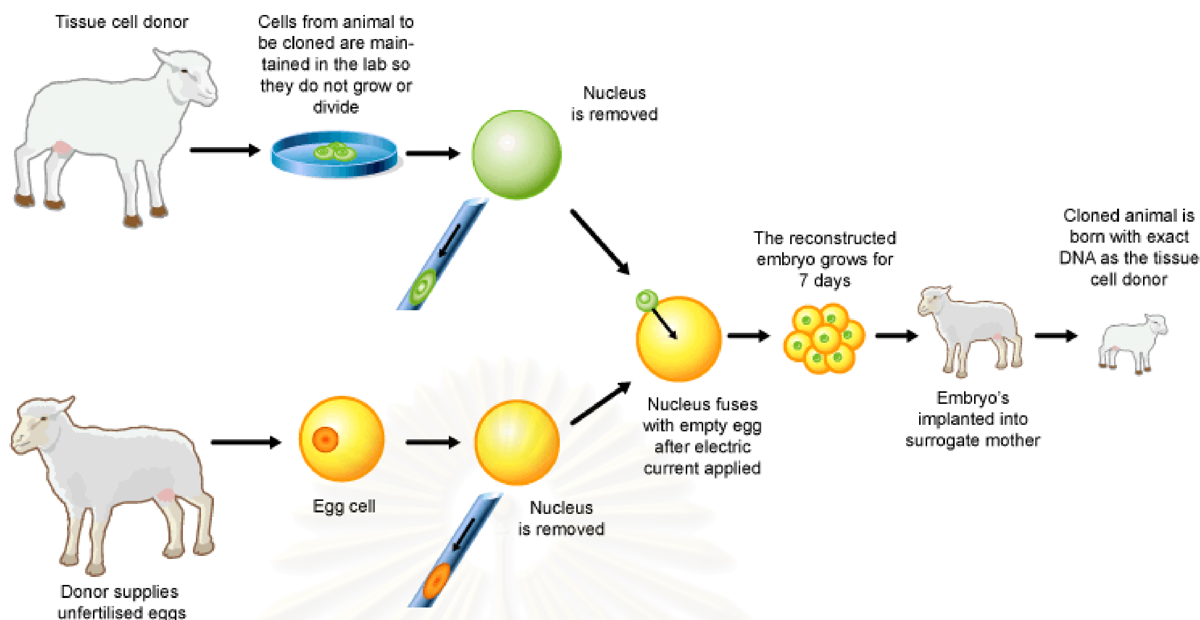

Figure 3. The SCNT animal is produced by transferring the nucleus from a diploid to an unfertilized oocyte from which the chromosomes have been removed. The chromosomes are remodeled and developed to a certain stage before being transferred to a surrogate mother. The cloned offspring is born with the exact DNA of the donor cell used (www.biotechnologyonline.gov.au/.../img_scnt.cfm).

SCNT in the felid species has been developed since year 2002 in domestic cats (Shin et al., 2002; Skrzyszowska et al., 2002) and has been used as a model for non-domestic cats. Many studies have been conducted to improve the poor development of cloned domestic cat embryos cultured *in vitro* (Kitiyanant et al., 2003; Yin et al., 2005). In endangered felids, due to a lack of oocytes from the wild, inter-species/generic NT or the process of transferring the donor cells from one species/genus into the oocytes of another (Fig. 4) has been performed to produce their embryos. The host oocytes from closely related and unrelated animals have been used as alternatives. The success of inter-species NT offspring production in endangered species has been shown in the gaur (Lanza et al., 2000), the mouflon (Loi et al., 2001), the banteng (Jansen et al., 2004) and the African wildcat (Gómez et al., 2003a), demonstrating the benefit of this technique in the conservation of endangered animals. The success of inter-generic NT embryo production has been demonstrated in the leopard cat (Lorthongpanich et al., 2004; Yin et al., 2006), using domestic cat oocytes as the recipient cytoplasm.

Also, rabbit oocytes namely universal recipient cytoplasm have been successfully used for producing domestic cat interspecies cloned embryos (Wen et al., 2003) and in other species i.e. the giant panda (Li et al., 2002), bovines (Techakumphu et al., 2005), elephants (Numchaisrika et al., 2005) and humans (Chen et al., 2003) for the study of embryology and embryonic stem cells (Chang et al., 2003; Chen et al., 2003).

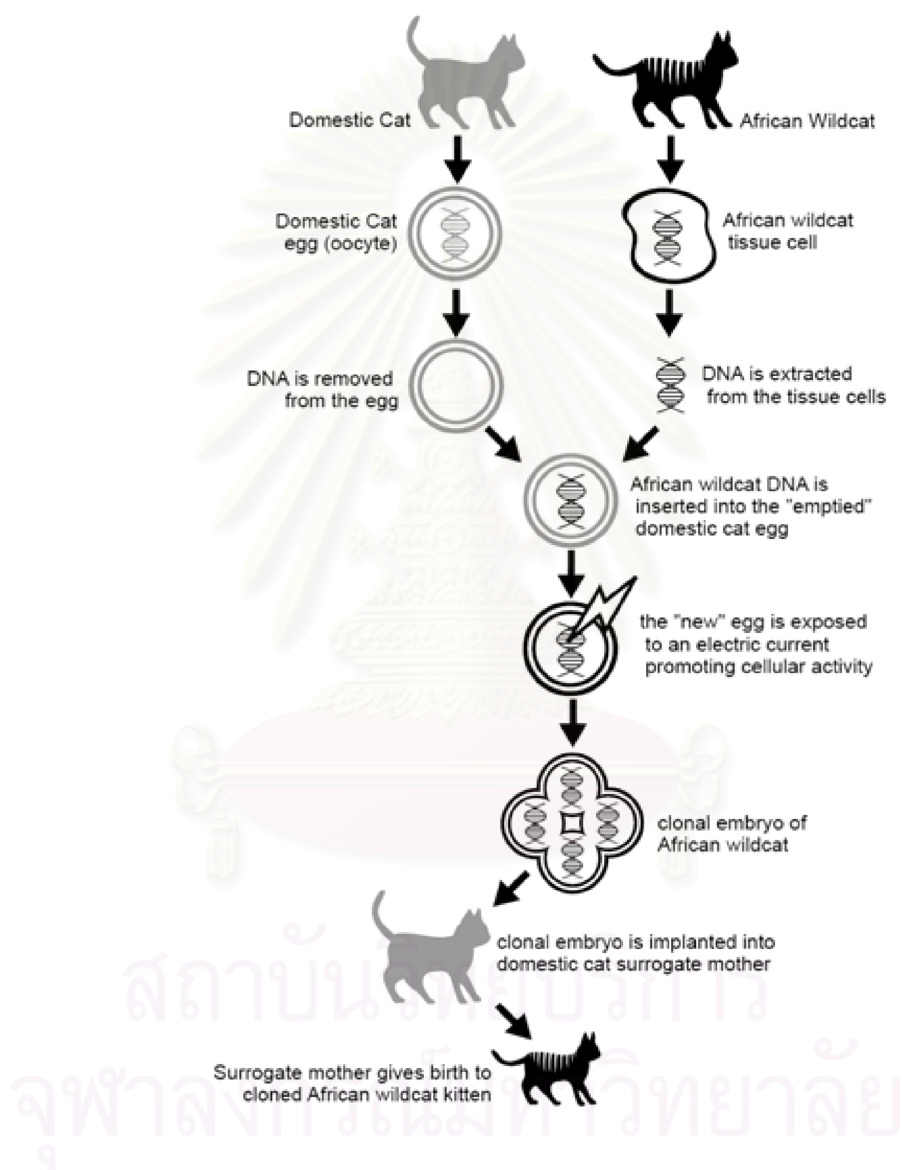

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Figure 4. Stages of inter-species cloning in endangered African wildcats (Audubon Nature Institute, 2003). Somatic cells are collected from an African wildcat and expanded *in vitro*. The cell is transferred into enucleated oocyte, collected from a physiogenetically related domestic cat. The cloned embryos are cultured *in vitro* until they develop to a certain stage and are transferred to a synchronized domestic cat surrogate, which finally delivers African wildcat kittens.

SCNT technology also provides an opportunity for the molecular tracking of nucleo-cytoplasmic interaction and the transmission of two cytoplasmic populations that will influence embryonic development and survival after implantation in endangered felids. According to previous studies, many attempts have been made to improve the efficiency of cat embryo production. However, producing cat embryos is restricted by their nature, unclear fundamental physiology and appropriate *in vitro* conditions. The *in vitro* maturation rate of cat oocytes varies between 40-60% (Farstad, 2000), and depends on many factors i.e. the composition of maturation medium, the stage of the reproductive cycle and supplementation in the medium (Freistedt et al., 2001; Johnston et al., 1989; Johnston et al., 1993; Karja et al., 2002; Kitiyanant et al., 2003; Merlo et al., 2005; Roth et al., 1994; Rungsiwiwut et al., 2002; Skrzyszowska et al., 2002; Spindler et al., 2002). Furthermore, two stages of embryo developmental arrest have been observed in cat embryos cultured *in vitro*, at 5-8 cell stage, which correspond to the transition period from maternal to embryonic control, and at morula to blastocyst stage (Kanda et al., 1995). Moreover, the fragmentation of reconstructed embryos that failed to undergo chromatin remodeling has been reported to have occurred in 18% of reconstructed embryos (Gómez et al., 2003a). Other limitations to produce felid NT embryos are an incomplete understanding of reprogramming, developmental block, poor results in cloned embryo production, the high number of cloned embryos (at least 30 embryos/recipient) required to establish pregnancy, the low number of offspring developed to term and developmental abnormalities of cloned offspring (Gómez et al., 2004a).

Although SCNT in felids has progressed, there is no report for marbled cats and flat-headed cats. Thus, research on SCNT in marbled cats and flat-headed cats is required to provide insights into embryo development and for the production of offspring in the future.

The overall objective of this dissertation is to observe the *in vitro* and in *vivo* development of marbled cat and flat-headed cat embryos after inter-generic NT.

1.2 Literature review

1.2.1 History and progress of somatic cell nuclear transfer

In 1996, Dolly the first lamb to be produced by nuclear transfer using a differentiated somatic cell as nuclear donor was created (Wilmut et al., 1997). Since then, somatic cell nuclear transfer (SCNT) has been recognized as an alternative tool for generating identical embryos and offspring by natural-fertilized imitated processes but without gamete genetic materials. SCNT has been widely applied to several domestic animals resulting in the birth of cloned offspring including cattle (Cibelli et al., 1998), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), cats (Shin et al., 2002), rabbits (Chesne et al., 2002), horses (Galli et al., 2003), rats (Zhou et al., 2003) and dogs (Lee et al., 2005). However, few reports have focused on the birth of wild animals because of the restricted numbers of their oocytes collected for research. Attempts through SCNT in wild animals provide the opportunity to developing “inter-species/generic NT”, which involves the transfer of donor cell nuclei from one species/genus to enucleated oocytes of another species/genus.

Inter-species/generic NT allows embryos carrying valuable genes to be developed in the surrogate ooplasm of other mammalian species. This technology has been established for many reasons including the production of embryos from species in which oocytes are difficult to obtain or where their collection is controlled. For reproduction proposes, inter-species/generic NT has been recognized as a valuable tool for the conservation of wildlife even though only a few success have been reported in guars (Lanza et al., 2000), mouflons (Loi et al., 2001), bantengs (Janssen et al., 2004), African wildcats (Gómez et al., 2004a) and ferrets (Li et al., 2006). In addition, patient-specific embryonic cell lines using inter-species/generic NT have been established for therapeutic or biomedical proposes (Chang et al., 2003 and Chen et al., 2003). Furthermore, inter-species/generic NT is a powerful tool for the understanding of the cellular and molecular aspects of nuclear reprogramming (Gómez et al., 2006) and nucleocytoplasmic interaction. An understanding of the factors influencing embryonic development and post-implantation survival enables the exploration of fundamental aspects of developmental biology in mammals.

Over the last decade, numerous scientific works on SCNT have been published, indicating stages of success in offspring production. These reports have involved the development of cloned embryos (at cellular and molecular level) in relation to the survival of offspring and searches for new procedures which may contribute to simplifying and improving its efficiency. In felids species, cloning in domestic cats (Shin et al., 2002) has been used as a model for non-domestic cats and cloned offspring of African wildcat have been successfully produced (Gómez et al., 2004). Recently, domestic cats, which have a closer genetic relationship to humans than other laboratory animals, have been used in transgenesis for biomedical proposes (Yin et al., 2008).

Although the successful generation of cloned animals and a better understanding of SCNT have been demonstrated, the efficiency of SCNT offspring production is very limited being less than 5% (Table 1). The production of animals by SCNT involves multiple steps including oocyte collection, maturation, enucleation, cell selection, culture and storage, embryo reconstruction, culture and transfer, care of surrogate mothers during pregnancy and parturition and postnatal care. Each of these steps influences the successful outcome of SCNT (Campbell et al., 2007). Furthermore, many crucial unknown factors relating to the molecular processes underlying normal and abnormal development require to be addressed.

Table 1. The efficiency of offspring production by somatic cell nuclear transfer in mammals

Species	Transferred embryos	Live clones/recipients	Efficiency (%)[a]	References
Sheep	29	1/13	3.4	Wilmut et al. (1997)
Cattle	3,435	148/935	4.3	Forsberg et al. (2002)
Goat	47	1/15	2.1	Baguisi et al. (1999)
Pig	110	1/4	0.9	Onishi et al. (2000)
Mouse	274	3/25	1.1	Wakayama and Yamaguchi (1999)
Rabbit	371	4/27	1.1	Chesne et al. (2002)
Domestic cat	87	1/8	1.1	Shin et al. (2002)
	675	3/10	0.4	Yin et al. (2005)
	812	4/18	0.5	Yin et al. (2007)
African wildcat	1552	10/50	0.6	Gómez et al. (2004a)
Leopard cat	1125	0/21	0	Yin et al. (2006)
Sand cat	1113	1/29	0.1	Pope et al. (2007)

[a] Efficiency (%): live-born clones/transferred embryos x 100

1.2.2 Factors affecting on SCNT

According to previous reports, many factors including recipient oocyte, donor cell, NT process, embryo culture, embryo transfer etc., are important to the success of NT embryos/offspring production. The main factors affecting NT results are reviewed as follows.

1.2.2.1 Recipient oocyte

In vitro and *in vivo* maturation and the period of oocyte maturation have an effect on NT success. Oocytes are the only cells that have the ability to change nuclei from the differentiated into the undifferentiated stage resembling pronuclei in freshly fertilized zygotes and later to complete the development of reconstructed embryos. However, the mechanism of nuclear reprogramming occurring in the cytoplasm remains unknown (Fulka et al., 2001). The quality and source of oocytes are key factors defining the proportion of oocytes development to the blastocyst stage and the efficiency at which live offspring are produced (Lonergan et al., 2003)

In vitro versus in vivo maturation

Although *in vivo* matured oocytes are a better source of cytoplasts for the production of cloned cat embryos, their production and collection is much more difficult and complicated than *in vitro* matured oocytes. Moreover, cat ovaries are abundant and obtainable from local veterinary clinics (Gómez et al., 2006). Therefore, *in vitro* matured oocytes are commonly used for the study of felid cloning. In the past decade, several studies of cat IVM have been conducted to improve maturation rate. Many factors influencing the meiotic competence and *in vitro* development of domestic cat oocytes i.e. morphological criteria for selecting good quality oocytes (Farstad, 2000; Wood and Wildt, 1997), culture period (Johnston et al., 1989; Pope et al., 1994), culture media supplementation (Goodrowe et al., 1991) and the season of the year (Freistedt et al., 2001) have been reported. In addition, growth factors such as an insulin-like growth factor I (IGF-I; 100 ng/ml) and epidermal growth factor (EGF; 25 ng/ml) have also been demonstrated to enhance the oocyte maturation rate (Kitiyanant et al., 2003) and cytoplasmic maturation, resulting in the increase in oocyte's ability to cleave and reach the blastocyst stage (Merlo et al., 2005). Currently, cat oocyte IVM has been improved from a consistent 40% (Jonhson et al., 1989) to 70-80% (Katska-Ksiazkiewicz et al., 2003; Nagano et al., 2008).

The delivered kittens produced by IVM/IVF demonstrate abilities equivalent to *in vitro* matured oocytes and those from *in vivo* oocytes (Pope et al., 1997).

A comparison of the effect of cytoplasts on the *in vitro* development of African wildcat cloned embryos has been reported (Gómez et al., 2003a). The fusion efficiency of donor nuclei and *in vivo* matured oocytes were superior to *in vitro* matured oocytes when used as recipient cytoplasts, (97 and 90%, respectively). However, the cleavage development after reconstruction using *in vitro* matured oocytes (85%) was higher than that of the *in vivo* matured oocytes (79%) and there was no significant difference in the blastocyst success between the two groups (27 vs. 23%) (Gómez et al., 2003a).

Regarding the achievement of cloned kitten production, *in vivo* and *in vitro*-matured oocytes have been used as recipient cytoplasts for the production of cloned domestic and non-domestic cats. The first cloned domestic cat kitten was produced after the transfer of embryos derived by the reconstitution of cumulus cells with enucleated oocytes matured *in vitro* for 26–30 h (Shin et al., 2002); whereas, African wildcat cloned kittens were produced after the transfer of cloned embryos derived from oocytes matured *in vivo* (Gómez et al., 2004a). In recent reports, an effective protocol of recipient oocyte collected from PMSG and hCG treated cats and partially matured *in vitro* for 4-12 h has been used to produce live cloned domestic cat kittens (Yin et al., 2005, 2007) but success has not yet been reported in leopard cats (Yin et al., 2006).

These data indicate that both *in vivo* and *in vitro* cytoplasts can be used for cat cloning; however, a comparison suggesting which type of cytoplast supports a higher rate of development to term remains to be elucidated. Furthermore, the successful establishment of IVM in the domestic cat has allowed the feasibility of genetic rescue in wild felids that may unexpectedly die in the forest and zoo.

Time of in vitro maturation

The mechanism of *in vitro* maturation of cat oocytes has become understood through the findings of Katska-Ksiazkiewicz et al. (2003), who found that the nuclear maturation of cat oocytes can be distinguished into two waves. The first wave takes place within 26 h and the other after 28-30 h of IVM. Most of the oocytes mature by 17–18 h during the first wave and the oocytes remain mature between 24 and 48 h by the second wave; indicating that the highest proportion of mature oocytes is observed between 42 and 45 h of culture. The mature oocytes collected when MII rises to its highest peak provide a high number of recipient oocytes suitable for cloning.

However, the oocytes collected at this period do not provide a satisfactory embryo developmental rate following the NT (Skrzyszowska et al., 2002). This observation demonstrates that the length of *in vitro* oocyte maturation has an influence on the *in vitro* development of reconstructed cat embryos; oocytes matured *in vitro* for 24-35 h are more suitable as recipient cytoplasm for exogenous nuclei than oocytes matured for 43 h, as indicated by a lower fusion rate (58%), a lower development to the morula stage (28%) and no development to blastocyst. In contrast, when using oocyte cytoplasts matured *in vitro* for 24 and 35 h, the fusion efficiency and percentage of cloned embryos developing to the morula and blastocyst stages were significantly higher those that of embryos reconstructed with oocyte cytoplasts matured for 43 h (71 vs. 72%, 56 vs. 34%, and 8 vs. 9%, respectively, Skrzyszowska et al., 2002). The authors suggested that a possible reason for the reduction in developmental potential is that oocytes matured for 43 h are post-matured, or aged with some morphological changes i.e. fragmentation in the polar body and ooplasm (Skrzyszowska et al., 2002). In accordance with other reports, similar rates of blastocyst development (2–10%) after maturation of the domestic cat oocytes *in vitro* for 24–34 h prior to the NT process have been demonstrated (Gómez et al., 2003a; Kitiyanant et al., 2003). In conclusion, young *in vitro* mature oocytes are better source of donor cytoplasts for cat cloning and the optimal time for IVM oocytes prior to cloning is 24 to 35 h.

1.2.2.2 Donor cell

Various studies have been carried out to find proper sources of donor cells and strategies to modify them for maximize the efficiency of NT. Most of these efforts have focused on source, type, gender and passage number of donor cells as well as cell cycle synchronization.

Source, type, gender and passage number of donor cell

Cell types such as cumulus and fibroblast cells, selected from various sources including fetus, skin and muscle have been used to produce cloned embryos in cats (Table 2). Fetal cells are believed to have less genetic damage and more proliferative ability, as measured by cell doubling, than adult somatic cells (Miyoshi et al., 2002). However, similar embryo development rates derived from fetus and adult fibroblast cells have been reported (Skrzyszowska et al. 2002; Kitiyanant et al., 2003; Yin et al.,

2006). No differences have been observed in the frequency of blastocyst development of embryos reconstructed either by cumulus cells or adult and fetal fibroblasts, and there is no evidence to indicate that embryo development to-term is improved by a specific cell type (Gómez et al., 2006a). In contrast to the fusion results when using cumulus or fibroblast cells; cumulus cells produced a lower fusion rate than fibroblast cells (Skrzyszowska et al., 2002), while no such difference has been reported by Kitiyanant et al. (2003). A low fusion rate has been reported to be obtained from adult fibroblasts collected from muscle (Wen et al., 2003). One possible reason for the low fusion rate obtained from muscle fibroblast may be due to a lower regenerative function compared to skin fibroblast.

The gender of donor cells has become one of the factors involved in the success of cloned embryo development since Gómez et al. (2003a) reported that African wildcat fibroblasts can be dedifferentiated in enucleated domestic cat oocytes to the blastocyst stage at a higher success rate than domestic cat fibroblasts (24.2% vs. 3.3%) and live offspring can be derived from the male cell line. One of the possible reasons for the conflicting results in embryo development may be that each cell line is derived from individuals of different genders. In the study of inter-species cloned development using male and female banteng cells, significantly higher cleavage, blastocyst and pregnancy rates have been found in embryos reconstructed from male fibroblasts than female. However, it is unclear whether X-chromosome inactivation and/or other epigenetic mechanism are affected by the sex of donor karyoplast (Sansinena et al., 2005). In contrast, no differences in embryo development have been found between the two genders of fibroblast in the cloning of leopard cats (Yin et al., 2006). Recently, viable cloned dogs have been produced from both male (Lee at al., 2005) and female donor cells (Jang et al., 2007), indicating that both genders of nuclei can be successfully reprogrammed.

The effect of the passage number (P) of donor cells in felid species has been demonstrated by Gómez et al. (2006b). This study shows that chromosomal abnormalities in African wildcat fibroblast cells increased progressively during culture *in vitro*: P1 (43%), P3 (46%), P4 (62%), and P9 (59%) and hypoploidy was the major defect. The incidence of chromosomal abnormalities in cloned embryos derived from those cells at P1, P3 and P4 was 45, 60 and 50%, respectively (Gómez et al., 2006b). Moreover, the incidence was higher for embryos reconstructed with donor fibroblasts at P9 (89%). Recently, however, two live kittens and 1 stillborn have been produced

from fetal fibroblast at P9 (Yin et al., 2005). This high ability of late passage cells to reprogram may be due to the fact that donor cell was collected from the fetus. Interestingly, bovine donor cells of later passage (up to 15) can develop from blastocyst to full term (Tian et al., 2003). Accordingly, the use of donor cells at late passage may be possible in producing cloned offspring, however, an analysis of the chromosomal stability of donor cells before use in NT could assist a subsequent increase in cloning efficiency (Gómez et al., 2006b).

Cell cycle synchronization

One important factor that is hypothesized as influencing the efficiency of NT is the cell cycle stage of the donor cell. Various strategies have been used to modify the stage of donor cells compatible to those of recipient oocytes. Serum starvation of the donor cells in the process of cloning of Dolly is believed to be the essential step in the success of SCNT (Wilmut et al., 1997). However, it is still controversial as to whether the induction of the quiescence of donor cells (G0 stage) is required for a successful nuclear transfer since four cloned calves have been obtained from 28 transferred cloned embryos derived from actively dividing bovine fibroblasts, which means starvation induction may not required (Cibelli et al., 1998).

Serum starvation induces the quiescence of cultured cells and arrests them at G0 stage. Most NT laboratories have utilized a serum starvation treatment. The methods for synchronizing cells in different cell-cycle phases have been well reviewed by Gómez et al. (2006a). The various methods of arresting cells in the G0 phase include: 1) serum deprivation, 2) contact inhibition, 3) reversible cycle inhibitors such as roscovitine. The methods for synchronizing cells at the G1 phase are; 1) culturing cells to early confluence, 2) the‘‘shake off’’ method, which is performed by physically shaking or vortexing a sub-confluent population of the cell culture to obtain newly divided couplets of cells with cytoplasmic bridges at the beginning of G1 phase, and 3) by serum deprivation to force the cells to enter in G0 phase and then stimulate re-entry in the G1 phase by culturing cells for 10 h in media containing serum.

In African wildcats and domestic cats, a higher percentage of nuclei are in the G0/G1 phase after serum starvation for 5 days than cycling cells, after contact inhibition or roscovitine treatment (Gómez et al., 2003a). Likewise, from the observation of Yin et al. (2007) in odd-eyed cats, based on flow cytometry, most fibroblast cells are in the G0/G1 phase (89.3%) after serum starvation.

In contrast, non-serum-starved cells contain a higher percentage of cells in the S and G2/M phases (8.3 and 15.2%, respectively) as compared with serum-starved cells (3.9 and 4.8%, respectively). However, synchronizing cells by serum starvation for a long time results in DNA fragmentation and apoptosis (Gómez et al., 2003a).

As shown in Table 2, the method used for cell synchronization affects the frequency of fusion and blastocyst development in domestic cat and African wildcat embryos (Gómez et al., 2003a) but not found in odd-eyed cloned embryos (Yin et al., 2007). However, in the synchronization of cells by the same method, a higher percentage of blastocysts is observed with African wildcat cells as compared to domestic cells (Gómez et al., 2003a). Thus, factors other than the method of donor nucleus cell-cycle synchronization, such as origin of the donor nucleus, are involved in nuclear reprogramming. However, the possibility of producing cloned offspring by using either cycling (Yin et al., 2007) or quiescence donor cells synchronized by serum starvation (Shin et al., 2002; Gómez et al., 2004; Yin et al., 2005) has been demonstrated. By using cycling and quiescence fibroblasts, one from nine (11%) and three from nine (33%) recipients successfully delivered a total of two and four kittens, respectively (Yin et al., 2007).

1.2.2.3 Reconstruction and culture

To date, cloned embryo production has developed rapidly by the modification and improvement of nuclear transfer techniques. Techniques i.e. fusion, activation and embryo culture have been reported that markedly affect the successful outcome of nuclear transfer.

Fusion

Fusion is an important process in SCNT. Low effectiveness of electrofusion of the somatic donor cell with the recipient oocyte limits cloning success in certain mammals. The induction of electrical pulses during fusion causes a reversible physical breakdown of bi-lipid membranes, resulting in the formation of temporary pores. When juxtaposed pores in the membranes of two cells are resealed, following the application direct current pulses, the cells become fused. The addition of an alternating current pulse before the inducing direct current pulses has produced a better alignment of the recipient cytoplast and donor cells (73–87% fusion rate) (Gómez et al. 2003a).

Table 2. *In vitro* development of domestic and non-domestic cat cloned embryos reconstructed with different cell types synchronized by different cell-synchronization methods

Species	Cell type of donor nucleus	Donor nucleus synchronization method	Fused %	No. developing to morula, blastocyst (%)	Reference
Domestic cat	Cumulus	Serum-starved 5 d	59	43, 4	Gómez et al. (2002)
	Fetal fibroblast	Cycling	60	32, 5	Skrzyszowska et al. (2002)
	Cumulus	Cycling	45	31, 8	Skrzyszowska et al. (2002)
	Fetal fibroblast	Cycling	60	48, 8	Kitiyanant et al. (2003)
	Adult fibroblast	Cycling	65	26, 5	Kitiyanant et al. (2003)
	Cumulus	Cycling	66	38, 3	Kitiyanant et al. (2003)
	Muscle	Cycling	43	42, 11	Wen et al. (2003)
	Adult fibroblasts	Serum-starved 5 d	79	8, 1	Gómez et al. (2003a)
		Contact inhibited	69	11, 3	Gómez et al. (2003a)
		Roscovitine	91	16, 3	Gómez et al. (2003a)
	Fetal fibroblast	Serum-starved 3 d	71	-, 4	Yin et al. (2005)
	Adult fibroblast	Serum-starved 3 d	67	-, 6	Yin et al. (2005)
	Adult fibroblasts	Serum-starved 4 d	61	-, 13	Yin et al. (2007)
		cycling	64	-, 10	Yin et al. (2007)
African wildcat	Adult fibroblasts	Serum-starved 5 d	89	39, 24	Gómez et al. (2003a)
		Contact inhibited	85	41, 20	Gómez et al. (2003a)
		Roscovitine	90	30, 27	Gómez et al. (2003a)

Modified from Gómez et al. (2006)

The protocol for the electrical fusion of a karyoplast into an enucleated oocyte varies among species and cell types. Many factors including the source of oocytes (*in vitro* vs. *in vitro*) (Gómez et al., 2003a), the type of donor cell (Lui et al., 2007), the method and equipment used (Lui et al., 2007) have been reported to affect fusion efficiency. Gómez et al. (2003a) found that *in vivo* matured oocytes are superior to *in vitro* matured oocytes, this being demonstrated by the higher percentage of fusion (97 and 90%, respectively). Regardless of the method used in laboratories, donor cells collected from muscle and cumulus cells provide a lower fusion rate compared to that from skin (43, 45-66 and 60-79%, respectively) (Table 2).

Fusion method is also a factor affecting fusion efficiency. The fusion rate increases by micro-electrode (Fig. 5A) compared with chamber fusion (Fig. 5B) from 72.2 to 89.0% for granulosa cells, 77.1 to 94.6% for fetal fibroblast cells and 51.2 to 78.0% for mammary gland epithelial cells. An orientation of donor cell and enucleated oocyte to the electrode and cell-to-cell contact are important factors to be monitored when fusion is performed (Miyoshi et al., 2001). A low fusion rate may occur due to the donor cell being frequently suspends in or sticking to the zona pellucida after NT, thereby, producing a little or no cell-to-cell contact between the donor cell and the oocyte. Chamber fusion and non-pressurized micro-electrode fusion cannot solve this problem. However, in pressurized fusion, membrane contact can be established and strengthened by pressurization on the couplet using a micro-electrode. It has been discovered that, for couplets whose membranes are not kept in touch, the donor cell can be pushed to and pressed onto the surface of the cytoplast with one micro-electrode. If the fusion pulse is applied, the donor cell can adhere tightly to the membrane of the cytoplast and thereafter, membrane fusion is achieved. The fusion rate increased by about 10% for each micro-electrode protocol used in this research and the results suggest that tightness of membrane contact is one of the critical factors affecting the efficiency of cell fusion (Lui et al., 2007).

These data indicate that fusion efficiency depends on various factors from oocytes, donor cells and methods used. To maximize the success rate *in vivo* mature oocytes and fibroblast cells from skin are preferred for reconstruction by micro-electrode method.

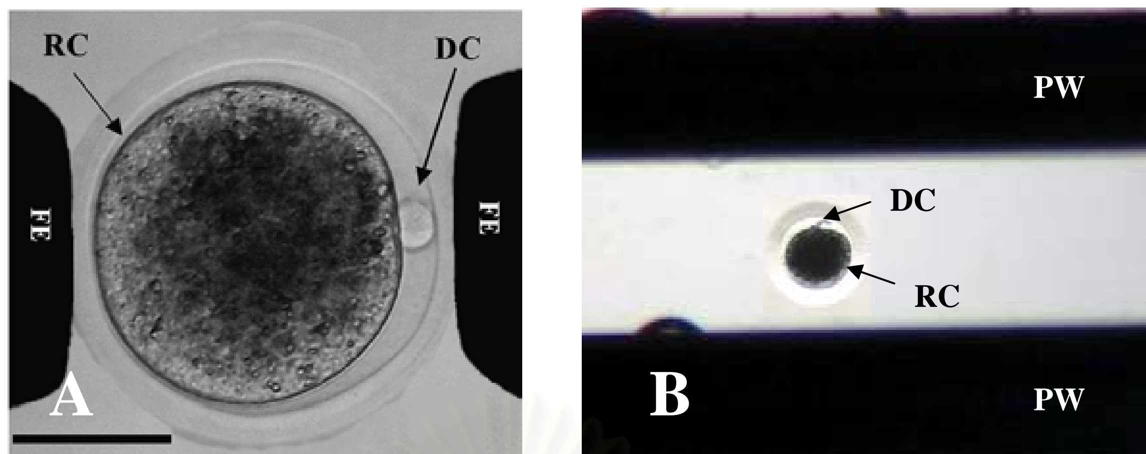

Figure 5. A karyoplast-cytoplast couplet aligned for micro-electrode fusion (A); FE, frustum-end microelectrode; DC, donor cell; RC, recipient cytoplast (Lui et al., 2007). (B) The couplet (arrows) is placed in a fusion chamber between electrical wires connected with electrodes; PW, platinum wire

Activation

Activation of the reconstructed embryos after NT is also one of the key stages in the cloning procedure. This process mimicking natural physiological fertilization provides a proper mechanism for embryo development. Ca^{2+} oscillations and a drop of maturation promoting factor (MPF) and mitogen activated protein kinases (MAPK) activity are the key triggers that initiate a series of biochemical events, leading to full activation including meiosis resumption, pronuclear formation, DNA replication, pronuclear apposition, nuclear envelop breakdown and chromatin condensation and readiness for the first cleavage division (Alberio et al., 2001).

Parthenogenesis or the production of embryos from the female gamete in the absence of any contribution from the male gamete, by activating oocytes during chromosome condensation with a high level MPF and MAPK has been commonly used as a control for SCNT study by reflecting the effectiveness of the activation protocol (Alberio et al., 2001). Electrical stimulation and chemicals are commonly used in the activation process for cat oocytes. Without electrical stimulus, inducing by calcium ionophore (Karja et al., 2006; Kitiyanant et al., 2003), ethanol and magnetic field (Grabiec et al., 2007) combined with other chemicals, has also been performed. The chemicals supplemented in the activation medium such as 6-dimethylaminopurine

(6-DMAP) (Kitiyanant et al., 2003) and cycloheximide (Skryszowska et al., 2002), have been used, resulting different success rates.

Embryo culture

There are two arrests in feline embryonic development under *in vitro* conditions. The first embryonic arrest happens at the 5- to 8-cell stage, which corresponds to the transition period from maternal to embryonic control and the second arrest happens between the morula and blastocyst stages (Kanda et al. 1995). Currently, embryo culture conditions have developed rapidly, aiming to overcome the embryo developmental block. The culture media for cat embryos are different among laboratories, i.e. B2 (Skryszowska et al., 2002), Tyrode's balanced salt solution (Pope et al., 2004), Ham F-10 (Kitiyanant et al., 2003), SOFaa (Freistedt et al., 2001) and CR1aa (Yin et al., 2006). Sequential changes of composition in the media by different concentrations of amino acid and fetal bovine serum supplementations have been demonstrated to enhance the embryo developmental rate (Karja et al., 2002). In addition, culture embryos co-cultured with BRL cells (Skrzyszowska et al., 2002), or oviductal epithelial cells (Kitiyanant et al., 2003) have been reported to have been able to overcome morula to blastocyst block. The supplementation of growth factors, particular epidermal growth factor and insulin-like growth factor during oocyte maturation has had positive effects in terms of further embryo development (Kitiyanant et al., 2003; Merlo et al., 2005). The co-culturing of cat embryos with heterospecific companion embryos (mouse) has been shown to dramatically improve the quality of cat embryos in terms of blastocyst cell numbers and hatching ability (Spindler et al., 2006).

1.2.2.4 Genetic distance of recipient oocyte and donor cell

The inter-species/genus NT is the transfer of the cell nucleus of one species/genus into an enucleated oocyte of another, offering a possible approach to clone endangered animals. A high percentage of inter-generic cleaved cloned embryos 65–84%, has been reported (Gómez et al., 2004b, Lorthongpanich et al., 2004; Yin et al., 2006) (Table 3). This indicates that domestic cat oocyte cytoplasm may contain certain chromatin remodeling factor(s) necessary to initiate the remodeling of the transferred nuclei of species of a different genus (Gómez et al., 2006a). However, lots of embryos failing to develop beyond blastocyst have been found.

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The cloned embryos derived from African wildcat somatic cell lines develop to the blastocyst stage *in vitro* (28%) at rates comparable to those of domestic cat embryos produced by ICSI (19–30%) (Gómez et al., 2000), whereas, only 13.9 and 3.3% of domestic cat cloned embryos reach morula and blastocyst (Gómez et al., 2003a), suggesting that inter-specific nuclear and cytoplasmic incompatibilities may not contribute to the developmental failure of the non-domestic felid inter-generic cloned embryos. However, abnormal nuclear remodeling of the differentiated nucleus has been suggested as a major constraint on the *in vitro* development of embryos (Gómez et al., 2006a). Leopard cat ig-NT embryos have been successfully generated with similar results of embryo development during cleavage to the morula stage of domestic cat. However, the blastocyst rate of inter-generic leopard cats was lower than those of domestic cats (Lorthongpanich et al., 2004). Six of 435 transferred leopard cat embryos developed to fetus at 30-45 days of observation but a full-term fetus was not obtained (Yin et al., 2006). The failure of pregnancy may have occurred because foreign mitochondria were introduced when conducting inter-species/generic NT procedures. Mitochondrial heteroplasmy may be a key factor that affects embryonic development and the *in utero* survivability of the nuclear transfer embryos (Gómez et al., 2006a).

Table 3. *In vitro* development of inter-species and inter-generic cloned embryos reconstructed by the fusion of somatic cells of non-domestic felids with enucleated domestic cat oocytes (Gómez et al., 2006a)

Species	No. of fused/injected (%)	No. of cleaved (%)	No. of blastocyst (%)	Reference
Korean tiger *(Panthera tigris altaica)*	N/A [a] (54)	N/A (65)	N/A (9)	Hwang et al. (2001)
African wildcat *(Felis silvertris lybica)*	425/484 (88)	357/425 (84)	101/357 (28)	Gómez et al. (2004a)
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. (2004b)
Black-footed cat *(Felis nigripes)*	170/170 (100)	144/170 (84.7)	144/170 (2.1)	Gómez (unpublished data)

[a] N/A: nubers not available.

Using bovine ooplasm, which can support differentiation of the introduced nuclei (Dominko et al., 1999), the genetic divergence between donor cells and recipient oocytes may indeed represent a limiting factor influencing NT outcome. In a study of minke whale NT, Ikumi et al. (2004) found that whale granulosa-cumulus cells have the ability to dedifferentiate in bovine and porcine oocytes. However, the inter-species NT embryos can not develop beyond the 4-cell stage. This might be related to a developmental cell block and a mitochondrial incompatibility between the recipient oocytes and donor cells.

It has been demonstrated that some factor(s) 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 study of Chen et al. (2002) is a good model for inter-genus NT, demonstrated by the implantation of panda-rabbit cloned embryos in domestic cat oviducts.

In summary, the host 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. At present, lived endangered cloned animals produced from closely genetic oocytes, different in species, have been achieved. This reveals 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.

1.2.2.5 Reprogramming

A reprogramming process of somatic cells after NT is suggested to involve changes in chromatin configuration and epigenetic DNA modification, such as DNA methylation. As shown in Fig. 6, in fertilized embryos, the sperm nuclear envelope breaks down after fertilization and the chromatin decondenses as the protamines are replaced with oocyte-specific histone H1FOO and core histones. Pronuclei form and transcription begins at mid-S phase, with apparent differences between maternal and paternal pronuclear transcriptional activity and probably a limit in total transcriptional activity. DNA replication is accompanied by changes in chromatin structure that permit transcription. The second round of DNA replication during the 2-cell stage is

accompanied by further changes in chromatin structure, allowing transcriptional regulation and increased transcription. The H1FOO is removed and replaced with somatic H1 variants, beginning during the 2-cell stage. A temporary paucity of H1 linker histone during the 2-cell stage may contribute to precocious transcriptional activity. Thereafter, the embryonic gene expression programme (late period), having been set in motion by the early events, directs normal development.

In cloned embryos, somatic cell nuclear envelope breakdown occurs and chromosomes condense before oocyte activation. Pseudopronuclei form, transcription begins during mid-S phase and, due to incomplete reprogramming, some genes are aberrantly expressed. Because there is no parental origin difference between pseudopronuclei and because the somatic genome may direct expression of an enhanced array of transcription factors, transcription may be elevated relative to fertilized zygotes. DNA replication permits chromatin remodelling. Histone H1 switching is recapitulated, along with a further transcriptional increase. Chromatin remodelling and nuclear reprogramming continue during cleavage but there continues to be a mixed embryonic–somatic gene expression profile. Some cells fail to acquire Dnmt1 from the ooplasm at the 8-cell stage, leading to imprinting defects in some of the daughter cells and possibly random X chromosome inactivation later in the developing somatic lineage.

For female donor cell nuclei, the previously silent X chromosome becomes reactivated. Cells with a suitable degree of reprogramming and retention of imprinting may become selectively allocated to the inner cell mass (ICM), while the other cells may become selectively allocated to the trophectoderm lineage. The latter process may contribute to placental defects. The number of clones that can develop to term will be determined, in part, by the fraction in which a minimum required number of cells arises with correct reprogramming and imprinting to contribute to the ICM. Nuclear reprogramming during the late period may be dependent on early reprogramming events. Reprogramming may continue after ICM formation, even up to the time of gastrulation. Embryos with inadequate reprogramming will generally fail around the time of gastrulation (Latham et al., 2005).

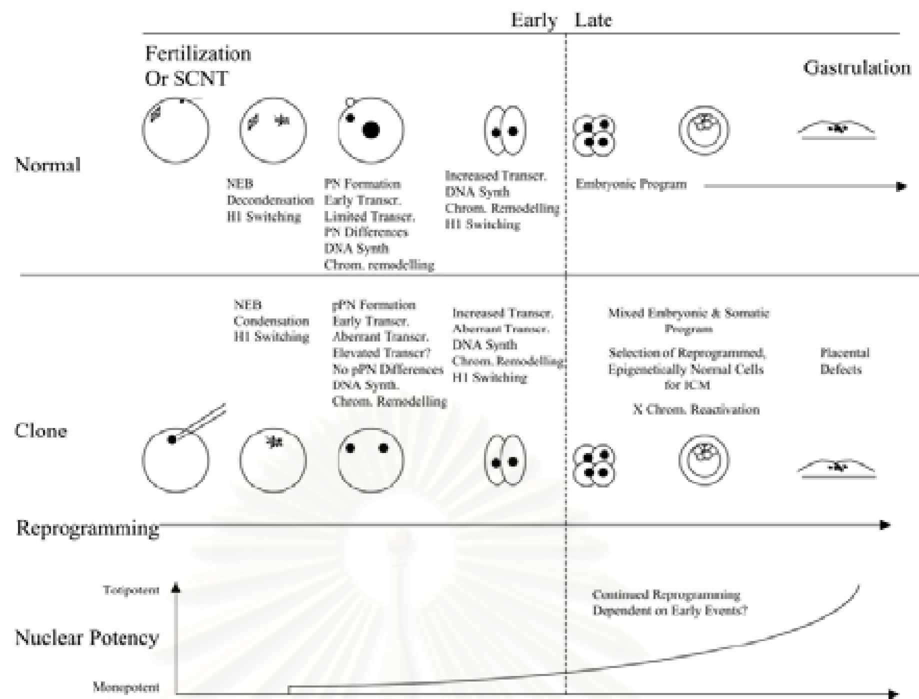

Figure 6. Summary of early and late events of nuclear reprogramming in normal fertilized and somatic cell nuclear transfer mouse embryos and its possible effects on development (Latham et al., 2005)

Successful SCNT requires erasing the cellular memory, or program, present in differentiated nuclei by disrupting the regulatory features controlling the expression of numerous genes. Erasing the memory in a differentiated nucleus or reprogramming with the molecules residing in the ooplasmin will allow for a switch in gene expression and enable conversion from a somatic cell function to embryonic development (Eilertsen et al., 2007). The inaccessible gene regions are often modified by methylation of the DNAbase cytosine and of the associated histone proteins. These methylations are passed on to the daughter cells but they may be modified by cytoplasmatic factors such as those occurring in the cytoplasm of oocytes. If reprogramming is very efficient, so that most or all somatic genes are silenced and the appropriate array of embryonic genes is programmed for expression, then transcriptional activation should result in cloned embryos with characteristics much like those of normal fertilized embryos. If, however, reprogramming is very slow or very inefficient, then transcriptional activation will lead to the expression of a variable array of genes previously expressed in the somatic cells (Latham, 2005).

In felid inter-species/generic NT, while a high percentage of cloned couplets undergo cleavage, most of them arrest at early cleavage stages. This can suggest that cat cytoplasm induces early remodeling of nuclei of a different species/genus and a high incidence of early developmental arrest may be caused by abnormal nuclear remodeling. The ability of donor nucleus reprogramming has been commonly evaluated by detecting the expression of the POU transcription factor Octamer-4 transcript (Oct-4), which is confined to totipotent and pluripotent cells (Surani, 2001; Yeom et al., 1996). While cat cytoplasm induces early nuclear remodeling of cell nuclei from a different genus and is able to develop to the blastocyst stage, a high incidence of early embryo developmental arrest occurrs and it is believed that this may be caused by abnormal nuclear reprogramming. In cat IVF blastocysts, Oct-4 has been detected only in the inner cell mass. Even this signal can be detected in most sand cat cloned blastocysts, some exhibiting aberrant expression in the trophectoderm (44%). The abnormality of this gene expression localized in the trophectoderm may contribute to the failure of cloned embryos to undergo postimplantation development (Pope et al., 2007).

Recently, analysis of the methylation patterns of repetitive sequences such as satellite DNA regions revealed different degrees in the placenta of normal and cloned kittens. The placental cells from three cloned kittens showed significantly higher DNA methylation (range, 76.7–80.5%) than placental cells from normal domestic cats (range, 64.2–74.9%). However, the somatic cells from normal domestic and Turkish Angora cats, the somatic cells from the 11 cloned cats (including stillborn and those dying early) showed an equal degree of hypermethylation. Whereas, methylation in somatic cells may not be responsible for stillbirth, early fetal death, or different eye and hearing attributes of cloned cats, hypermethylation in the placenta of cloned cats may be responsible for low success rates in cloning cats (Cho et al., 2007). Moreover, attention has subsequently turned to include the functional aspect of methylation changes and its role on the imprinted genes. Imprinted genes are particularly interesting since they are related to the balance of growth in the fetus versus fetal membranes. Imprinted genes show a parentage-specific, stable silencing of one of the two alleles and silencing is often related to differential methylation of the two alleles. Since disturbances in placental as well as fetal, growth has been repeatedly associated with *in vitro* culture and SCNT; these genes are key candidates for a study of epigenetic alterations due to SCNT. For example, IGF2R is an imprinted gene and

changes in its fetal expression could therefore be due to alteration of its methylation programme induced by the high serum level during *in vitro* development and thus cause of the overgrowth of fetus and placenta (Young et al., 2001).

1.2.2.6. Embryo transfer

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). 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., 2004a). The high number of domestic cat cloned embryos derived from fetal (140 one cell) and adult donor cells (65 two to four cell) has been transferred to recipients, resulting in 1 stillborn and 2 lived kittens obtained from fetal donor cells and 1 kitten from adult donor cells (Yin et al., 2006). Recently, the number of transferred embryos has been reduced to about 16 embryos at 2-4 cell stage per recipient which are able to establish pregnancy and deliver live kittens (Yin et al., 2007).

A high implantation and survival rate has been observed (20%), after the transfer of five domestic cat cloned embryos (three derived from cumulus cells and two derived from fibroblasts cells) to a recipient that subsequently produced the first live domestic cat cloned kitten (Shin et al., 2002). Micro-satellite analysis of cells collected by oral swab confirmed that the cloned kitten was derived from the cumulus cell line. Conversely, the implantation rate (1.2%) without pregnancy to term after the transfer of domestic cat cloned embryos derived from fibroblasts has been reported (Shin et al., 2002). This result was similar to the transfer of black-footed cat or African wildcat embryos derived from fibroblast cells (Gómez et al., 2004a; 2005). Whereas 24% (7/29) of recipients receiving sand cat cloned embryos became pregnant but live kittens could not be obtained; one kitten died *in utero* and one died 2 h after caesarean section (Pope et al., 2007).

Meanwhile inter-generic leopard cat cloned embryos have been transferred to a domestic cat surrogate mother, 4.8% (21/435) of the embryos were implanted,

1.4% (6/435) developed to fetus but not to term (Yin et al., 2006). In research by the same team, recipients received transgenic odd-eyed cat embryos giving a greater pregnancy rate (27.3%; 3/11) and 18.2% (2/11) of recipients delivered live offspring (Yin et al., 2007).

The major limitation of *in vivo* development is the embryonic loss found in several species that may be caused by incomplete reprogramming of the differentiated nucleus (Yin et al., 2006; Pope et al., 2007). The aberrant of Oct-4 expression in the trophectoderm found in black-footed cat and sand cat embryos may be one of the causes of early embryonic loss resulting in a cessation of development occurring between day 22 to 29 of gestation. Even though the embryos are able to be implanted, the fetus can not develop to term (Gómez et al., 2005; Pope et al., 2007).

A single domestic cat cloned embryo that was implanted ceased development by day 44 of gestation and at least one-third of the African wildcat cloned fetuses ceased development between day 30 and 50 of gestation. It appears that placental atrophy may be the principal cause of the loss of African wildcat fetuses, as similarly reported in cloned mice, cattle and sheep. In addition, placental dysfunction results in premature separation before delivery and, secondarily, respiratory failure due to lung immaturity (Gómez et al., 2004a).

Although, several live domestic and African wildcat cloned kittens have been born that are seemingly normal and healthy, abnormalities such as abdominal organ exteriorization, respiratory failure and immune dysfunction have been reported and suggested to be the main causes of cloned neonatal death (Gómez et al., 2004a; Yin et al., 2006, 2007). In sheep, cows and mice, an abnormality such as "Large Offspring Syndrome" (LOS) refers to increased birth weight, which is caused by the significantly altered levels of the fetal growth factor IGF2R and of simultaneous alterations in the methylations status of the regulatory part of this gene (Young et al., 2001). However, this kind of abnormality has not been yet reported in felid cloned offspring, and this may be because the cloned embryos are normally transferred at an early stage.

Other factors that may relate to developmental anomalies are inappropriate donor cell or/and recipient oocyte, inappropriate synchrony between the cell cycle phase of the donor nucleus and recipient cytoplasm, inappropriate handling of oocytes, somatic cells and embryos during maturation, various manipulations and cultural techniques causing mechanical, osmotic, electrical, toxic, thermal and other types

of damage and incomplete reprogramming (Vajta and Gjerris, 2006). In addition, it is necessary for a better understanding of cloned offspring development i.e. its growth, fertility, other abnormalities and lifespan to be investigated in the future.

According to the above review, many factors influence SCNT success in terms of the capability of embryo development to the blastocyst and to-term pregnancy. Current knowledge demonstrates the possibility of using inter-species/generic NT for producing wild felid species. However, up to now, there has been no SCNT study reported in marbled cats and flat-headed cats. Thus, SCNT research in these species is required for providing an insight into embryo development and as application for offspring production in the future.

1.3 Objectives of the thesis

1. To establish an appropriate SCNT protocol including IVM, fusion and activation in felid species.
2. To investigate *in vitro* development of marbled cat and flat-headed cat cloned embryos using domestic cat and rabbit oocytes as recipient cytoplasm.
3. To investigate *in vitro* development of domestic cat IVF embryos and their transfer to recipients and pregnancy establishment of recipients receiving flat-headed cat cloned embryos.

1.4 Hypothesis

1. IVM process, fusion and activation protocols affect the efficiency of felid embryo production.
2. Marbled cat and flat-headed cat cloned embryos can be produced from domestic cat and rabbit oocytes as recipient oocytes**.**
3. Recipients receiving domestic cat IVF embryos and flat-headed cat and domestic cat cloned embryos can become pregnant.

1.5 Key words: somatic cell nuclear transfer, embryo development, marbled cat, flat-headed cat

1.6 Research merits:

1. Appropriate procedures of SCNT including IVM, fusion and activation to be used in felid species
2. Information on the *in vitro* development of domestic cat, marbled cat and flat-headed cat cloned embryos using domestic cat and rabbit oocytes as recipient cytoplasm
3. Techniques of embryo transfer and *in vitro* fertilization in felid species.
4. The possibility of producing flat-headed cat and domestic cat offspring by SCNT

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CHAPTER II

ESTABLISHMENT OF SOMATIC CELL NUCLEAR TRANSFER PROTOCAL IN MARBLED CAT AND DOMESTIC CAT

2.1 Abstract

The objective of the study was to evaluate the use of Somatic Cell Nuclear Transfer (SCNT) techniques in marbled cats (MC; *Pardofelis marmorata*), using enucleated domestic cat (DC) oocyte as a recipient cytoplasm. The recipient oocytes were obtained from ovariohysterectomized cats. Donor cells were obtained from a male MC died in captivity. Experiment I was conducted to observe the development of cloned MC-DC embryos (donor cells- recipient oocytes), derived from oocytes matured *in vitro* for 24, 36 and 42 h. The results showed that the developmental success of MC-DC cloned embryos at the 4-8 cell and the morula stages, were greater (P<0.05) in group of oocytes cultured for 24 h than those cultured for 36 and 42 h. Experiment II was conducted to compare the fusion rate of MC-DC couplets, fused by inducing different fusion voltages and pulses (2 direct current pulses of 2.1 and 2.4 kV/cm for 80 µs and 3 pulses of 2.4 kV/cm for 50 µs). The results showed that there were no differences in fusion efficiencies in among fusion protocols (P>0.05). Experiment III was conducted to compare the developmental kinetics of MC-DC and DC-DC cloned embryos using IVF cat embryos as a control. The development of MC-DC and DC-DC cloned embryos to the 4-8 cell, morula and blastocyst stages was not different (P>0.05). However, the development rates at morula and blastocyst stages in the control group were greater than those of cloned embryos (P<0.05). Experiment IV was conducted to evaluate activation protocols using parthenogenic embryos. Cat oocytes matured for 24-27 h were activated by 3 direct current pulses of 1.2 kV/cm for 50 µs and incubated in 10 µg/ml cycloheximide or 10 µg/ml cycloheximide in combination with 5 µg/ml cytochalasin B. The results demonstrated that blastocyst debelopment was significantly improved (P<0.05) in group of oocytes cultured in activation medium containing cycloheximide and cytochalasin B (8.3 vs. 35%). In conclusion, DC oocyte matured *in vitro* for 24 h could be used as recipient ooplasm for cloned MC embryos. The MC-DC couplets can be fused successfully by the introducing of 3 pulses of 2.4 kV/cm for 50 µs. Furthermore, the MC fibroblast cells demonstrate the ability to be reprogrammed in the DC oocytes, resulting in the

development to the morula stage. Exposure of mature DC oocytes to cycloheximide and cytochalasin B after electrical stimulation improves the rate of parthenogenic development and blastocyst formation.

2.2 Introduction

The success of somatic cell nuclear transfer (SCNT) in domestic cat (DC; *Felis catus*) has been reported since 2002 (Skrzyszowska et al., 2002). In the previous study, low numbers of cloned embryos are obtained due to the low IVM success of cat oocytes and unclear SCNT protocols tested and used in felid species. Thereafter, the delivery of the first cloned DC has been assured the feasibility to produce genetically valuable identical cat offspring by SCNT (Shin et al., 2002). Then SCNT has been extensively studied in the DC and has been major research interest in many laboratories including those in the USA (Gómez et al., 2003a), Thailand (Kitiyanant et al., 2003; Lorthongpanich et al., 2004), South Korea (Yin et al., 2005) and Japan (Karja et al., 2006).

For endangered felids, the inter-species/genus NT defined as the technique of transferring of cell nucleus of one species/genus into an enucleated oocyte of different inter-species, offers a possible approach to clone endangered animals. The oocyte collected from genetically closely related animals, the DC are used to produce African wildcat-DC (donor cell-recipient oocyte) (Gomez et al., 2004a) and leopard cat-DC embryos (Lorthongpanich et al., 2004; Yin et al., 2005). The birth of cloned African wildcats demonstrates the feasibility to produce offspring for the preservation of endangered species (Gomez et al., 2004a). Among nine felid species in Thailand, the marbled cat (MC; *Pardofelis marmorata*) is one of the most least known wild cat species and is at critical risk to extinction. The information regarding wild population of MC is note available. Furthermore, there was no MC in captivity collection at any zoo in Thailand. Fortunately, MC cell lines have been established and frozen in the cryobank under the collaboration of the Zoological Park Organization and Chulalongkorn University, and are made available for this experiment. Thus, the inter-generic NT (ig-NT) is a valuable tool to produce MC-DC embryos for studies of embryology and allow experimental transfer to recipients to generate live offspring in the future. However, although the DC (Shin et al., 2002) and African wildcat (Gómez et al., 2004a) offspring can be produced by the SCNT, the embryo production and pregnancy remain low. Attempts to overcome the failure of embryo development are required.

Several steps involved in the SCNT, i.e. oocyte *in vitro* maturation (IVM), fusion with donor cell and activation of reconstructed embryos, influence SCNT success. The maturation of oocytes *in vitro* is an initial step in both *in vitro* embryo production and SCNT. This step aims to prepare nucleus and cytoplasm of oocyte to be fertilizable. These two main events are known as nuclear and cytoplasmic maturation. The IVM oocytes are commonly used for producing cat embryo because they are reliable alternative, abundant and easily obtained (Gómez et al., 2006). This technique may eventually allow the rescue of genetic materials from endangered felids species (Donoghue et al., 1990). The overall IVM efficiency of cat oocytes (40-60% of maturation rates) is generally lower than that of livestock (Farstad, 2000). In the past decade, several studies of cat IVM have been conducted to optimize culture media and conditions that maximize maturation success. Many factors influencing the meiotic competence and *in vitro* development of DC oocytes i.e. morphological criteria for selecting good quality oocytes (Farstad, 2000; Wood and Wildt, 1997), culture period (Johnston et al., 1989; Pope et al., 1994), culture media supplementation (Goodrowe et al., 1991) and season (Freistedt et al., 2001) have been reported. Although, the IVM oocytes have been successfully used for generating DC cloned offspring (Shin et al., 2002), the optimal condition for IVM of recipient oocytes to be used for cloning has not been systematically investigated.

Fusion is the process of electrical pulses induction causing a reversible physical breakdown of bi-lipid membranes, resulting in a formation of temporary pores between donor cell and plasma membrane. When juxtaposition pores in the membranes of two cells reseal, the cells become fused. Factors including the source of oocytes (*in vitro* vs. *in vivo*; Gómez et al., 2003a), type of donor cells (review by Gómez et al., 2006), methods and equipments (Lui et al., 2007) have been reported affecting fusion efficiency. Thus, the fusion protocol is needed to be optimized.

Activation of the reconstructed embryos after NT is also one of the key steps in the cloning procedure. This process mimics the physiology of fertilization and provides a proper mechanism for early embryo development. The event during activation involves the level of Ca^{2+}, maturation promoting factor (MPF) and the mitogen activated protein kinases (MAPK). Parthenogenesis or production of embryos from female gamete in the absence of any contribution from male gamete, by activating oocytes during chromosomes condensation with high level MPF and MAPK has been commonly used as control for SCNT study by reflecting success of activation

protocol (Alberio et al., 2001). Beside electrical stimuli, calcium ionophore (Karja et al., 2006; Kitiyanant et al., 2003), ethanol and magnetic field (Grabiec et al., 2007) combined with other chemicals, have been investigated. The chemicals used in activation medium such as 6-dimethylaminopurine (6-DMAP; Kitiyanant et al., 2003) and cycloheximide (Skryszowska et al., 2002), have been shown to produce different successes. Cytochalasin B supplementation in activation medium is targeted to generate the correct ploidy embryos by inhibiting the second polar body extrusion. It has been used alone (Yin et al., 2005) or in combination with cycloheximide (Gómez et al., 2003a). However, the mechanism of the transduction is not clearly understood and it varies among species. Furthermore, it has not been proven that cytochalasin B has a positive effect on the activation as well as further embryo development.

Due to fundamental insights and information of MC cloned embryo development that have not been studied, the establishment of optimal procedure of NT is required. Therefore, the overall goals of present studies attempt to optimize conditions for producing MC-DC cloned embryos including; 1) suitable duration for maturation of recipient oocytes (Experiment I); 2) fusion efficiency of MC-DC couplets from different fusion voltages (Experiment II); 3) developmental kinetics of MC- and DC-DC cloned embryos (Experiment III); and 4) optimal activation protocol for cat oocytes (Experiment IV).

2.3 Materials and methods

All chemicals were purchased from Sigma Co. (St Louis, MO) unless otherwise stated. Media were prepared weekly, filtered (0.2 μ, # 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 at least 4 h before use.

2.3.1 Preparation of recipient cytoplasm

Domestic cat ovaries were obtained after ovariohysterectomy and stored at room temperature in phosphate buffer saline (PBS; Gibco, USA), supplemented with 5% fetal bovine serum (FBS; Gibco, USA), and 10 IU/ml penicillin and streptomycin (Gibco, USA). Oocytes were collected within 4 h after ovaries were removed by mincing the ovaries in an oocyte collecting medium composed of Dulbecco's modified eagle medium (DMEM; Gibco, USA) supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% bovine serum albumin (BSA fraction V), 100 IU penicillin, 100 μg/ml streptomycin and 10 mM hepes buffer. Only oocytes with compact cumulus cells of more than 2 layers and homogenous dark ooplasm were selected (Fig. 7A, B).

Oocytes were washed in the oocyte collecting medium 3 times, and once in an oocyte culture medium composed of DMEM supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin, 100 μg/ml streptomycin, 1 μg/ml porcine luteinizing hormone (LH), 1 μg/ml porcine follicle stimulating hormone (FSH) and 1 μg/ml oestradiol (Wood and Wildt, 1997). Group of 5 to 10 oocytes were cultured in a 50 μl drop of oocyte culture medium, at 38.5°C, under 5% CO_2 in air, for 24, 36 and 42 h (Experiment I) and for 24 h (Experiments II, III and IV) (Fig. 7C). At the end of cultured, cumulus cells were removed from the oocytes by gentle pipetting in tissue culture medium 199 (TCM199) contained 25 mM hepes buffer, 5% FBS and 0.1% hyaluronidase. Oocytes presenting the 1st polar body were classified as matured (Fig.7D) and later confirmed by nuclear staining using 15 μg/ml Hoechst 33342. Mature oocytes were selected and enucleated in a handling medium; TCM199 supplemented with 25 mM hepes containing 7.5 μg/ml cytochalasin B. Enucleation was performed by aspirating the 1st polar body and metaphase II (MII) chromosomes with a small volume of surrounding cytoplasm visually under UV light (Fig. 8A-D).

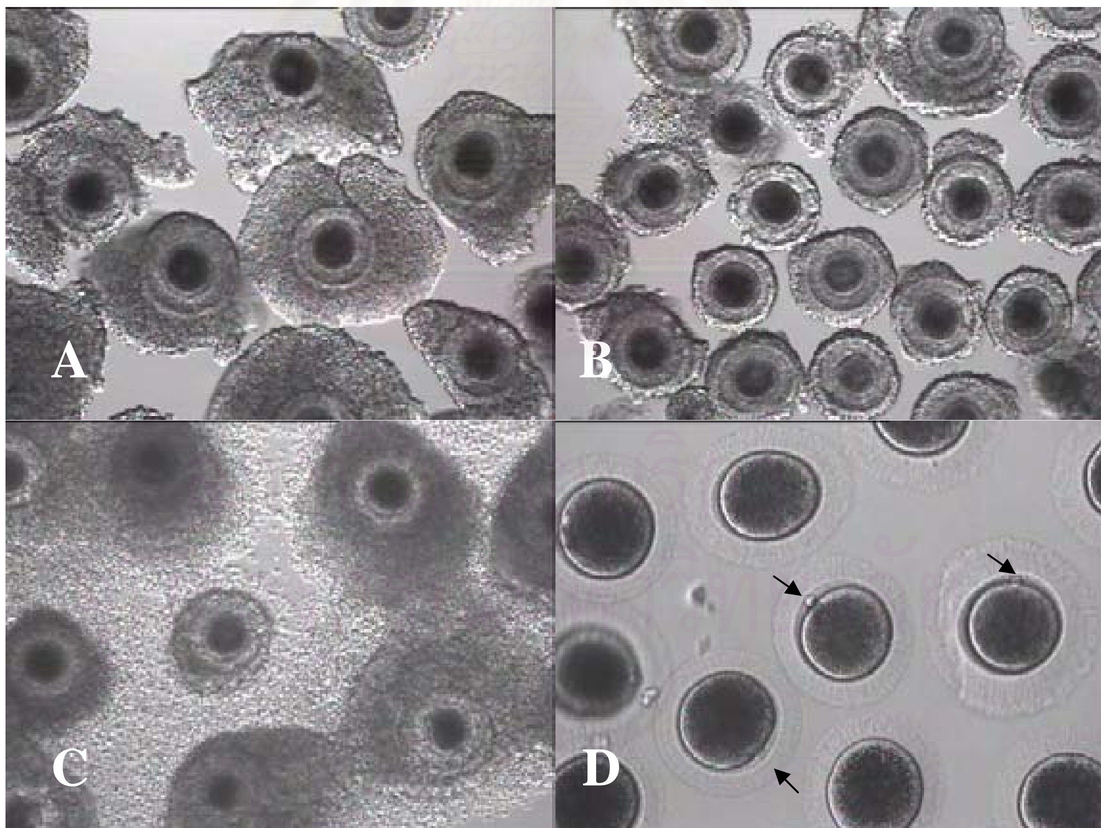

Figure 7. Immature and mature DC oocytes; DC oocytes with compact cumulus cells of more than 2 layers and homogenous dark ooplasm were selected (A, B) and cultured for 24 h (C) (X80). Mature oocytes presenting the 1st polar body (arrows) were selected for NT (D) (X100).

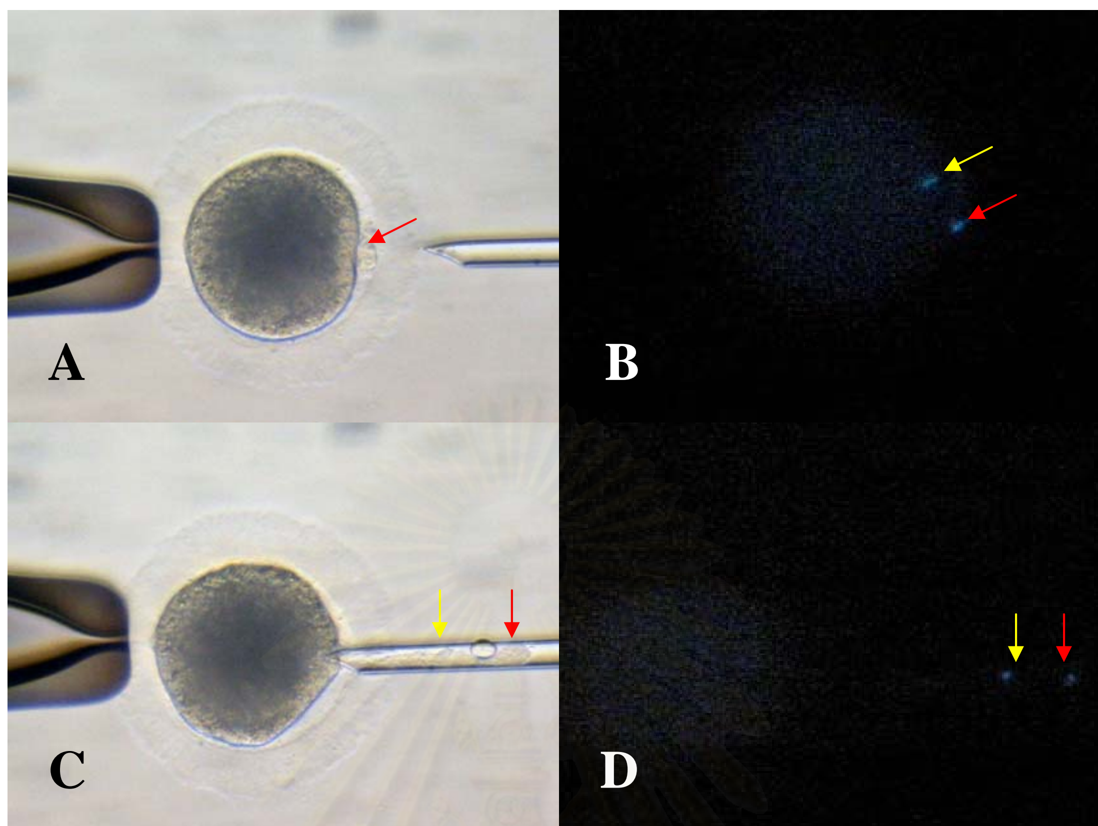

Figure 8. Enucleation of DC oocyte; The MII chromosomes (yellow arrows) and 1st polar body (red arrows) of mature oocyte (A) stained by Hoechst 33342 (B) enucleated by aspirating the 1st polar body and MII chromosomes with a small volume of surrounding cytoplasm (C) located under UV light (D) (X200).

2.3.2 Preparation of donor nuclei

Muscle tissue from a dead MC from the Khao Kheow Open Zoo in Chonburi province, and a domestic cat were stored in DMEM supplemented with 5% FBS, penicillin and streptomycin, at 4°C, within 12 h after collection. Tissues were washed in DMEM, sliced into small pieces and cultured in a 30 mm petri-dish containing DMEM supplemented with 10% FBS, penicillin and streptomycin, at 38.5°C, under 5% CO_2 in air. Fibroblast cells (Fig. 9A) were sub-cultured by washing with PBS, trypsinised by 0.25% trypsin and washed in DMEM, supplemented with 5% FBS and centrifuged at 1,000 g for 5 min. Fibroblast cells were cultured in DMEM, supplemented with 10% FBS, penicillin and streptomycin, in a 60 mm petri-dish, at 38.5°C, under 5% CO_2 in air. Confluent cells were frozen with 10% DMSO in FBS and stored in liquid nitrogen for future use. Frozen cells, between passages 4-10 of the

culture, were thawed and used as a source of donor nuclei. Fibroblast cells were cultured as previously described, until reaching 60% confluency, then starved by culturing in DMEM, supplemented with 0.5% FBS, at 38.5°C, under 5% CO_2 in air, for 1-5 days prior to the NT procedure. The starved cells were trysinised and kept in DMEM supplemented with 10% FBS at room temperature until used (Fig. 9B).

2.3.3 Nuclear transfer, fusion and activation

Nuclear transfer (NT), fusion and activation methods were performed following Skrzyszowska et al. (2002). An individual donor cell was transferred into the perivitelline space of an enucleated oocyte (Fig. 10A,B). The NT oocytes were washed and incubated in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.05% fatty acid-free BSA) for 1 min. The NT oocytes were placed in a fusion chamber between 2 platinum electrodes and overlaid with fusion medium (Fig. 11A,B). Then the NT oocytes were induced by 2 direct current pulses of 2.0 kV/cm for 15 µs (experiment I); 2.1 kV/cm (Kitiyanant et al., 2003) or 2.4 kV/cm (Gómez et al., 2003a) for 80 µs or 3 pulses of 2.4 kV/cm for 50 µs (experiment II) and 2.4 kV/cm for 80 µs (experiment III). The couplets were evaluated for the success of fusion 1 h later by observing the absence of the donor cell (Fig. 12A-D).

Thereafter, they were incubated in B2 medium (INRA, France; Skrzyszowska et al., 2002) supplemented with 10% FBS at 38.5°C, in 5% CO_2, for 2 h. The reconstructed NT embryos were activated by exposing them to 7% ethanol for 5 min in experiment I or by inducing them with 2 pulses of 1.2 kV/cm for 40 µs in experiments II and III. They were then incubated in B2 medium, supplemented with 10% FBS and 10 µg/ml cycloheximide, at 38.5°C, in 5% CO_2, for 4 h. In experiments II, and III, a synthetic oviductal fluid plus 1% essential and 1% non essential amino acids (SOFaa) (Freistedt et al., 2001) was used to replace B2 medium. In experiment IV, reconstructed embryos were induced by 3 pulses of 1.2 kV/cm for 50 µs and incubated in SOFaa medium, supplemented with 5% FBS, 10 µg/ml cycloheximide with or without 5 µg/ml cytochalasin B, at 38.5°C, in 5% CO_2, for 4 h (Table 4).

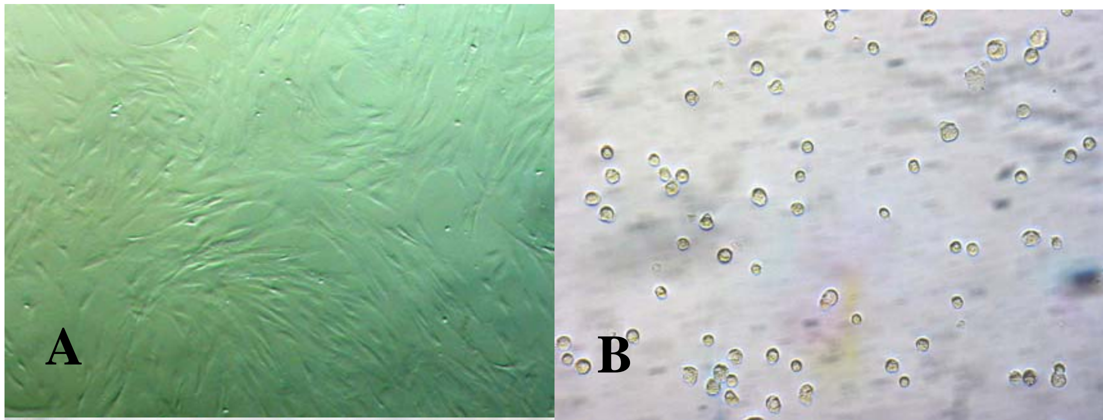

Figure 9. The MC fibroblast cells; the monolayer of MC fibroblast cells (A) cultured from muscle tissue of marbled cat. Donor cells were trypsinised before NT process (B) (X100).

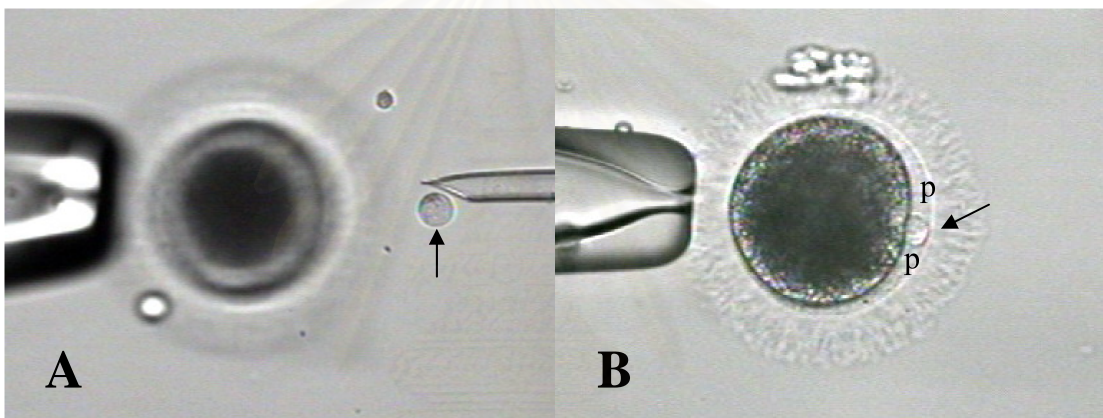

Figure 10. Nuclear transfer method; an individual donor cell (arrows) was selected (A) and transferred into the perivitelline space (p) of an enucleated oocyte (B) (X200).

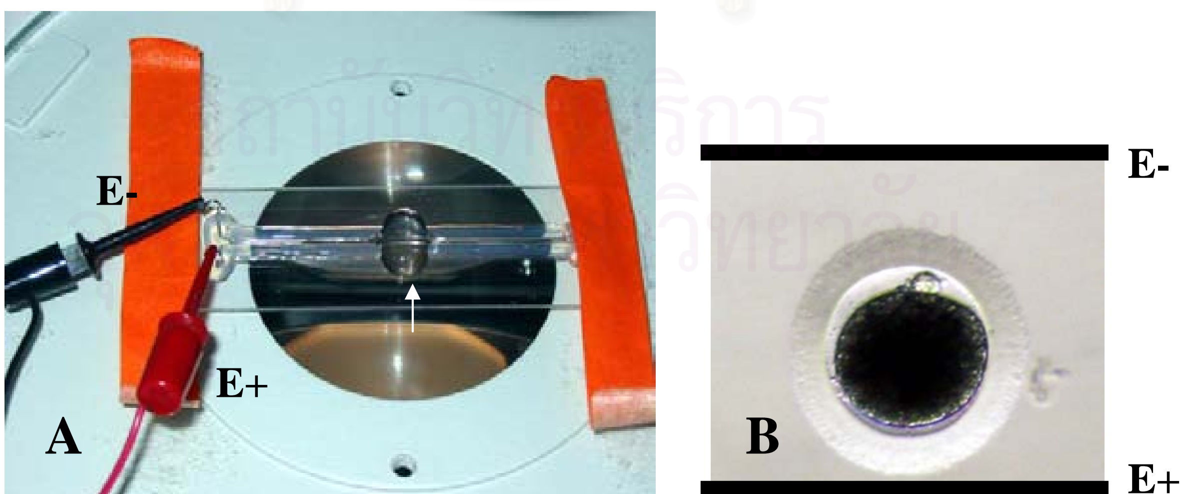

Figure 11. Fusion of NT couplets by electrical pulses; a fusion chamber overlaid with fusion medium (arrow) (A), NT couplets placed between 2 platinum electrodes (E+, E-) (B)

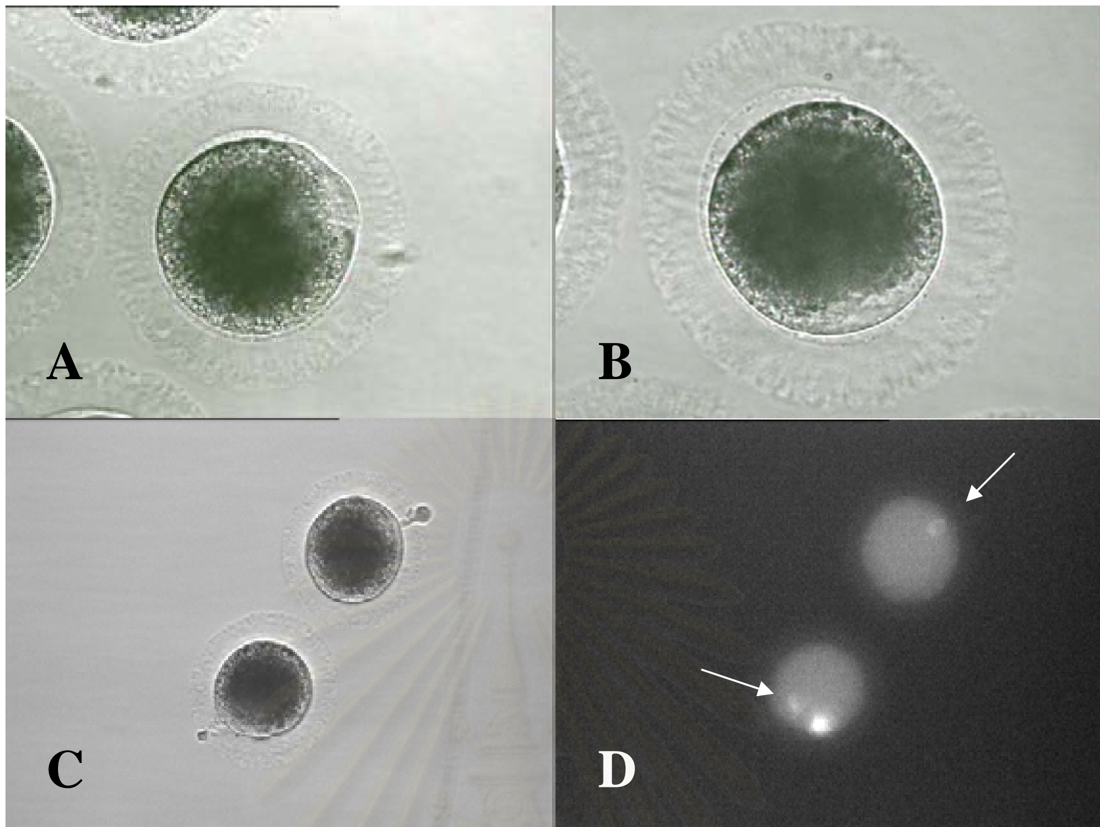

Figure 12. The evaluation of fusion; success of fusion confirmed by the absence of the donor cells as the occurrence of incomplete (A) (X180) and complete (B) (X200) fused membrane between ooplasm and donor cell. The donor nucleus (arrows) inside ooplasm (C) observed after Hoechst staining (D) (X100).

2.3.4 *In vitro* culture of embryos

Reconstructed embryos were cultured in a 50 µl drop of SOFaa medium supplemented with 10% FBS, at 38.5°C, under 5% CO_2, for 7 days, but in experiment I, they were cultured in B2 supplemented with 10% FBS for 2 days and then co-cultured with Vero cells until day 7.

2.3.5 *In vitro* fertilization

In vitro fertilization (IVF) was conducted as controls in experiment III. Semen was collected from an adult DC tom using electro-ejaculation. The semen was frozen in 0.25 ml straws according to Axner et al. (2004). On the day of *in vitro* insemination, the frozen semen was thawed and spermatozoa were prepared by a swim-up method in M199 supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin and 100 µg/ml streptomycin, at 38.5°C, for 15 min. Total of 4 x 10^4 spermatozoa were subsequently co-incubated with 5-10 DC oocytes (cultured *in vitro*

for 24 h), at 38.5°C, under 5% CO_2, in a 100 µl drop of IVF medium (swim up medium but the 0.4% BSA was changed to 0.6%). At 18 h post-insemination, the oocytes were washed in IVF medium and later subsequently cultured in SOFaa medium, supplemented with 10% FBS for 7 days.

2.3.6 Parthenogenesis

In experiment IV, activation protocols were evaluated using parthenogenic embryos (DCP). DC oocytes matured for 24-27 h were activated by 3 pulses of 1.2 kV/cm for 50 µs and then randomly incubated in 10 µg/ml cycloheximide (Skryszowska et al., 2002) or 10 µg/ml cycloheximide in combination with 5 µg/ml cytochalasin B (Gómez et al., 2003) in SOFaa medium supplemented with 10% FBS, at 38.5°C, under 5% CO_2 in air, for 4 h. Then they were transferred into SOFaa supplemented with 5% FBS, at 38.5°C, under 5% CO_2 in air. At day 5, the embryos were removed to fresh SOFaa supplemented with 10% FBS and cultured at 38.5°C in air, under 5% CO_2 for day 7.

2.3.7 Statistical analysis

The fusion rate and developmental success of embryos were compared according to the different culture periods (24, 36 and 42 h), fusion protocols, donor cell used and activation protocol, using chi-square analysis. Data with number of observation less than 5 were analyzed by Fisher's exact test. P-values < 0.05 were considered statistically significant.

2.3.8 Experimental design (Table 4)

Experiment I: The *in vitro* development of cloned MC-DC embryos derived from 24, 36 and 42 h matured oocytes

The DC oocytes matured for 24, 36 or 42 h were used as recipient cytoplasm for producing MC-DC cloned embryos (14 replicates).

Experiment II: The fusion and developmental capacity of cloned MC-DC embryos derived from different fusion voltages

Three fusion protocols; 2 direct current pulses of 2.1 kV/cm or 2.4 kV/cm for 80 µs or 3 direct current pulses of 2.4 kV/cm for 50 µs were performed to investigate the fusion efficiency of MC-DC couplets (4 replicates).

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Experiment III: Comparison of the development of MC- and DC-DC cloned embryos

MC and DC fibroblast cells were used as donor nuclei for MC- and DC-DC cloned embryo production. *In vitro* fertilized cat embryos served as controls (10 replicates).

Experiment IV: The effect of cytochalasin B in activation medium on development of parthenogenic embryos

Two activation conditions; exposure to cycloheximide with or without cytochalasin B were done to compare the development of parthenogenic embryos (6 replicates).

Table 4. Procedures and conditions in each experiment

Exp.	IVM period	Donor cell*	Fusion protocol	Activation protocol	Culture condition
I	24 h 36 h 42 h	MC	2 pulses (p) of 2 kV/cm, 15 µs	7% ethanol for 5 min	D 1-2: B2 + 10% FBS D 3-7: B2 + 10% FBS coculture with Vero cell
II	24 h	MC	2 p 2 .1 kV/cm, 80 µs 2 p 2 .4 kV/cm, 80 µs 3 p 2 .4 kV/cm, 50 µs	-	-
III	24 h	MC DC	3 p 2 .4 kV/cm, 50 µs	1.2 kV/cm for 40 µs cycloheximide	SOFaa + 10% FBS
IV	24 -27 h	-	-	1.2 kV/cm for 50 µs cycloheximide +/- cytochalasin B	D 1-5: SOFaa + 5% FBS D 6-7: SOFaa + 10% FBS

*MC: marbled cat, DC: domestic cat, p:pulses

2.4 Results

2.4.1 Experiment I: *In vitro* development of cloned MC-DC embryos derived from 24, 36 and 42 h *in vitro* mature oocytes

As shown in Table 5, the number of cloned embryos at the 2-4 cell stage derived from the oocytes cultured for 24 h, 36 h and 42 h was not different (P > 0.05). However, the developmental success to the 4-8 cell stage and the morula stages of oocytes cultured for 24 h was greater than those cultured for 36 and 42 h (the 4-8 cell stage; 27.7, 11.6 and 9.9% and the morula stage; 9.2, 4.3 and 0%, respectively) (P<0.05).

Table 5. The development of cloned MC-DC embryos derived from 24, 36 and 42 h mature cat oocytes

Culture period (h)	n	2-4 cell	>4-8 cell	Morula	Blastocyst
		n (%)			
24	119	53 (44.5)	33 (27.7) [a]	11 (9.2) [a]	0
36	138	50 (36.2)	16 (11.6) [b]	6 (4.3) [b]	0
42	203	75 (37.0)	20 (9.9) [b]	0 (0) [c]	0

Values with different superscripts in the same column are significantly different (*P*<0.05).

2.4.2 Experiment II: Fusion of cloned MC-DC embryos derived from different fusion voltages

As shown in Table 6, the fusion efficiency of the couplets using the 2 pulses of 2.1 and 2.4 kV/cm for 80 μs and 3 pulses of 2.4 kV/cm for 50 μs was not different (46, 48.5 and 67.7%) (P > 0.05).

Table 6. The comparison of fusion rate of cloned MC-DC embryos

Pulse n	Voltage kV/cm	Duration μs	Reconstructed embryo (n)	Fused n (%)
2	2.1	80	37	17 (46.0)
2	2.4	80	33	16 (48.5)
3	2.4	50	31	21 (67.7)

2.4.3 Experiment III: Comparison of the development of MC- and DC-DC cloned embryos

As shown in Table 7, the development of IVF embryos to morula and blastocyst stages was greater than those of cloned MC- and DC-DC embryos. The development of cloned MC- (Fig. 13A-D) and DC-DC embryos (Fig. 14A,B) to the 4-8 cell, morula and blastocyst stages was not different (the 4-8 cell; 56 vs. 50%, the morula; 8 vs. 8.3% and the blastocyst stages; 0 vs. 4.2%, respectively) (P<0.05).

Table 7. The development of MC-, DC-DC cloned and IVF embryos

Donor cell-oocyte	n	Fused	4-8 cell	Morula	Blastocyst
		n (%)			
MC-DC	63	25 (40.0)	14 (56.0)	2 (8.0)[a]	0[a]
DC-DC	60	24 (40.0)	12 (50.0)	2 (8.3)[a]	1 (4.2)[a]
IVF (control)	53	-	28 (52.8)	12 (22.6)[b]	5 (9.4)[b]

Values with different superscripts in the same column are significantly different (*P*<0.05).

2.4.4 Experiment IV: Effect of cytochalasin B in activation medium on development of parthenogenic embryos

As shown in Table 8, the development of DCP embryos (Fig. 15A-E) at cleavage to morula stages was not affected by supplementing cytochalasin B in activation medium (at cleavage-premorula; 82 vs. 85% and morula; 73 vs. 75%) (P>0.05). Addition of the cytochalasin B in the activation medium improved blastocyst development compared to that without the cytochalasin B (8.3 vs. 35%) (*P*<0.05).

Table 8. The development of DCP activated in without (- CB) or with cytochalasin B (+ CB) in activation medium

Condition	Oocyte n	2-4 cell	>4-8 cell	>8-16 cell	Morula	Blastocyst
		n (%)				
- CB	60	49(82.0)	49(82.0)	46(82.0)	44(73.0)	5(8.3)[a]
+ CB	60	51(85.0)	51(85.0)	51(85.0)	45(75.0)	21(35.0)[b]

Values with different superscripts in the same column are significantly different (*P*<0.05).

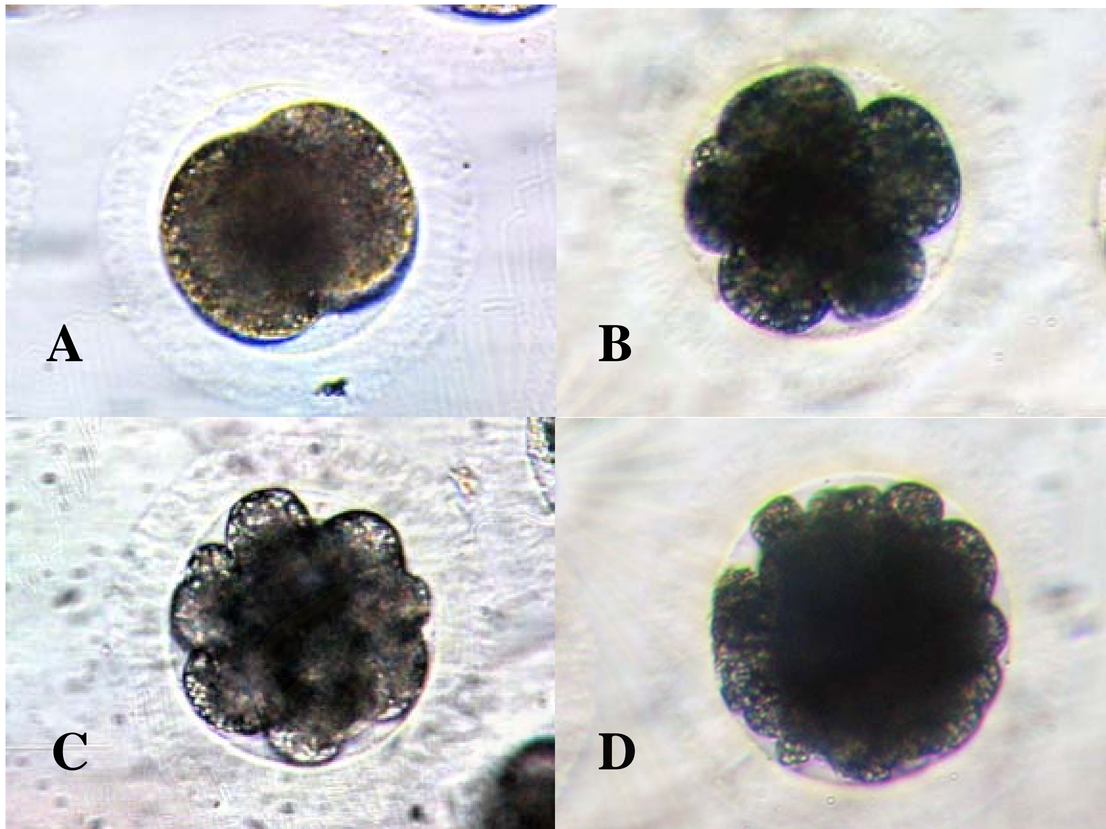

Figure 13. Development of MC-DC cloned embryos; at 2-cell (A), 8-cell (B), 16-cell (C) and compact morula stages (D), observed at 24 , 72, 96 and 120 h after cultured, respectively (X200).

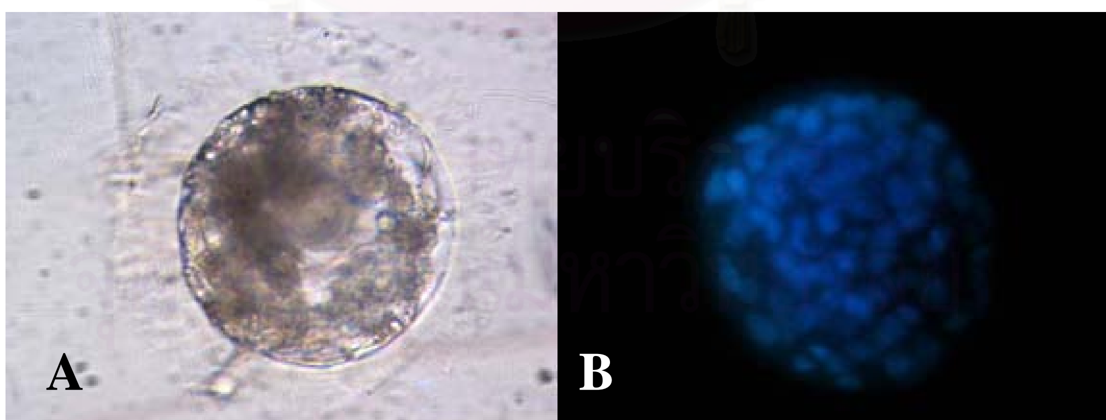

Figure 14. Steriomicrograph (A) and fluoromicrograph (B) of a DC-DC cloned blastocyst (X200)

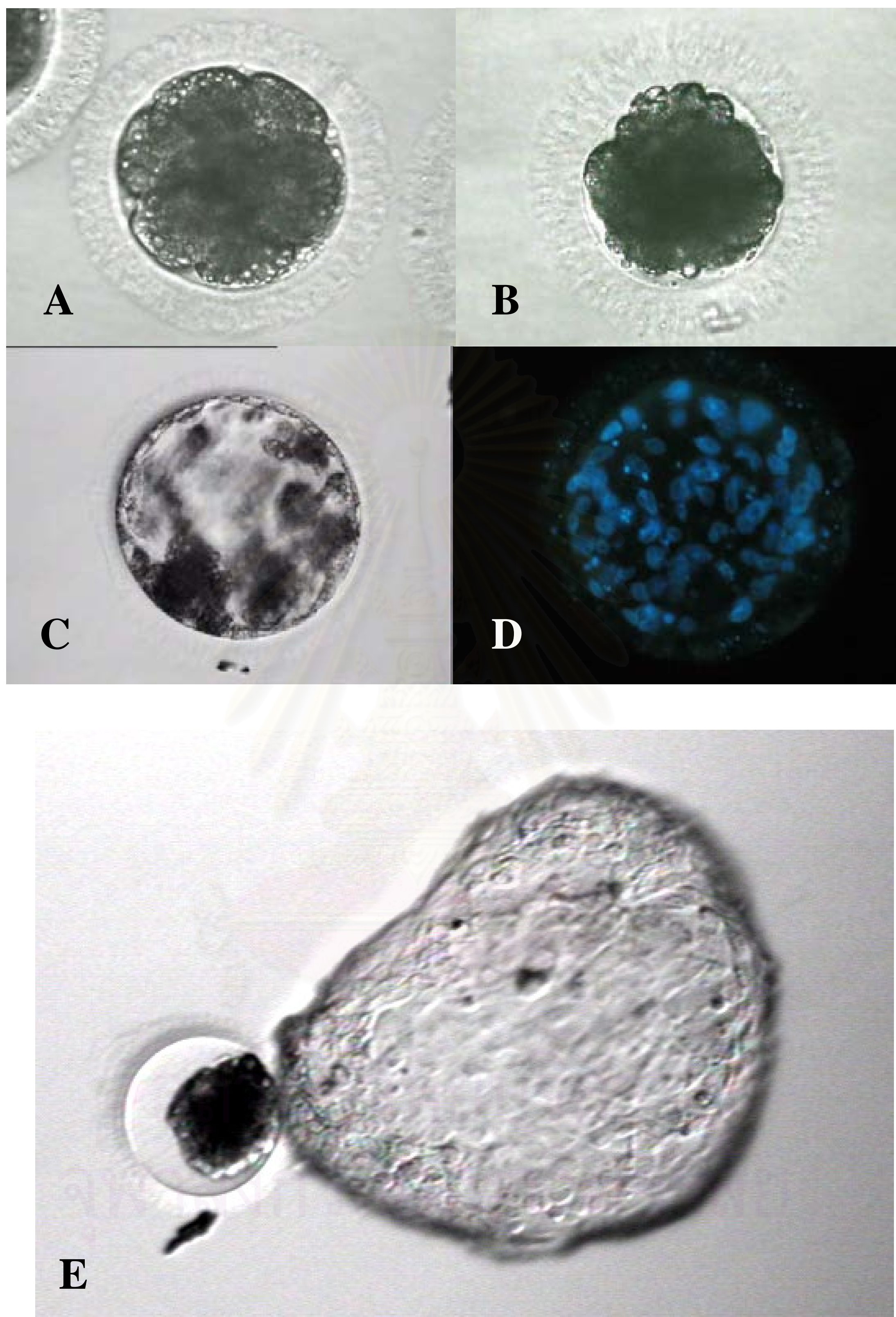

Figure 15. Steriomicrograph of DCP embryos development at 8-cell (A), compact morula (B), blastocyst (C), blastocyst stained with Hoechst 33342 (D) (x200) and hatching blastocyst stages (E) (X100)

2.5 Discussion

Effects of recipient oocyte maturation duration on the developmental success of cloned MC-DC embryos

Developmental success of cloned MC-DC embryos was higher when recipient oocytes matured *in vitro* for 24 h were used as cytoplasm, than those of 36 and 42 h. These results indicated that DC oocytes matured *in vitro* for 24 h were more capable of developing to 8 cell stage than those cultured with longer duration. Maturation time has been reported to influence level of the MPF and the MAPK in oocytes which necessary for initiating germinal vesicle breakdown, meiotic progression and arrest of oocyte development at the MII stage, which subsequently allows donor chromosomes to condense properly and enhance correct ploidy (Hayes et al., 2005). Bogliolo et al. (2004) found that after 24 h of incubation, matured oocytes had higher MPF and MAPK levels than those cultured for 40 h. The low levels of MPF and MAPK may compromise the development and reprogramming competent of the embryos, as in the 36 and 42 h IVM oocytes (Bogliolo et al., 2004). Our findings are in agreement with that reported by Skrzyszowska et al. (2002), who show that the prolonged culture period for cat oocytes up to 40 h results in a decreased development of the reconstituted embryos. In the cattle, Miyoshi et al. (2002) suggested that rapidly matured oocytes (18-24 h) represent a novel way to improve the developmental rates of cloned offspring.

The oocyte culture periods had a slight effect on the maturation rate in our study. The oocyte culture periods of 24, 36 and 42 h result in similar maturation rates (52, 53 and 56%) (data not shown), which corresponded with the previous reports (Farstad, 2000; Rungsiwiwut et al., 2005). In contrast, the greater number of cat oocytes reaching the MII stage (66-70%) was obtained from the oocytes cultured for 42-45 h in the other study (Katska-Ksiazkiewicz et al., 2003). In this study, oocytes cultured for 42 h showed deterioration characteristics, such as aging-like fragmentation of the polar body, debris in the perivitelline space and clumping of the chromosomes at the metaphase plate, as described in the previous studies in the oocytes cultured for 42-45 h (Katska-Ksiazkiewicz et al., 2003; Skrzyszowska et al., 2002). The findings from in this study suggested that prolonged culture of oocytes (longer than 24 h) is not suitable condition for producing good quality recipient cytoplasm for embryo development using NT protocol.

Effects of different electrical stimuli on the fusion efficiency of MC-DC couplets

Results from this study showed that the induction of 2 direct current pulses of either 2.1 or 2.4 kV/cm for 80 μs to reconstructed embryos resulted in a similar fusion efficiency. The difference between both electrical voltages (0.3 kV/cm) may not be significantly different in the MC-DC fusion process. There was a tendency that higher pulse up to 3 pulses might slightly improve the fusion efficiency. Fusion efficiency observed in this experiment was slightly higher than that reported by Wen et al. (2003) (42.7%), using the electrical voltage of 1.4 kV/cm. The fusion efficiency was greater (60-66%) when the number of inducing pulses was increased to four times (Kitiyanant et al., 2003). Moreover, the addition of an alternating current (AC) pulse before the inducing DC pulses, has been shown to be beneficial for fusion and resulted in a fusion success of 73-87% (Gómez et al., 2003a). The AC pulses seem to provide a better alignment of the recipient cytoplast and donor cells. The fusion equipments (fusion chamber and micro-electrodes) are additional important factors affecting fusion efficiency (Lui et al., 2007). The pressurized fusion protocol carried out by a pair of tip-end micro-electrodes has been demonstrated as being optimumal to improve the fusion efficiency in the goat SCNT (Lui et al., 2007).

Comparison of the developments between MC-DC and DC-DC cloned embryos

Cat embryos obtained from IVF was used as a control in the present study to evaluate the ability of the oocytes to fertilize and subsequently cleave *in vitro*. This step was designed to assure that these oocytes could be fertilized and developed to the morula and blastocyst stages (23 and 9%, respectively). Although the development of interspecies MC-DC and intraspecies DC-DC cloned embryos to the morula stage was similar, but only the cloned embryo using DC nuclei demonstrated the ability to develop to the blastocyst stage. It indicated a species specific response between the donor nucleus and the recipient cytoplasm that needs further investigation. In contrast, Gómez et al. (2003) demonstrated that African wildcat fibroblasts can be differentiated at greater rates than domestic cat fibroblasts, in the enucleated domestic cat oocytes. The limitation of cloned MC- and DC-DC production is not only due to incomplete reprogramming but also embryonic blocking, causing low embryo development. Report on cat embryos cultured *in vitro* demonstrated that the first embryonic arrest happens at the 5-8 cell stage, which corresponded to the transition period from maternal to embryonic control and the second arrest happens between the morula and the blastocyst stages (Kanda et al., 1995). Moreover, the fragmentation of

cloned embryos that fail to undergo chromatin remodeling has shown to occur in 18% of reconstructed embryos (Gomez et al., 2003).

Effects of cytochalasin B on the development of parthenogenic embryos

Results from present study demonstrated the effectiveness of the activation protocols, applying multiple electrical stimuli together with cycloheximide and cytochalasin B treatment. Although the development of parthenogenic embryos from cleavage to morula stages was not affected by supplementing cytochalasin B to activation medium, the presence of cytochalasin B enhanced embryo development to the blastocyst stage (35%). These findings are in accordance with a reported of in pig oocytes (Suzuki et al., 2002). After the activation of reconstructed NT embryos, there are the occurrences of nuclear envelop breakdown, subsequent chromatin condensation and new spindle formation (Latham et al., 2005). In parthenogenesis, oocytes being activated during chromosomes are condensed at MII or mature stage which is presenting of high levels of MPF and MAPK in ooplasm (Bogliolo et al., 2004). The Ca^{2+} oscillations and the drop of MPF and MAPK activity by electrical or chemical stimuli are the key triggers that initiate series of biochemical events leading to full activation including meiosis resumption, pronuclear formation, DNA replication, pronuclear apposition, nuclear envelop breakdown and chromatin condense and ready for the first cleavage division (Alberio et al., 2001). Accordingly, the activation by electrical or chemical stimulation is necessary for further development.

The potential mechanism of activation in the study was firstly induced by the Ca^{2+} influx through the plasma membrane, and periodically repeated electrical stimuli mimicking the pattern of the oscillation-like that observed during fertilization. Then the activated oocytes were exposed to activation medium containing cycloheximide, a kind of protein synthesis inhibitor aiming to inhibit MPF activity. Secondly, inhibition of the second polar body extrusion that permitted a correct ploidy, by using cytochalasin B, an inhibitor of microfilament polymerization. The higher incidence of diploid parthenotes was probably caused altered microfilament organization of ooplasm caused by the cytochalasin D treatment (Suzuki et al., 2002). Regardless of age of oocytes and types of medium used, cat oocytes treated with cycloheximide and cytochalasin B provided a greater parthenogenic blastocyst formation rate than those treated with cycloheximide and cytochalasin D alone (13%; Lorthongpanich et al., 2003), calcium ionophore and 6-dimethylaminopurine (12%; Kitiyanant et al., 2003), cycloheximide alone (31%; Karja et al., 2005) and calcium

ionophore and cycloheximide (11.8%; Karja et al., 2006). Thus, our results indicate that adding cytochalasin B in activation medium resulted in greater blastocyst development.

In conclusion, DC oocytes matured at 24 h is suggested to be used as recipient ooplasm for cloned MC embryos. The MC-DC couplet can be fused successfully by introducing 3 direct current pulses of 2.4 kV/cm for 50 µs. Furthermore, The MC fibroblast cells demonstrate the possibility to be reprogrammed in the domestic cat oocytes, resulting in the development to the morula stage. Exposure of mature DC oocytes to cycloheximide and cytochalasin B after electrical stimulation improves the rate of parthenogenic blastocyst formation rate. The study provides the optimal protocol and useful information for cloning of the MC-DC embryos, which can be used as a model for other felid species.

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CHAPTER III

IN VITRO DEVELOPMENT OF MARBLED CAT AND FLAT-HEADED CAT CLONED EMBRYOS RECONSTRUCTED FROM DOMESTIC CAT AND RABBIT OOCYTES

3.1 Abstract

The objectives of the study were to investigate 1) the development of marbled cat (MC; *Pardofelis marmorata*) and flat-headed cat (FC; *Prionailurus planiceps*) embryos reconstructed from domestic cat (DC) and rabbit (RB) oocytes by interspecies/generic nuclear transfer (NT) and 2) the effect of individual cell line and gender on FC cloned embryo production. Experiment I was conducted to investigate the development of MC- and FC-DC cloned embryos (donor cell-recipient oocyte), DC-DC reconstructed embryos served as controls. The fusion efficiency of the DC-DC couplets (38%) was lower than those of the MC- (74%) and the FC-DC couplets (79%; $P<0.05$). The FC-DC couplets yielded a greater percentage of morula (53%) than those of the MC- (23%) and the DC-DC couplets (11%; $P<0.05$). However, the number of couplets achieving blastocyst expansion did not differ (MC; 5, FC; 8.3 and DC; 8.5%, $P>0.05$). Experiment II was conducted to investigate the development of MC-, FC- and DC-RB cloned embryos, RB-RB reconstructed embryos served as controls. The cleavage development of the MC-, FC-, DC- and RB-RB couplets was 100, 87, 85 and 96.4%, respectively. The blastocyst success of the FC- (8.5%) and the DC-RB embryos (7.7%) was lower than those of the RB-RB (14.3%; $P<0.05$) but not the MC-RB embryos (11.5%; $P>0.05$). Experiment III was conducted to observe the effect of individual cell lines and gender on the success of FC-DC cloned embryo production. The variations of fusion and embryo developmental success but not at the blastocyst were observed between three cell lines. The development of the embryos deriving from female and male donor cells at subsequent stages was not different ($P>0.05$). These results indicate that 1) the MC and FC embryos can be produced successfully by NT of either DC or RB oocytes and they can develop to the blastocyst stage *in vitro* and 2) the individual cell line but not the gender of donor cells influences the development of cloned FC embryos.

3.2 Introduction

Marbled cat (MC; *Pardofelis marmorata*) and flat-headed cat (FC; *Prionailurus planiceps*), the small wild cats of south-east Asia, are currently at risk of becoming extinct. Little is known about the wild populations of these two species. No MC and fewer than ten individual FC exist in captivity in Thailand. There are many problems involved in their breeding management in captivity such as ageing, infertility, the inability to pair and single sex existence. Assisted reproductive biotechnology is thus advantageous when captive breeding is unsuccessful. Over the decades, the technique of somatic cell nuclear transfer (SCNT) has been introduced with the ultimate goal of generating live offspring. However, its limitation is that the retrieval of oocytes from endangered animals is a life-threatening process due to animal restraint, anesthesia and manipulation. Consequently, species and generic related animals are deemed to be useful donors of oocytes for recipient cytoplasm.

Inter-generic nuclear transfer (ig-NT) is a useful technique based on the NT process but the donor cell nucleus and enucleate oocytes are from a different genus. Its application is not only useful to the understanding of embryo chronology but also for sustaining the genetics of rare species (Gómez et al., 2003a). Host oocytes from both closely or unrelated animals have been applied for NT studies. The success of NT offspring production using the oocytes from closely related animals has been shown in gaur (Lanza et al., 2000), mouflon (Loi et al., 2001) and banteng (Jansen et al., 2004). In the Felidae, domestic cat (DC) oocytes have been used for generating DC (Shin et al., 2002; Yin et al., 2005) and African wild cat cloned offspring (Gómez et al., 2004a). In addition, DC oocytes are capable of remodeling the donor nuclei collected from the different genus, i.e. leopard cats (Lorthongpanich et al., 2004). Even though the birth of live offspring from leopard cats has not been reported, implanted leopard cat cloned fetuses have been obtained after the transferring of their ig-NT embryos to DC (Yin et al., 2006). This indicates the feasibility of producing viable ig-NT offspring in other species in the future.

The oocytes of unrelated animals such as rabbits are valuable as the universal recipient cytoplasm for NT. Using rabbit (RB) oocytes for inter-species cloned embryo production is interesting especially for wild species and the species whose oocytes are difficult to obtain. The use of rabbit oocytes has been shown in many reports to enable the production of giant panda (Li et al., 2002), bovine (Techakumphu et al., 2005), elephant (Numchaisrika et al., 2005), human (Chen et al., 2003), and cat (Wen et al.,

2003) cloned embryos. Even though the success of using RB oocytes for producing inter-species live offspring has not been reported, the cloned embryos generated from RB oocytes have provided valuable information about embryo chronology, nuclear reprogramming and implantation (Li et al., 2002; Wen et al., 2003) as well as the pluripotency of human stem cells (Chen et al., 2003). Thus, this study hypothesized that the donor nuclei of MC and FC can be reprogrammed by the recipient cytoplasm of genetically related (DC) and unrelated species (RB). Moreover, the factors including the ability of individual cells line and gender which may be involved in the success of NT are investigated.

The aims of the study were to investigate the development of MC-, FC- and DC-DC (donor cell-recipient oocyte) embryos (Experiment I) and MC-, FC-, DC- and RB-RB embryos (Experiment II), and the effect of individual cell lines and the gender of FC on NT success (Experiment III).

3.3 Materials and methods

All chemicals were purchased from Sigma Co. (St Louis, MO, USA) unless otherwise stated. Media were prepared weekly, filtered (0.2 μ, # 16534 Sartorius, Minisart) and kept in sterile tubes. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air at least 4 h before use.

3.3.1. Preparation of recipient cytoplasm

The DC ovaries were obtained after ovariohysterectomy. Within 4 h of the ovaries being removed, the oocytes were collected by mincing the ovaries in an oocyte collecting medium, composed of M199 supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% bovine serum albumin (BSA fraction V), 100 IU penicillin, 100 μg/ml streptomycin and 10 mM hepes buffer. Only oocytes with compact cumulus cells of more than 2 layers and homogenous dark ooplasm were selected. The oocytes were cultured in an oocyte culture medium, composed of M199 supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin, 100 μg/ml streptomycin, 1 μg/ml porcine lutinizing hormone, 10 μg/ml porcine follicle stimulating hormone (FSH; Follotropin-V®; Canada) and 1 μg/ml oestradiol, at 38.5°C, under 5% CO_2 in air, for 24 h (Wood and Wildt, 1997). Matured oocytes characterized by the presence of the 1st polar body and confirmed by 7.5 μg/ml Hoechst 33342

staining, were enucleated in a handling medium; M199 with hepes buffer containing 7.5 µg/ml cytochalasin B.

The RB oocytes were obtained from superovulated New Zealand White rabbit does which were given 21 mg FSH, 100 IU human chrorionic gonadotrophin (hCG; Chorulon®; The Netherlands) and then mated with a vasectomized male. The mature oocytes were flushed from the oviducts using PBS solution at16 h post coitus. The cumulus cells were removed and the oocytes were enucleated in the same way as the cat oocytes.

3.3.2 Preparation of donor nuclei

Skin tissue from dead female MC collected from the Khoa Kheaw Open Zoo in Chonburi province, muscle tissue from live female FC collected from the Khao Patap Chang Wildlife Rescue Centre at Ratchaburi province and muscle tissue from live female DC and RB collected from the Small Animal Hospital, Chulalongkorn University were used in experiment I and II. In experiment III, the tissues of three FC collected from two females and one male were used as donor cells in order to evaluate the effect of individual ability and gender on NT success. Details of FC individuals are described as follows;

- Cell line 1 (L1): collected from the muscle of a live female from the Khao Patap Chang Wildlife Rescue Centre in Ratchaburi province
- Cell line 2 (L2): collected from the skin of a dead female from the Khoa Kheaw Open Zoo in Chonburi province
- Cell line 3 (L3): collected from the skin of a live male from the Songkla Zoo in Songkla province

The tissues were sliced into small pieces and cultured in Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (FBS; Invitromex), penicillin and streptomycin, at 38.5°C, under 5% CO_2 in air. Fibroblast cells of MC at passage 4 and 9 and FC at passage 2-4 were used as donor cells. The donor cells were starved by culturing in DMEM supplemented with 0.5% FBS, at 38.5°C, under 5% CO_2 in air, for 2-5 days prior to the NT procedure.

3.3.3 Nuclear transfer, fusion and activation

An individual donor cell was transferred into the perivitelline space of an enucleated oocyte, by manipulation under an oil-covered handing medium. NT couplets were washed and placed in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.05% fatty acid-free BSA) at room temperature for 1 min. The couplets were transferred to a fusion chamber placed between two platinum electrodes and overlaid with a fusion medium. The couplets were then induced by 3 direct current pulses of 2.4 kV/cm for 50 µs. Thereafter, the couplets were washed in a handling medium and incubated in a synthetic oviductal fluid plus 1% essential, 1% non-essential amino acids (SOFaa) (Freistedt et al., 2001) supplemented with 10% FBS at 38.5°C, in 5% CO_2 in air, for 2 h. The fused NT embryos were activated by inducing 3 direct current pulses of 1.2 kV/cm for 50 µs, and incubated in SOFaa supplemented with 10 µg/ml cycloheximide, 5 µg/ml cytochalasin B and 5% FBS for 4 h. The couplets reconstructed by the RB oocytes were fused by inducing 3 direct current pulses of 3.2 kV/cm for 20 µs in the fusion medium (Chesne et al., 2002). Fused oocytes were subsequently held in SOFaa, supplemented with 10% FBS for 1 h. For activation, the fused couplets were induced using the same procedure of fusion and then were incubated in SOFaa supplemented with 5 µg/ml cycloheximide, 2 mM 6-DMAP and 5% FBS for 1 h.

3.3.4 *In vitro* culture of embryos

By using DC oocytes, the couplets were cultured in a SOFaa medium supplemented with 5% FBS, at 38.5°C, under 5% CO_2 in air. At day 5, the embryos were transferred to a fresh SOFaa medium supplemented 10% FBS and cultured at 38.5°C in air, under 5% CO_2 for another 3 days.

The RB couplets were cultured in SOFaa supplemented with 10% FBS, at 38.5°C, under 5% CO_2 in air for 5 days.

Embryo development was monitored once daily at 8.00 am. In order to find the blastocyst formation time of FC embryos derived from the DC and RB oocytes after they had reached the morula stage, they were monitored twice daily at 8.00 am and 4.00 pm until reaching the blastocyst stage. The time of blastocyst formation was identified at the beginning of culture to blastocyst forming.

The FC-DC and FC-RB blastocysts (Day 7 and 5, respectively) were stained with Hoechst 33342, fixed on slides with glycerol and were immediately counted for cell numbers.

3.3.5 Parthenogenesis

The DC parthenogenic embryos (DCP) served as a control. Twenty five to 27 h-mature DC oocytes derived from the method described above were activated by inducing of 3 direct current pulses of 1.2 kV/cm for 50 µs and then incubated in SOFaa supplemented with 10% FBS, 10 µg/ml cycloheximide and 5 µg/ml cytochalasin B at 38.5°C, under 5% CO_2 in air, for 4 h (Gómez et al., 2003a). They were cultured in the same way as cloned embryos.

3.3.6 Statistical analysis

The fusion and developmental rates of the cloned and parthenogenic embryos were compared across treatment groups by Chi-square analysis. The times of blastocyst formation and the blastocyst cell numbers of FC was presented as mean ± SD and compared by ANOVA. Significant differences were set at $P<0.05$.

3.3.7 Experimental design

Experiment I: The developmental capacity of MC and FC cloned embryos reconstructed from DC oocytes

Ten replicates were conducted to investigate the early development of MC- and FC-DC cloned embryos by transferring individual MC and FC fibroblast cells to DC enucleated oocytes. The development of embryos was monitored for 7-8 days. The DC-DC cloned and parthenogenic embryos served as controls.

Experiment II: The developmental capacity of MC, FC and DC cloned embryos reconstructed from RB oocytes

Seven replicates were conducted to investigate the early development of MC-, FC- and DC-RB cloned embryos by transferring individual MC, FC and DC fibroblast cells to RB enucleated oocytes. The development of embryos was monitored for 5-6 days. The RB cloned embryos served as control.

The times for cavity formation and cell numbers of FC blastocyst reconstructed from domestic cat and rabbit oocytes were compared.

Experiment III: The developmental ability of FC-DC embryos reconstructed from different cell lines and genders

Three cell lines (L1-3) of FC collected from two females and one male were used as donor cells in order to evaluate the individual ability of NT success. The developments of embryos generated from two females were grouped and compared to male cell line.

3.4 Results

3.4.1 Experiment I: The development of MC- and FC-DC cloned embryos

As shown in Table 9, the fusion efficiency of the DC-DC couplets (38%) was lower than those of the MC- (74%) and the FC-DC couplets (79%; P<0.05). No difference in the cleavage development of MC-, FC- and DC-DC fused couplets was found (93, 97, 87 and 89% respectively). The cleavage development of parthenogenic embryos (86%) was slightly lower than that of cloned embryos. The FC-DC couplets yielded a greater percentage of morula (53%) than that of the MC- (23%) and DC-DC couplets (11%; P<0.05). However, the number of couplets that achieved blastocyst expansion did not differ (MC; 5, FC; 8.3 and DC; 8.5%, *P*>0.05). The morula and blastocyst success of parthenogenetic embryos yielded a greater number than those of cloned embryos (75 and 43%; P<0.05) (Fig. 16).

Table 9. The development of MC-, FC- and DC-DC cloned embryos reconstructed from DC oocytes and DCP embryos

Donor cell	Reconstructed couplet (n)	Fused	2-4 cell	Morula	Blastocyst
		n (%)			
MC	81	60 (74)^{a}	56 (93)^{ab}	14 (23)^{a}	3 (5.0)^{a}
FC	76	60 (79)^{a}	58 (97)^{a}	32 (53)^{b}	5 (8.3)^{a}
DC	94	36 (38)^{b}	32 (89)^{ab}	4 (11)^{a}	3 (8.5)^{a}
DCP	72	-	62 (86)^{b}	54 (75)^{c}	31 (43)^{b}

Values with different superscripts in the same column are significantly different (P<0.05).
MC: marbled cat, FC: flat-headed cat, DC: domestic cat, DCP: domestic cat parthenogenic

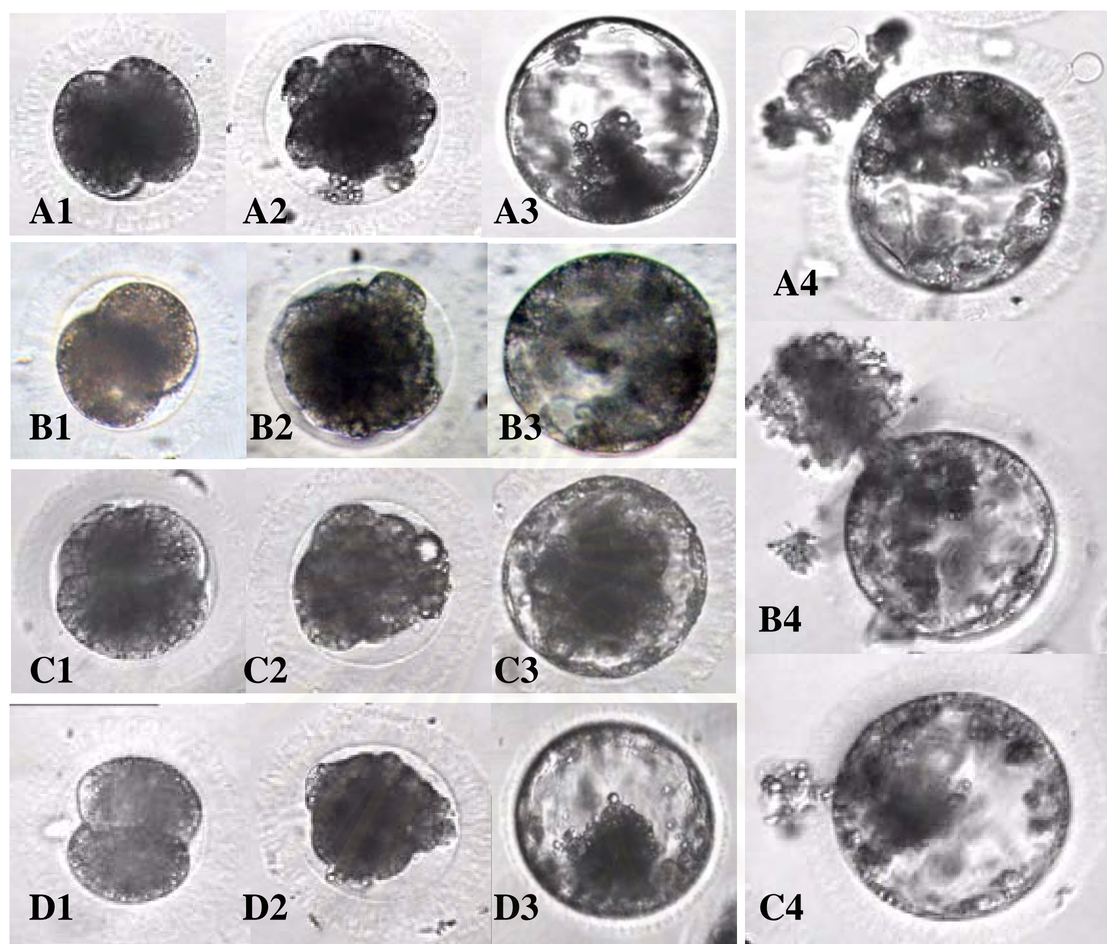


Figure 16. The development of MC-, FC- and DC-DC cloned and DCP embryos; the MC- (A1-4), FC- (B1-4) and DC-DC (C1-4) cloned and DCP embryos (D1-3) at 2-cell (1), compact morula (2), blastocyst (3) and hatching blastocyst stages(4) (X200) after being cultured for 1, 6, 7 and 8 days respectively.

3.4.2 Experiment II: The development of MC-, FC-, DC- and RB-RB cloned embryos

As shown in Table 10, the fusion efficiency of the FC-RB couplets (78%) was greater than that of the MC- (46.4%), DC- (49.1%) and RB-RB couplets (51%; $P<0.05$). The cleavage development of MC-, FC-, DC- and RB-RB couplets was (100, 87, 85 and 96.4%, respectively). There was no difference in the ability of embryos to reach the morula stage in all groups (19.2, 25.5, 23.1 and 35.7%, respectively, $P<0.05$) (Fig. 17). The blastocyst rate of the FC- (8.5%) and DC-RB embryos (7.7%) (Fig. 18) was lower than those of the RB-RB (14.3%) but not the MC-RB (11.5%; $P<0.05$).

Table 10. The development of MC-, FC-, DC- and RB-RB cloned embryos

Donor cell	Reconstructed couplet (n)	Fused	2-4 cell	Morula	Blastocyst
		n (%)			
MC	56	26 (46.4)[a]	26 (100) [a]	5 (19.2)	3 (11.5)[ab]
FC	60	47(78.0)[b]	41 (87.0)[ab]	12(25.5)	4 (8.5)[a]
DC	53	26 (49.1)[a]	22 (85.0)[b]	6 (23.1)	2 (7.7)[a]
RB	55	28 (51.0)[a]	27 (96.4)[ab]	10 (35.7)	4 (14.3)[b]

Values with different superscripts in the same column are significantly different ($P<0.05$).
MC: marbled cat, FC: flat-headed cat, DC: domestic cat, RB: rabbit

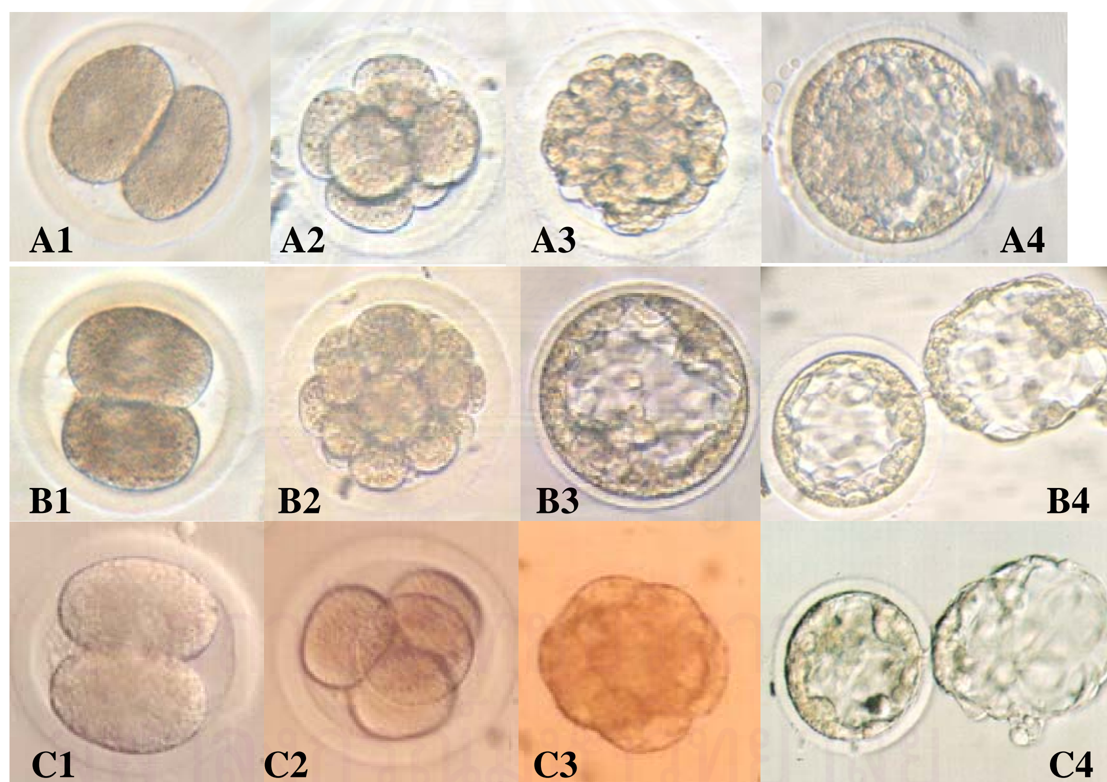


Figure 17. The development of MC-, FC-, DC-and RB-RB cloned embryos; MC-RB cloned embryos at 2-cell (A1), 8-cell (A2), morula (A3), hatching blastocyst stages (A4); FC-RB cloned embryos at 2-cell (B1), 16-cell (B2), blastocyst (B3) (X200), hatching blastocyst stages (B4) (X100); and RB-RB cloned embryos at 2-cell (C1), 4-cell (C2), compact morula (C3) (X200) and hatching blastocyst stages (C4) (X100)

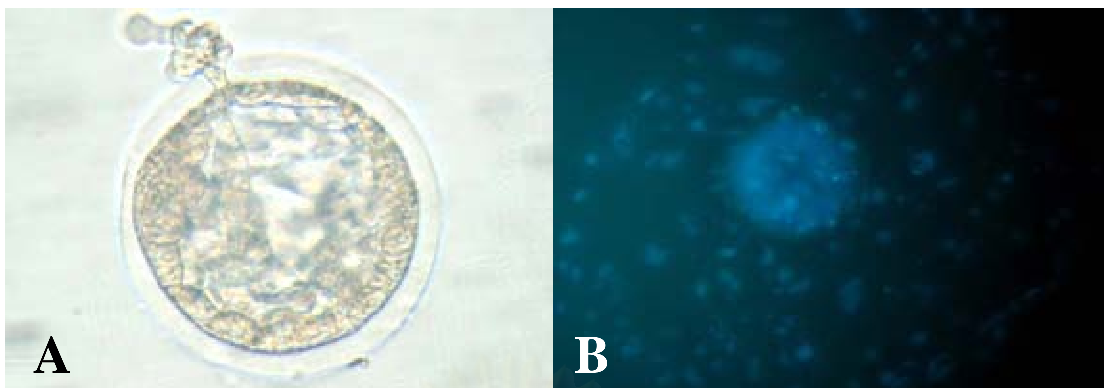


Figure 18. A DC cloned blastocyst reconstructed from RB oocytes under an inverted microscope (A) (x300) and a Hoechst stained hatching blastocyst, under UV light (B) (X100)

The blastocyst formation time and cell numbers of FC blastocyst reconstructed by DC and RB oocytes

The time of blastocyst formation of FC embryos reconstructed from DC (n=5) was longer than that reconstructed from RB oocytes (n=4) (142 ± 13.2 vs. 99 ± 13.9 h) ($P<0.05$).

The cell numbers of FC blastocyst reconstructed from DC (n=5) (Day 7) were lower than those reconstructed from RB oocytes (n=3) (Day 5), which were 70 ± 21.5 and 127 ± 33.4 cells, respectively ($P<0.05$) (Fig. 19).

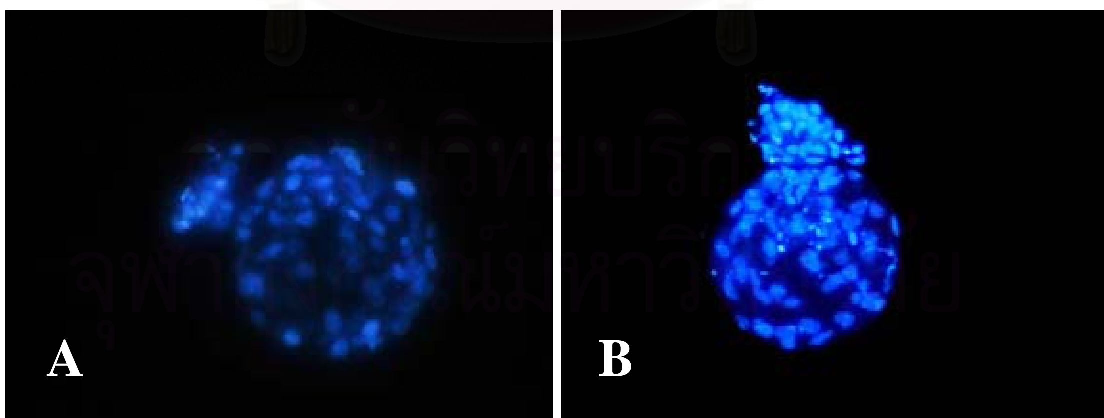


Figure 19. Hoechst stained FC-DC (A) and FC-RB blastocysts (B), demonstrating 72 and 90 cells, respectively (x200)

3.4.3 Experiment III: The development of FC-DC embryos reconstructed from different cell lines and genders

As shown in Table 11, the fusion efficiency of L3 was lower than L1 and L2 (57.7, 75.5 and 78.9%, respectively; P<0.05). The L2 and L3 gave a greater cleavage number than L1 (81.1, 80 and 73.5%, respectively, P<0.05). The morula developmental success of embryo reconstructed from L1 (53.3%) provided a difference greater than L3 (26.7%) but not L2 (35.1%; P<0.05). However, there was no difference in the blastocyst formation rate among cell line (L1; 8.3, L2; 8.1 and L3; 6.7%, P>0.05).

As shown in Table 12, the fusion efficiency of the couplets reconstructed from female lines (77.6%) had a greater rate than that reconstructed from male line (57.7%; P<0.05). The development of the embryos derived from the female and male donor cells at cleavage (90.7 vs. 80%), morula (46.4 vs. 26.7%) and blastocyst (8.3 vs. 6.7%) was not different.

Table 11. The development of FC-DC embryo reconstructed from different cell lines

Cell Line*	Reconstructed couplet (n)	Fused	2-4 cell	Morula	Blastocyst
		n (%)			
L1	76	60 (78.9)^{a}	58 (73.5)^{b}	32 (53.3)^{a}	5 (8.3)
L2	49	37 (75.5)^{ab}	30 (81.1)^{a}	13 (35.1)^{ab}	3 (8.1)
L3	52	30 (57.7)^{b}	24 (80.0)^{a}	8 (26.7)^{b}	2 (6.7)
Total	177	127 (71.8)	112 (88.2)	53 (41.7)	10 (7.9)

Values with different superscripts in the same column are significantly different (P<0.05).
* Flat-headed cat cell lines; L1: cell line 1 collected from the muscle of a live female at Khao Pratub Chang, Ratchaburi province, L2: cell line 2 collected from the skin of a dead female at the Khoa Kheaw Open Zoo, Chonburi province, L3: Cell line 3 collected from the skin of a live male at Songkla Zoo, Songkla province

Table 12. The development of FC-DC embryos reconstructed from donor cells derived from different genders

Cell line	Reconstructed couplet (n)	Fused	2-4 cell	Morula	Blastocyst
		n (%)			
Female	125	97 (77.6)[a]	88 (90.7)	45 (46.4)	8 (8.3)
Male	52	30 (57.7)[b]	24 (80.0)	8 (26.7)	2 (6.7)

Values with different superscripts in the same column are significantly different (P<0.05).

3.5 Discussion

The present study demonstrated that MC and FC ig-NT embryos enabled cleaving with high percentages which were similar to intra-species. This result is in accordance with the interspecies/generic-NT embryos in studies of Korean tiger (65%; Hwang et al., 2001), African wildcats (84%; Gómez et al., 2004a), leopard cats (83; Lorthongpanich at al., 2004), Rusty spotted cats (81%; Gómez et al., 2004b) and black foot cats (85%; reviewed by Gómez et al., 2006). The quality of the oocytes and the effectiveness of the activation protocol used in the study could be supportive factors. The FC-DC couplets yielded a greater morula rate than the MC- and DC-DC embryos. However, the number of cloned embryos achieving blastocyst expansion did not significantly differ. This may be because of the high ability of FC donor cells to be reprogrammed in DC ooplasm but being unable to overcome embryonic block in the same way as MC- and DC-DC embryos.

The MC- and FC-DC blastocyst rates were similar to the leopard cat (7%) (Lorthongpanich et al., 2004) and were in the range of DC-DC cloned embryos (1-11%) (Gómez et al., 2003a; Skrzsowska et al., 2002; Kitiyanant et al., 2003; Wen et al., 2003) and higher than those in black-footed cat (2.1%), and rusty spotted cat cloned embryos (2.4%) (reviewed by Gómez et al., 2006). Although, NT in several felid species has been successful and the blastocysts of such species can be produced, the developmental block at morula to blastocyst stage seems to occur in all species, demonstrated by the fact that 2.5-44.7% (in this study) and 21-40% (Skrzsowska et al., 2002; Kitiyanant et al., 2003; Wen et al., 2003) of morula could not develop to

blastocyst. An inappropriate culture system is believed to be the crucial factor in the incomplete reprogramming leading to developmental block. Accordingly, transferring the embryos at cleavage stage or after activation may be the appropriate way to avoid improper conditions during culture *ex vivo*. However, when early stage embryos are transferred, this does not ensure the achievement of development in the later stage according to low pregnancy rates in all felid species. Since abnormal gene expression associated with incompatibilities between the nucleus and ooplasm may inhibit the normal progression of epigenetic events required to create a functional genome (Vrana et al. 1998). Thus, the embryo blocking at morula to blastocyst in cloned felids needs to be more accurately specified and in order to improve NT success.

Even though the phylogenetic distance of RB is far from felid species, producing inter-species cloned embryos from RB oocytes is possible. As shown in the present study either DC or RB oocytes are able to reprogram MC and FC nuclei and support the development of embryos to blastocyst. Moreover, our previous studies have demonstrated the feasibility of using RB oocytes as recipient cytoplasm for producing elephant blastocysts (Numchaisrika et al., 2005). This may be because the developmental biology of RB embryos and fetuses more closely resembles that of large farm animals and rodent species, including the transition from maternal to embryonic control of embryo development (Dinnyes et al., 2002). For wild animals, previous reports have show the possibility of producing endangered species; gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Jansen et al., 2004) and African wild cats (Gómez et al., 2004) by inter-species cloning using oocytes of closely related species. However, up to now, the birth of cloned animals derived from unrelated species oocytes has not been yet reported. The inter-species NT using rabbit oocytes is currently useful for the *in vitro* study of endangered animals whose oocytes can not be obtained and will open new avenues to generate embryonic stem cells, which may provide beneficial genetic information in the future.

The timing of preimplanted embryogenesis, especially blastocyst formation, is species-specific and varies between species in mammals. In inter-species NT, a difference of nucleo-cytoplasmic interaction between species has been found in the present study showing a significant difference in the time of blastocyst formation of FC embryos reconstructed from DC and RB oocytes. The time of blastocyst formation of FC-DC (142 h) and FC-RB embryos (99 h) corresponds to that of cat-cat (144 h) cat-rabbit (135 h) and rabbit-rabbit (90 h) cloned embryos in the previous study

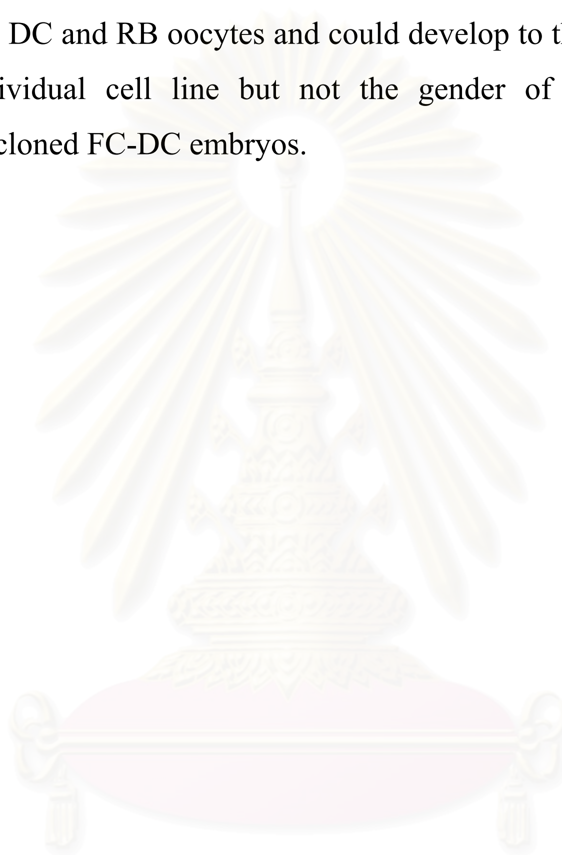
(Wen et al., 2003). The unlikely embryogenesis time of cloned embryos reconstructed from the oocytes of different species implies that unknown factor(s) regulating the cell division exist in ooplasm. The possible explanation for this evidence is the difference in the mathernal-zygotic transition (MZT) of DC and RB. The first embryonic arrest of feline embryos happens at the 5- to 8-cell stage and the second arrest (2-3 d) happens between the morula and blastocyst stages (Kanda et al. 1995), while MZT of RB embryos occurs at 8-16 cell stage (Henrion et al., 1997).

Among FC cell lines, the fusion efficiency and embryo development throughout the morula stage of the couplets is varied. According to previous reports, many factors, including the source of oocytes (*in vitro* vs. *in vitro*) (Gómez et al., 2003a), the type of donor cells (Lui et al., 2007; reviewed by Gómez et al., 2006), methods and equipment used (Lui et al., 2007) affected fusion ability. In the present study, fusion efficiency was not influenced by the source of the tissues collected because similar fusion rates of L1 and L2 (collected from muscle and skin, respectively) were found. In the FC-DC couplets, based on fusion success rate, fibroblast cells from both muscle and skin are able to be donor cells but vary in their success rates (58-79%). It is suggested that fusion ability depends on the quality of individual cell lines, which have to be evaluated before use as karyoplast of NT. However, no differences in the frequency of blastocyst development of embryos reconstructed from L1, L2 and L3 have been observed, indicating that all FC cell lines used in this study were equally capable of reprogramming.

The present study did not find differences in embryo development derived from female and male flat-headed cats. However, the gender factor may affect the success of cloned embryo development since Gómez et al. (2003a) reported that African wildcat fibroblasts can dedifferentiate in the nucleated DC oocytes at a significantly greater rate than those of DC (blastocyst rates; 24.2 vs. 3.3%) and live offspring are derived from the male cell line (Gómez et al., 2004a). One of the possible reasons for the contrasting results in embryo development may be that each cell line was derived from individuals of different genders. Moreover, in the study of inter-species cloned banteng, a significantly higher cleavage, blastocyst and pregnancy rate for cytoplast reconstructed with adult male fibroblasts compared with female has been reported (Sansinena et al., 2005). However, the reason for the difference is unclear be it X-chromosome inactivation and/or other epigenetic mechanism affected by the sex of donor karyoplast (Sansinena et al., 2005). In contrast, no differences of embryo

development were found when comparisons were made between the two genders of leopard cats. Moreover, recently viable cloned dogs have been produced from both male (Lee at al., 2005) and female donor cells (Jang et al., 2007). Accordingly, it is suggested that the nuclei of both genders are able to serve as donor cells to produce embryos and/or offspring but the success rate stemming from each gender may be different according to the species.

In conclusion, 1) MC and FC embryos could be produced successfully by ig-NT from both DC and RB oocytes and could develop to the blastocyst stage *in vitro* and 2) the individual cell line but not the gender of donor cells affected the development of cloned FC-DC embryos.



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CHAPTER IV

IN VIVO DEVELOPMENT OF FLAT-HEADED CAT AND DOMESTIC CAT CLONED AND *IN VITRO* FERTILIZED EMBRYOS

4.1 Abstract

The production of endangered flat-headed cat (FC) inter-generic cloned offspring was the ultimate goal of this study. The domestic cat (DC) IVF served as a control for *in vitro* and *in vivo* developments and was used as model for developing the technique of a 2-cell stage embryo transfer of cloned embryos. The objectives of the study were to investigate 1) the *in vitro* development of domestic cat IVF embryos and their transfer to recipients and 2) the pregnancy establishment of recipients receiving FC-DC (donor cell-oocyte recipient) cloned embryos. Experiment I was conducted to investigate the *in vitro* development of DC IVF embryos. The 24-27 h *in vitro* mature oocytes were co-cultured with sperm collected by an artificial vagina. The cleaved rates of inseminated oocytes observed at 18-, 27- and 42 h post insemination (pi) were 37, 54.5 and 68.4%, respectively. The blastocyst rate and cell numbers of blastocysts derived from cleaved embryos collected at 27 h were greater than those collected at 42 h (61.4 vs. 18.6% and 106±43 vs. 60±27 cells, respectively) ($P<0.05$). Experiment II was conducted to observe the pregnancy establishment of recipients receiving DC IVF embryos. A total of 150 cleaved embryos were transferred to the oviducts of 6 recipients (mean 25 ± 9). Ultrasonography at 30 days of gestation demonstrated that all recipients became pregnant resulting in five to-term kittens delivered from three recipients at day 60-63 of gestation. Experiment III was conducted to observe the pregnancy establishment of recipients receiving 1) FC-DC cloned embryos 2) FC- and DC-DC cloned embryos and 3) FC-DC cloned and DC IVF embryos. None of recipients receiving reconstructed FC cloned reconstructed embryos (mean 41.4 ± 13) and FC- and DC-DC cloned embryos (mean 29.8 ± 20.8; 23.4 ± 15 of FC-DC and 26.4 ± 7 DC-DC cloned embryos) became pregnant. One out of four recipients receiving FC-DC cloned and DC IVF embryos (mean 55 ± 15; 43.3 ± 15 of FC-DC cloned and 10.8 ± 1.5 DC IVF embryos) became pregnant and delivered 2 still births and 6 live IVF kittens. In conclusion, 1) pregnancy could be established after DC IVF embryo transfer and embryos cleaved early at ≤27 h pi would rather be used for transfer than those cleaved later due to their greater developmental

rate and quality, 2) FC-DC ig-NT and DC-DC cloned offspring were not able to be produced which may have been caused by the low development and, quality of the embryos and 3) by co-transfer of FC-DC cloned and DC IVF embryos to recipients, pregnancy could be established and live IVF but not cloned kittens were obtained.

4.2 Introduction

The feasibility of producing viable offspring in felid species by somatic cell nuclear transfer (SCNT) has been demonstrated in the domestic cat (DC) (Shin et al., 2002) and the African wildcat (Gómez et al., 2004). This technique may apply to efficiently produced cloned offspring of other non-domestic felids, i.e. flat-headed cat (FC; *Prionailurus planiceps*), the critically endangered species in Southeast Asia. The success of FC embryo production by inter-generic NT (ig-NT) has been demonstrated in chapter III. In accordance with previous reports in other felid species, the limitation of *in vitro* development of FC-DC (donor cell-recipient oocyte) ig-NT embryos i.e. a low developmental rate due to embryonic blocking at morula to blastocyst, has been recorded (Lorthongpanich et al., 2004; Yin et al., 2006). Many attempts to improve the SCNT technique have been made by establishing comparative studies using the *in vitro* fertilized (IVF) technique (Ammath et al., 2007). Since the IVF technique imitates the natural fertilization process it provides the opportunity of observing abnormalities in terms of chronology, cellular and molecular aspects that may occur in the laboratory conditions in which the SCNT embryos are produced. Indeed, comparative studies have been widely performed on several species, i.e. feline (Pope et al., 2007), bovine (Ammath et al., 2007) and porcine (Katayama et al., 2006).

The ultimate goal of the *in vitro* procedure is to transfer produced embryos with subsequent pregnancies. Successes in embryo transfer (ET) of the IVF-derived DC embryos at morula and blastocyst into the uterus have been reported (Goodrowe et al., 1988; Pope et al., 1998; Tsutsui et al., 2000). Thereafter, it has been used as a model for producing kittens of many endangered felid species, i.e. Indian dessert cat (Pope et al., 1993), African wildcats (Pope et al., 2000) and caracal (Pope et al., 2001). The NT-derived feline embryos are usually transferred at an early stage (1 to 2 cell stages) to overcome the embryo developmental block which generally occurs at the morula to blastocyst stage (Kanda et al., 1998) and cytoplasmic fragmentation caused by the *in vitro* process (Gómez et al., 2003a). The survival of African wildcat cloned

embryos is higher after laparoscopic oviductal transfer on day-1 than after uterine transfer on day 5-7 (Gómez et al., 2004a). However, a few studies have focused on *in vitro* derived-embryo transfer at the 2-cell stage (Swanson et al., 1998). Moreover, a low pregnancy rate (50%) of recipients receiving *in vivo* matured/IVF embryos at the 2-cell stage has been reported (Swanson et al., 1998). A low embryo survival rate after the transfer of *in vitro*-derived embryos has been demonstrated by the low normal pregnancy rates of recipients and the fewer number of kittens born per litter (Pope et al., 2006). There are many factors involved in the pregnancy establishment of recipients receiving *in vitro*-derived embryos, i.e. the developmental ability of the embryos, the techniques used for the transference of embryos, the responses of hormonal induced recipients and the acceptance of the recipients to the cloned embryos. Thus, this study aimed to develop the technique of ET at the 2-cell stage for *in vitro* matured (IVM)/IVF and FC-DC cloned embryos. The established IVM/IVF/ET technique and the recipient preparation will assure the efficiency of the processes involved in the pregnancy establishment of recipients receiving FC cloned embryos. Therefore, DC IVF embryos were used as controls for the *in vitro* and *in vivo* development of FC-DC embryos. Moreover, the co-transfer of FC-DC cloned with DC IVF embryos was hypothesized as being able to improve pregnancy establishment due to their better developmental competence than those derived from ig-NT. We expected that the DC IVF embryos would help with maternal recognition, and uterine preparation for implantation that might also involve the success rate for cloned embryos.

The objectives of the study were to investigate the *in vitro* development of DC IVF embryos (Experiment I) and their transfer at the 2-cell stage to recipients (Experiment II) and 2) the pregnancy establishment of recipients receiving FC-DC cloned embryos (Experiment III).

4.3 Materials and methods

All chemicals were purchased from Sigma Co. (St Louis, MO) unless otherwise stated. Media were prepared weekly, filtered (0.2 μ, # 16534 Sartorius, Minisart) and kept in sterile tubes. The bicarbonate-buffered cultured media were incubated at 38.5°C under 5% CO_2 in air at least 4 h before use.

4.3.1 *In vitro* fertilized embryos

Oocyte collection and maturation

Domestic cat ovaries were obtained after ovariohysterectomy. Within 4 h of the ovaries being removed they were minced in an oocyte collecting medium, composed of M199 medium supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% bovine serum albumin (BSA fraction V), 100 IU penicillin, 100 µg/ml streptomycin and 10 mM hepes buffer. Oocytes with compact cumulus cells of more than 2 layers and homogenous dark ooplasm were selected. The oocytes were cultured in an oocyte culture medium which was composed of M199 supplemented with 0.292 g/ml glutamine, 0.026 g/ml pyruvate, 0.4% BSA, 100 IU penicillin, 100 µg/ml streptomycin, 1 µg/ml porcine lutinizing hormone, 10 µg/ml porcine follicle stimulating hormone (FSH; Follotropin-V; Canada), 1 µg/ml oestradiol and 25 ng/ml epidermal growth factor, at 38.5°C, under 5% CO_2 in air, for 24-27 h (Wood and Wildt, 1997).

Semen collection

Semen was collected from an adult tom cat by artificial vagina. Semen was evaluated and washed by centrifugation at 1,000 rpm, for 10 min, in an IVF medium comprised of synthetic oviductal fluid plus 1% essential and 1% non-essential amino acids (SOFaa) (Freistedt et al., 2001) supplemented with 0.6% BSA, penicillin and streptomycin. The supernatant was discarded and the spermatozoa were allowed to swim up the IVF medium overlaid on the sperm pellet, at 38.5°C for 2-3 h. The supernatant was collected and subsequently adjusted to a final concentration of 5 x 10^5spermatozoa/ml.

***In vitro* fertilization and culture**

The 24-27 h-IVM oocytes were washed in the IVF medium 2 times and were then co-incubated with the sperm for 18 h, at 38.5°C, under 5% CO_2 under humidified atmosphere. After that the cumulus cells surrounding the oocytes (day-1 embryos) were removed and washed 3 times before being cultured in SOFaa supplemented with 0.4% BSA, penicillin and streptomycin, at 38.5°C, under 5% CO_2 under humidified atmosphere. The cleavage rate was evaluated twice; at 18 h post insemination (pi) and 27 h pi. At 27 h pi, the cleaved embryos were randomly collected for ET and the remainder was cultured separately from uncleaved oocytes. At 42 h pi, the uncleaved

oocytes were investigated for their development again and cleaved embryos were collected and cultured.

The embryo culture condition was performed at 38.5°C, under 5% CO_2 in a humidified atmosphere and the culture medium was changed on subsequent days as follows;

- days 1 to 2: SOFaa supplemented with 0.4% BSA, penicillin and streptomycin
- days 3 to 4: SOFaa supplemented with 5% fetal bovine serum (FBS; Invitromex), penicillin and streptomycin
- days 5 to 7: SOFaa supplemented with 10% FBS, penicillin and streptomycin

The blastocysts derived from 27-h cleaves and 42-h cleaves were stained by Hoechst 33342 for cell number count.

4.3.2 The FC- and DC-DC cloned embryo production

The production of FC-DC and DC-DC cloned embryos was processed according to the methods in Chapter III. The procedures are described in brief as follows;

Preparation of recipient cytoplasm

The recipient oocytes were prepared as described above in the IVF embryo production. At 18-24 h of IVM, the cumulus cells surrounding the oocytes were removed. Matured oocytes characterized by the presence of the 1st polar body and confirmed by 7.5 µg/ml Hoechst 33342 staining, were enucleated in a handling medium, M 199 with hepes buffer containing 7.5 µg/ml cytochalasin B.

Preparation of donor nuclei

Fibroblast cells of a male FC from the Songkla Zoo at the Songkla province and a female DC at passage 2-5 were used as donor cells. The donor cells were starved prior to the NT procedure.

Nuclear transfer, fusion, activation and culture

An individual FC donor cell was transferred into the perivitelline space of an enucleated oocyte. The NT couplets were fused by inducing 3 direct current pulses of

2.4 kV/cm for 50 µs. Thereafter, the couplets were incubated in SOFaa supplemented with 5% FBS at 38.5°C, in 5% CO_2 in air, for 2 h. The fused NT embryos were activated by inducing 3 direct current pulses of 1.2 kV/cm for 50 µs and were incubated in SOFaa supplemented with 10 ug/ml cycloheximide, 5 ug/ml cytochalasin B and 5% FBS, for 4 h. Thereafter, the couplets were cultured in SOFaa supplemented with 5% FBS, at 38.5°C, under 5% CO_2 in air prior to ET.

4.3.3 Estrus induction, embryo transfer and pregnancy assessment

Animal care and use

DCs of mixed breed aged 1-5 years, weighing 3-4 kg were used as embryo recipients. The cats were group-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 Faculty of Veterinary Science, Chulalongkorn University.

Estrus and ovulation inductions

The estrus and ovulation inductions were performed according to Yin et al. (2006). The cats were administered with 100-150 IU of equine chorionic gonadotrophin (eCG) (Folligon®, Intervet, The Natherlands), subcutaneously (s/c). The response to eCG 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. eCG-unresponsive cats were to be excluded. At 96 days later, ovulation was induced by administering 100 IU of human chrorionic gonadotrophin (hCG) (Chroluron®, Intervet, The Natherlands) (s/c).

Embryo transfer technique

The eCG-hCG treated cats were deprived of food and water for 9-12 h before the operation. Forty eight hours after 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,

Hungry), intramuscularly. Laparotomy was performed and the ovarian response was evaluated by the number of corpus hemorragicum (CH) (Fig. 20).

The IVF (Fig. 21A) and/or cloned embryos (Fig. 21B) at the 1 to 2 cell stage were transferred into the oviducts with the fimbria being held using tissue forceps. A polyethylene feeding tube (1 mm in diameter) containing embryos attached with a tuberculin syringe was inserted into the oviducts until the tip reached the ampulla. A volume of 0.2 ml air was flushed to deposit the embryos into the ampulla (Fig.22).

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 (Fig. 23) 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 45 after the embryo transfer by using real-time, B-mode ultrasonography. In cases where the pregnancy was negative, ovariohysterectomy was performed at day 45 after ET. The cats with implantation lesions characterized by reddish zonary bands on the endometrium were noted.

4.3.4 Statistical analysis

The developmental rate of IVF embryos cleaved at ≤27 and >27-42 h pi was compared by Chi-square analysis. The cell number of IVF derived from blastocyst at 18-27 and >24-42 h cleaved was presented as mean ± SD and compared by ANOVA. Significance was established at $P<0.05$. The number of CH and transferred embryos was presented as mean ± SD

4.3.5 Experimental design

Experiments I and II: *In vitro* development and the transfer of IVF embryos

The DC embryos were observed in their *in vitro* development to blastocyst (five replicates). IVF embryos cleaved at ≤27 h were selected for transferring into six recipients.

Experiment III: Transfers of FC-DC cloned embryos alone, FC-DC and DC-DC cloned embryos and FC-DC cloned and IVF embryos

FC-DC cloned embryos alone, FC-DC and DC-DC cloned embryos and FC-DC cloned and IVF embryos were transferred into the oviducts of five, five and four DC recipients, respectively.

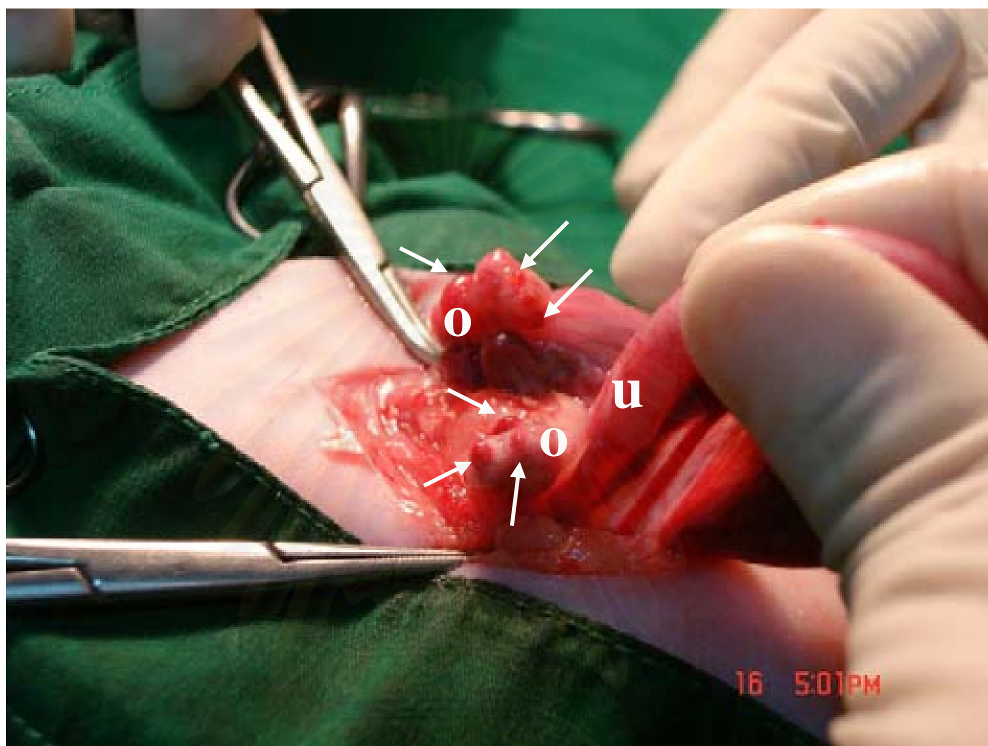

Figure 20. Evaluation of the ovarian responses of eCG-hCG treated recipients; after mid-ventral abdominal incision, the uterus (u) and ovaries (o) were observed in terms of ovarian response regarding the number of fresh CH (arrows)

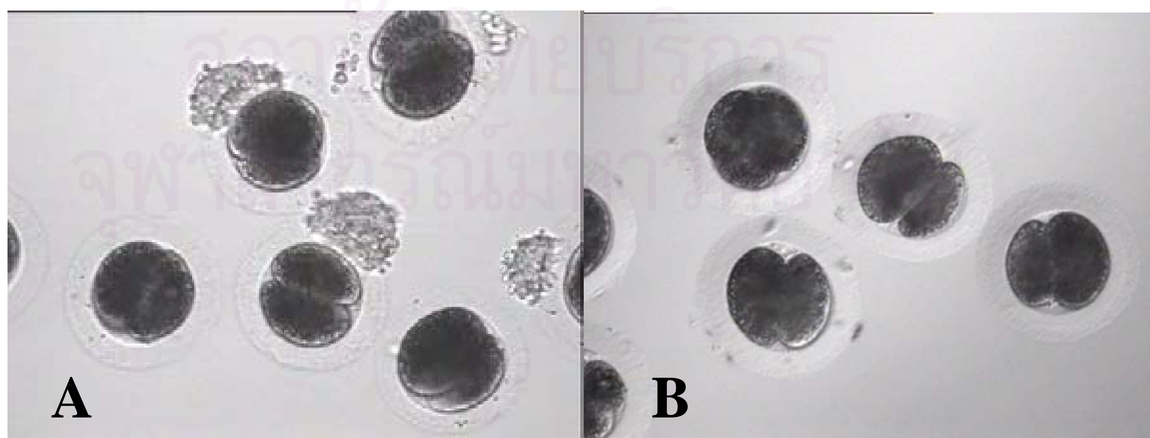

Figure 21. Transferred DC IVF and FC cloned embryos; two-cell stage DC IVF embryos at 27 h post insemination (A) and FC cloned embryos at 24 h of culture (B) displayed a similar architecture (x100)

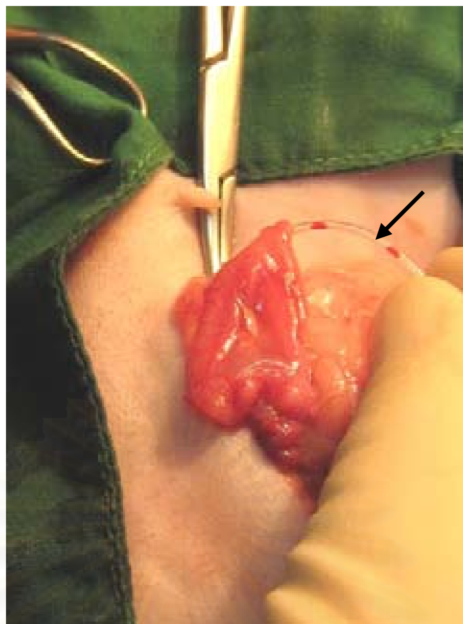

Figure 22. Embryo transfer by catheter (arrow); embryos were loaded in a polyethylene catheter with approximately 0.02 ml culture medium. The catheter was inserted into the oviduct via the fimbria.

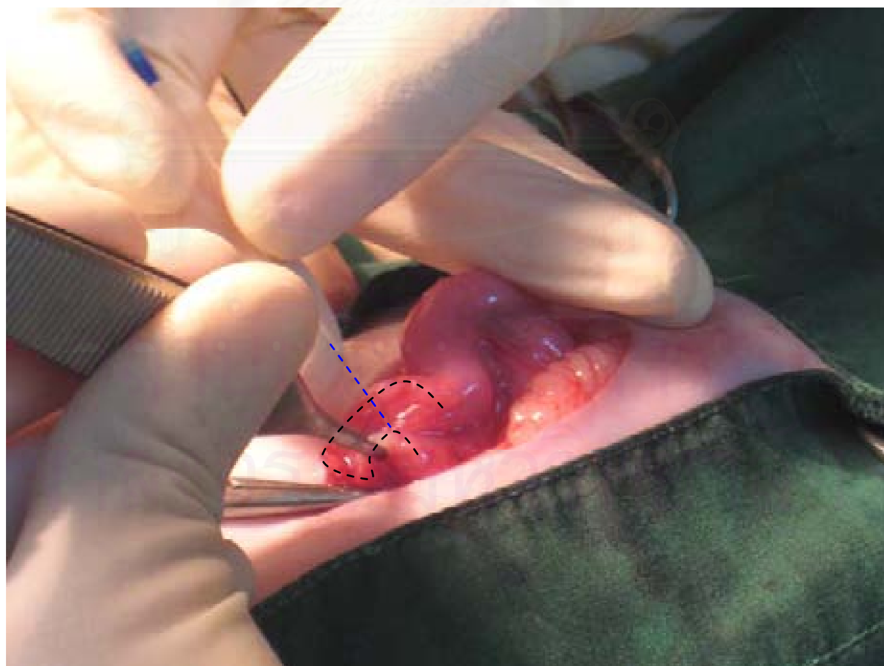

Figure 23. Embryo transfer by glass pipette (-----); the pipette containing embryos was inserted through the hole made by a needle and the embryos were deposited into the lumen of the oviduct (-----).

4.4 Results

4.4.1 Experiment I: *In vitro* development of IVF embryos and their transfer to the recipients

In vitro development of IVF embryos

The cleaved development of inseminated oocytes observed at 18-, 27- and 42 h pi was 37, 54.5 and 68.4%, respectively (Fig. 24). Cleaved embryos cleaved at ≤27 (n = 171) and >27-42 h pi (n = 59) reached the 8-cell stage equally (98.3%). However, the morula and blastocyst successes of the ≤27 h pi cleaved embryos were greater than those of >27-42 h pi cleaved embryos (92.4 vs. 59.3 and 61.4 vs. 18.6%, respectively; P<0.0001) (Fig. 25). The overall 8-cell, morula and blastocyst development of cleaved embryos (≤42 h pi) was 98.3, 84 and 50.4%, respectively (Fig. 26). The total cell number of the blastocyst derived from ≤27 h cleaved embryos (106 ± 43; n = 22) was greater than that of >27-42 h cleaved embryos (60 ± 27; n = 7) (P<0.05) (Fig. 27).

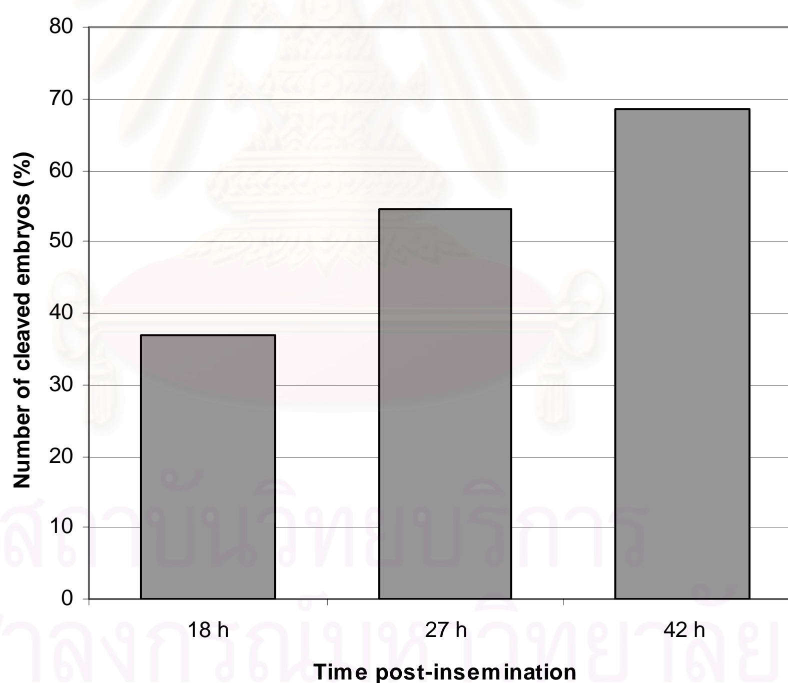

Figure 24. Development of IVF embryos evaluated at 18, 27 and 42 h post-insemination. Data represents percentages of cleavage based on n = 5 replicates (total 620 oocytes).

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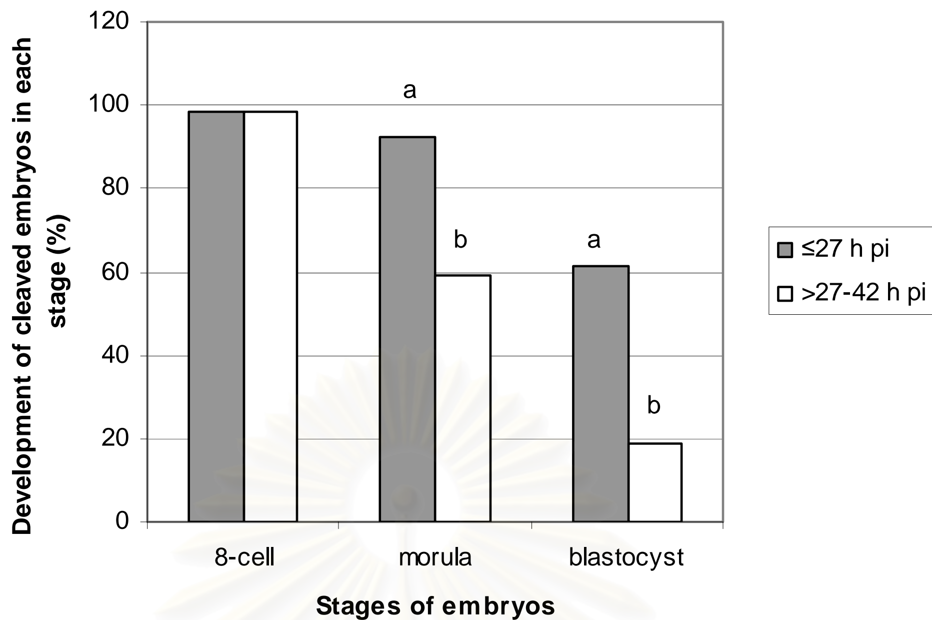

Figure 25. Development to the 8-cell, morula and blastocyst stages of embryos derived from 2-cell stage embryos cleaved at ≤27 (n=171) and >27-42 h (n=59) post-insemination (4 replicates); a, b P<0.0001.

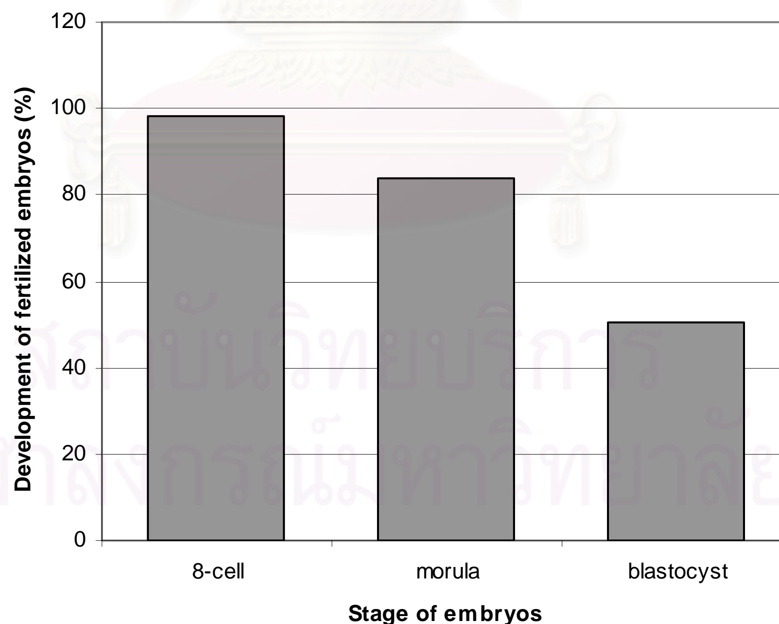

Figure 26. Development of fertilized embryos to 8-cell, morula and blastocyst observed at day 3, 5 and 7, respectively (n= 230 obtained from 4 replicates).

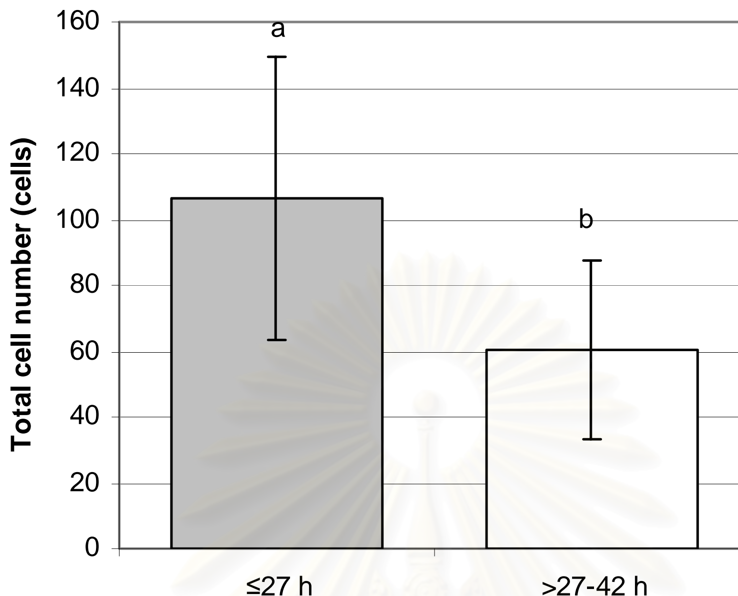

Figure 27. Total cell numbers of day 7 blastocyst derived from 2-cell stage IVF embryos cleaved at ≤27 (n=22) and >27-42 h (n=7) post-insemination. Data represents means ± S.E.M. based on 3 observations with 22 and 7 blastocysts, respectively; a, b P<0.05.

4.4.2 Experiment II: The pregnancy of recipients receiving IVF embryos

As shown in Table 13, a total of 150 cleaved embryos were transferred to 6 recipients (mean 25 ± 9). All recipients responded to eCG-hCG administration demonstrated by 9-20 CH on the ovaries (mean 11.8 ± 5.7). Ultrasonography at 30 days of gestation demonstrated that all (6/6) recipients became pregnant. Three recipients delivered five to-term kittens at day 60-63 of gestation. The cat IVF01 (Fig. 28A) delivered two live kittens (Fig. 28B) but then died 2 and 3 days post-parturition due to inadequate colostrums intake, 1 stillbirth (Fig. 28C) and 3 mummies (Fig. 28D-F). IVF05 (Fig. 29A) gave 2 healthy male kittens (Fig. 29B). IVF06 had one kitten that was cannibalized after birth. The other 3 recipients had severe respiratory infection during gestation and the pregnancy could not be maintained. The fetuses of IVF02, 04 were reabsorbed during midterm to late gestation. IVF03 aborted 2 fetuses on day 49 of gestation.

4.4.3 Experiment III: FC-DC cloned embryo transfer into recipients

The FC-DC cloned embryo transfer

As shown in Table 14, a total of 207 reconstructed FC-DC cloned embryos were transferred into the ampulla of the oviducts of five recipients (mean 41.4 ± 13). Three recipients responded well to the eCG-hCG treatment, demonstrated by the 6-16 CH on the ovaries (mean 11.3 ± 5). The cat NT02 responded slightly to eCG-hCG, showing only 1 CH and one follicular cyst on the left ovary. The NT04 showed a hyper-ovulated response to eCG-hCG administration, having 38 CHs. None of the recipients receiving flat-headed cat cloned embryos became pregnant.

The FC-DC and DC-DC cloned embryo transfer

As shown in Table 15, a total of 249 reconstructed cloned embryos (117 FC- DC and 132 DC-DC cloned embryos) were transferred to 5 recipients (mean 29.8 ± 20.8; 23.4 ± 15 of FC-DC and 26.4 ± 7 DC-DC cloned embryos). FC/DC05 had a cyst (3 mm in diameter) on the left fimbria but responded well to PMSG-hCG administration. An impairment of the oviduct junction and a cyst (1 mm in diameter) on the left oviduct were found in FC/DC06. None of the recipients receiving FC- and DC-DC cloned embryos became pregnant.

The FC-DC cloned and DC IVF embryo transfer

As shown in Table 16, a total of 220 embryos (177 FC-DC cloned and 43 DC IVF embryos) were transferred to 4 recipients (mean 55 ± 15; 43.3 ± 15 of FC-DC cloned and 10.8 ± 1.5 DC IVF embryos). NT/IVF01 delivered 2 stillbirths and 6 live IVF kittens (Fig. 30). NT/IVF04 did not respond to PMSG-hCG administration which presented 1 CH and old corpus luteum on the ovaries.

Table 13. The ability to establish pregnancy in gonadotrophin-treated recipients after IVM/IVF embryos in varying numbers were transferred into oviducts

Cat ID	Ovulation* R/L (n)	Transferred embryos** R/L (n)	Pregnancy/Implantation	Note
IVF 01	3/6 (9)	20/15 (35)	+/+	R: glass pipette transfer 2 live kittens, 1 stillbirth and 3 mummies at day 60 current status: died at day 2 and 3 after birth
IVF 02	n/3	15/15 (30)	+/+	reabsorbed fetus and 6 placentas †
IVF 03	11/9 (20)	15/15 (30)	+/+	2 aborted fetuses at day 49 †
IVF 04	12/3 (15)	20/5 (25)	+/+	reabsorbed fetus and 4 placental sites †
IVF 05	4/9 (13)	10/10 (20)	+/+	2 live kittens at day 63 current status: healthy
IVF 06	5/6 (11)	3/7 (10)	+/+	glass pipette transfer, cannibalized kitten at day 63
Total	11.8 ± 5.7	25 ± 9		

* Number of corpora heamorragica on the right and left ovaries
** Number of transferred embryos in right and left oviducts
† Respiratory disease infection
n: not observed

Table 14. The ability to establish pregnancy in gonadotrophin-treated recipients receiving FC cloned embryos

Cat ID	Ovulation* R/L (n)	Transferred embryos** R/L (n)	Pregnancy/Implantation	Note
NT 01	8/4 (12)	27/27 (54)	-/-	
NT 02	0/1 (1)	-/34 (34)	-/-	follicular cyst on left ovary
NT 03	4/2 (6)	18/39 (57)	-/-	
NT 04	23/15 (38)	7/23 (30)	-/-	
NT 05	9/7 (16)	32/- (32)	-/-	
Total	14.6 ± 14.3	41.4 ± 13		

* Number of corpus heamorragica and aspirated follicles on the right and left ovaries
** Number of transferred embryos in right and left oviducts

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Table 15. The ability to establish pregnancy in gonadotrophin-treated recipients receiving FC- and DC-DC cloned embryos

Cat ID	Ovulation* R/L (n)	Transferred embryos** R/L (n)	Pregnancy/Implantation	Note
FC/DC 01	7/13 (20)	DC22/FC22 (44)	-/-	
FC/DC 02	4/7 (11)	-/DC22 (22)	-/-	
FC/DC 03	15/8 (23)	DC38/ FC39 (77)	-/-	right oviduct using glass pipette transfer
FC/DC 04	5/10 (15)	DC28/FC34 (62)	-/-	cyst Ø 3 mm on left fimbria
FC/DC 05	12/10 (22)	FC22, DC22/- (44)	-/-	left oviduct: no UTJ + cyst Ø 1 cm
Total	18.2 ±5.1	49.8 ± 20.8 (DC 26.4 ± 7, FC 23.4 ± 15)		

* Number of corpor heamorragica on right and left ovaries
** Number of transferred embryos in right and left oviducts
UTJ: uterine tubule junction, DC: domestic cat, FC: flat headed cat

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Table 16. The ability to establish pregnancy in gonadotrophin-treated recipients receiving FC-DC cloned and DC IVF embryos

Cat ID	Ovulation* R/L (n)	Transferred embryos** R/L (n)	Pregnancy/Implantation	Note
NT/IVF 01	2/3 (5)	NT40, IVF2/NT15, IVF10 (NT 55, IVF12)	+/+	
NT/IVF 02	4/7 (11)	NT48, IVF5/NT10, IVF5 (NT58, IVF10)	-/-	right oviduct using glass pipette transfer
NT/IVF 03	5/3 (8)	NT23, IVF5/NT15, IVF4 (NT38, IVF9)	-/-	
NT/IVF 04	-/1 (1)	NT5, IVF6/NT21, IVF6 (NT26, IVF12)	-/-	show slight estrus signs old corpus luteum observed
Total	6.3 ± 4.3	55 ± 15 (NT 44.3 ± 15, IVF 10.8 ± 1.5)		

* Number of corpora heamorragica right and left ovaries
** Number of transferred embryos in right and left oviducts
NT: flat-headed cat nuclear transfer, IVF: domestic cat *in vitro* fertilization

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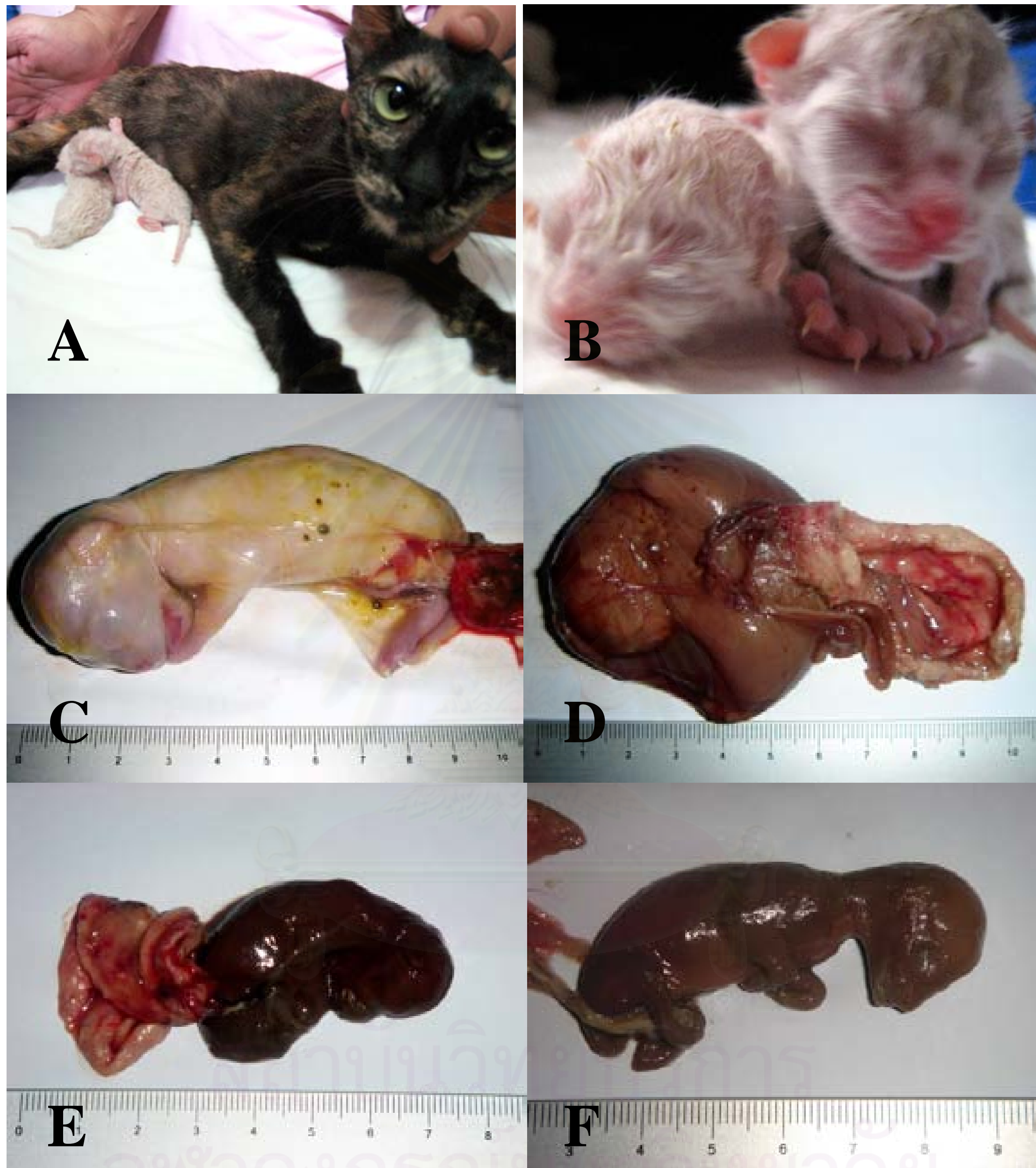

Figure 28. IVF01 (A) receiving 35 IVF embryos delivered 2 live kittens (B), still birth (C) and mummies (D-F)

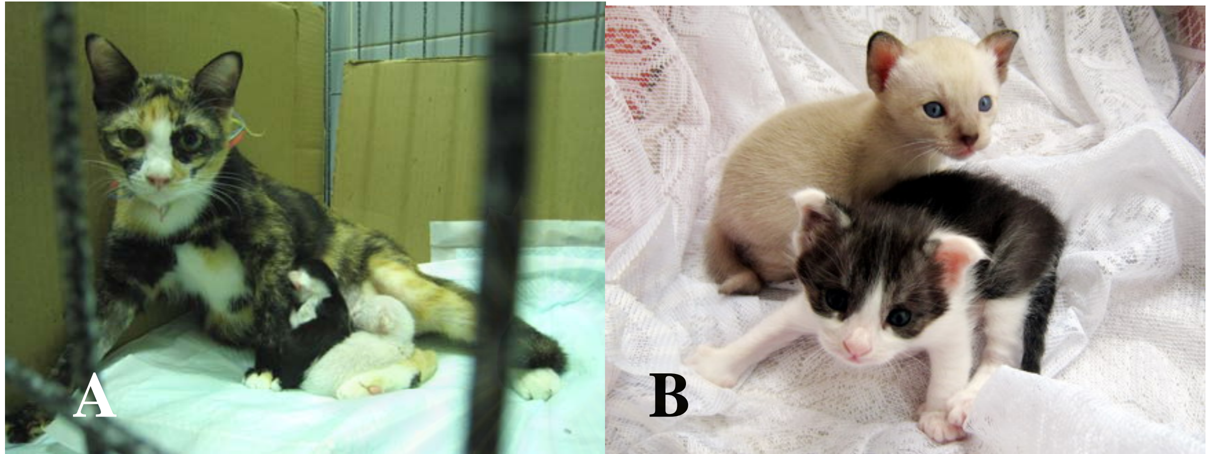

Figure 29. IVF05 (A) receiving IVF embryos delivered 2 healthy male kittens (B) at day 63 of gestation.

Figure 30. NT/IVF 01 receiving FC-DC cloned and DC IVF embryos delivered 2 stillbirths and 6 live IVF kittens at day 60 of gestation

4.5 Discussion

The present study demonstrated that pregnancy in recipients receiving IVF embryos could be established with a high success rate, resulting in 11 live IVF kittens from 4 recipients. However, the recipients receiving FC-DC cloned embryos did not become pregnant, which may be due to many factors relating to both the embryo and the recipient. Using the same FC cell line as in the previous study (chapter III), blastocyst development of FC-DC embryos in our previous study showed an evidently lower rate than that of DC IVF embryos observed in this study (6.7 vs. 61.4%). In addition, FC-DC cloned blastocysts had a lower cell number than that of the IVF ones (70 vs. 106 cells). It is suggested that the developmental competence and quality of embryos are the crucial factor affecting pregnancy establishment.

According to our previous data in chapter III, the *in vitro* development to blastocyst of FC-DC cloned embryos was similar to that in leopard cat (Yin et al., 2006) and higher than that in black-footed cat and rusty spotted cat cloned embryos (Gómez et al., 2004b). However, the birth of cloned offspring to those species has not been yet reported. It is not surprising to see this occurrence when a comparison of the blastocyst rate and cell numbers has been made with that of African wildcat cloned embryos, which were able to develop to term (blastocyst rate = 24% and cell number = 218 cells) (Gómez et al., 2004a). Besides this, the acceptance of pregnancy, in fact, involves the number of placenta and fetuses that can produce sufficient progesterone to maintain pregnancy (Feldman, 1996). As the investigation of Gómez et al. (2004a) demonstrated more than 30 African wildcat cloned embryos were required for pregnancy establishment. However, the number of transferred embryos depends on their development and quality, for example in domestic cat SCNT which had 4-6% blastocyst rate and 22-24 cells in blastocyst, 140 one-cell or 65 two to four-cell embryos were required to obtain live cloned kittens (Yin et al., 2006). Thus, if the culture system is standardized by IVF process, the *in vitro* development will reflect the competence of the cloned embryo to develop further *in utero*.

Our strategy to overcome the above limitations is by co-transfer of cloned embryos with IVF ones. We hypothesized that IVF embryos would support the *in vivo* development of cloned embryos, the acceptance of pregnancy and survival to term. However, this attempt has not been succeeded since only IVF embryos could develop to offspring. The possible explanation is that embryos created by ig-NT have a lower developmental capacity than that observed in embryos created by IVF. Moreover, many constraints limit the *in vivo* development of cloned embryos including karyotypically abnormal (Gómez et al., 2006b)

and inadequate nuclear remodeling or incomplete reprogramming. Furthermore, the aberrant of Oct-4 expression in trophectoderm found in black-footed cat and sand cat cloned embryos has been considered as the cause of early embryonic loss and an inability to maintain pregnancy (Gómez et al., 2005; Pope et al., 2007). Since up to now there is no report of success in the production of offspring derived by ig-NT, 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; Yin et al., 2006). Mitochondrial heteroplasmy may responsible for the developmental arrest in inter-species/generic reconstructed embryos (Thongphakdee et al., 2008) which perhaps from altered respiration in the mitochondria and influences survivability *in utero* of the cloned embryos (Gómez et al., 2006a). According to the previous report, 6 out of 435 leopard cat embryos were able to develop to day 30-45th fetus in DC uterus but could not develop to full-term (Yin et al., 2006). This failure may be due to a DC ooplasm which could not support the development of leopard cat fetuses, by abnormal gene expression or incompatibility between the mitochondrial DNAs of the donor cell and host oocyte.

Moreover, many factors in recipients affect the pregnancy establishment of recipients receiving *in vitro* derived embryos such as the response to estrus and ovulation induction and the genetic distance between embryo and recipient. A suitable surrogate mother for inter-species/generic cloned embryos of endangered animals should be considered because the genetic background of the surrogate mother and the cloned embryos is one of the crucial factors of pregnancy acceptance (Loi et al., 2007). However, the inter-species/generic ET between endangered species and domestic surrogates is limited by a low success rate and feto-maternal recognition is unclear (Andrabi and Maxwell, 2007). In the present study, the recipients responded to 100-150 IU eCG and 100 IU hCG administration by presenting fresh corpora haemorrhagica (mean 11.8±5.7) on the ovaries, and recipients receiving IVF embryos maintained pregnancy to term. In fact, the feline ovary is very sensitive to eCG but response depends on age, the estrus cycle induced and individual threshold (Colby, 1970). However, some of the recipients did not respond well to eCG-hCG administration. Whereas most of PMSG-hCG treated cats ovulated in average numbers, hyper- and non-response cats could be found. This indicated that administration of 100-150 IU eCG and 100 IU hCG was effective for the induction of estrus and ovulation for ET.

The IVF/ET procedures used in this study provided a high pregnancy rate. In addition, insights into embryo development can be used as an application for wild felids. The present study found that the time of the first division of the embryo indicates the ability

of further development and quality of embryos, as is shown by the significantly greater developmental competency and blastocyst cell numbers in embryos cleaved early (≤27 h pi) than those cleaved later (>27-42 h pi). Regarding the number of transferred embryos, this study found that the transfer of 35 cleaved IVF embryos per recipient caused over-crowding and resulted in high rates of stillbirth and mummification. Recipients receiving 10-20 cleaved-IVF embryos delivered 1-2 kittens. However, when FC-DC cloned and 12 cat IVF embryos were co-transferred to a recipient, 6 live kittens and 2 stillbirths were delivered. Thus, the number of kitten delivered was not influenced solely by the number of embryos transferred but was also influenced by the acceptance of the recipients individually. It suggests that embryos cleaved early are preferable for transfer for establishing a precise pregnancy. Pregnancy can be established whether the embryo is transferred by catheter or glass pipette injection. The proper number of transferred embryos at the 2-cell stage is between 10-12 embryos and the number of kittens derived varies depending on individual recipients.

In conclusion, 1) pregnancy could be established after domestic cat IVF embryo transfers and embryos cleaved early at 18-27 h pi were used for transfer rather than those cleaved later due to greater development and quality, 2) FC-DC ig-NT and DC-DC cloned offspring were not able to be produced which may have been caused by the low development and quality of the embryos and 3) by the co-transfer of cloned and IVF embryos to recipients, pregnancy could be established and live IVF but not FC kittens were obtained. Future research should focus on defining the factors affecting the development of ig-NT embryos and understanding the mechanism involved in nucleo-cytoplamic interaction, reprogramming and acceptance of pregnancy after ig-NT embryo transfer. More attempts are needed to improve the efficiency of SCNT and be this can then applied to the preservation of endangered felids.

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CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

Inter-generic nuclear transfer of endangered felids: feasibility and reality

When the somatic cell nuclear transfer (SCNT) issue is discussed, one important consideration is its application in the production of live offspring. Attempts using SCNT have been made intensively aiming for wild animal preservation. Successes in cloned offspring production have been reported, in guar (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Janssen et al., 2004), African wildcats (Gómez et al., 2004a) and ferrets (Li et al., 2006). On the other hand, several attempts for using SCNT in wild animals have failed to achieve this ultimate goal of nuclear transfer although various obstacles have been discovered. The key question of whether it is feasible for SCNT technology to be applied in reality remains to be investigated. Moreover, the results of this study indicate a hope for saving endangered felids by SCNT technology but future steps towards success also remain to be discussed. This chapter includes cloning multi-facets based on our investigation and other reports including prospective and realistic and practical solutions for the conservation of endangered felids.

At present, few reports are available on the birth of cloned wild felids but most studies deal with early embryonic development following NT. The major obstacle in advancing cloning success is the limited knowledge of basic reproductive physiology in the endangered species of interest. Producing embryos from cryobank-derived cells collected from alive or dead animals using NT is an alternative approach for the conservation of rare species. However, unlike AI or IVF, cloning is far from being useful as a valuable conservation tool because it does not alleviate the problem of loss of genetic diversity. Offspring will be identical genetically but are still impaired by the main factors i.e. the biological complexity of nuclear reprogramming and the availability of host oocyte donors and/or suitable recipients. Moreover great skill is needed together with, expensive laboratory facilities, scientific equipment and supplies and animal colonies. Also, long-term commitment is needed for improving efficiency and standardizing NT techniques.

From the embryologists' point of view, the development of SCNT for the production of embryos has been simplified and rapid progress in this field has been reported in several species. This technology can be successfully applied to wild animals that are closely related to their domestic counterparts. Studies including oocyte maturation, NT processes and embryo culture environment have laid the foundation for further research in domestic cat models. For example, low oocyte maturation success was identified in the past decade to be a crucial limitation of SCNT in felids. Currently, it has been improved by optimizing IVM conditions and thereby has increased IVM success up to 10-20%. Likewise, factors involved in NT processes (i.e. fusion, activation and embryo culture) have been extensively studied. As shown in this study (Chapter II), maturation time, electro-fusion intensity and chemical activation certainly influence SCNT success.

The possibility of embryo production in several non-domestic felids species has been reported, allowing further intensive investigation in other selected species. In Thailand, the conservation status of marbled cats and flat-headed cats is critical. Cryobanking of gametes, embryos and somatic cells collected from both live and dead animals has been established aiming to maintain genetic diversity by producing genetically desired offspring. Currently, fibroblast cells of several endangered felid species including marbled cats and flat-headed cats are successfully collected and preserved in cell bank for further study including SCNT. This study (Chapter III) shows that marbled cat and flat-headed cat cloned embryos produced by inter-generic nuclear transfer (ig-NT) have potentially developed *in vitro* as shown in domestic cat counterparts. Embryo development success in felids is also comparable with reports from other laboratories. However, a lower rate of blastocyst development of ig-NT embryos than that observed in IVF embryos was observed. In addition, no pregnancy of recipients receiving flat-headed cat and domestic cat NT embryos was observed in this study (Chapter IV).

The major constraints on the development of ig-NT embryos are likely to include chromosomal abnormalities derived from karyotypically abnormal somatic cells, inadequate nuclear reprogramming and inter-generic differences. Furthermore, other external factors such as 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*. Consequently, the use of fibroblast cells at late passage results in an increased

incidence of chromosomal abnormalities in cloned embryos (Gómez et al., 2006b). Abnormal nuclear reprogramming could be another cause of developmental arrest occurring at the embryonic stage (Gómez et al., 2003a). In addition, abnormal gene expression could be associated with incompatibilities between the nucleus of one genus and the cytoplasm of another genus that may inhibit the normal progression of epigenetic events required to create a functional genome (Vrana et al., 1998).

Although manipulation procedures for SCNT have been simplified, leading to the production of a large number of cloned embryos using straightforward methodologies, such technical progress has not been paralleled by an improved knowledge of the molecular mechanism underlying nuclear reprogramming. Such epigenetic alteration is still controversial but is probably responsible for low success rates and may be the first molecular sign of the placental abnormalities reported in many species. Unfortunately, this issue has not been investigated in inter-specific SCNT embryos. Our study did not investigate these factors in-depth to molecular level. However, such challenging issues in ig-NT will be overcome in the future.

Controversial issues such as methylaion and mtDNA heteroplasmy in SCNT clones still require further investigation. Whereas methylation in somatic cells has been found not to be involved in abnormalities in cloned cats, i.e. stillbirth, early fetal death, different eye and hearing attributes, the hypermethylation found in the placenta of cloned cats may be responsible for low success rates in cloning cats (Cho et al., 2007). Inevitably, a side effect of this approach is the production of cloned animals bearing mtDNA from the host oocyte and genomic DNA from the nucleus donor. Little is known about the effects of heteroplasmy in inter-species/generic SCNT. However, our recent report on feline-bovine embryos shows that developmental block is likely due to nuclear transcription failure and therefore affects the control of the mtDNA concentration (Thongphakdee et al., 2008). Nevertheless, the functional implication of mitochondrial heteroplasmy of inter-species/generic cloned embryos needs to be thoroughly investigated. An additional problem related to the use of host oocytes from different genera has still occurred. Our recent study on marbled cat and flat-headed cat ig-NT (Chapter II and III) demonstrated that some groups of embryos developed to the blastocyst stage but underwent developmental block at the zygotic transitional stage. This observation indicates that crucial developmental steps might have been impaired in ig-NT.

At present, many mechanisms of nuclear reprogramming contributing to normal development of cloned embryos remain unclear. However, the feasibility of producing normal live offspring by SCNT has been demonstrated in the domestic cat and African wildcat (Gómez et al., 2004a). Certainly, these findings allow for further investigation involving molecular mechanisms that might improve successful embryo production in other felids using SCNT and to evaluate its potential as a tool for preserving endangered felids.

On the other hand, the production of live kittens IVF is more efficient than NT. This study (Chapter IV) demonstrates a high pregnancy and delivery rate of recipients receiving IVF embryos. Many obstacles of NT-derived *in vivo* development i.e. embryonic loss by aberrance of gene expression, neonatal death by placental atrophy and dysfunction, respiratory failure due to lung immaturity, abdominal organ exteriorization, respiratory failure and immune dysfunction have been reported (Gómez et al., 2004a). However, some cloned cats with normal growth and reproductive fertility have been demonstrated (Choi et al., 2007). To date, only a small number of cloned felids has been investigated. Therefore, continuous evaluation throughout their lives with the examination of their capability for natural reproduction and other developmental abnormalities is required.

Wildlife conservation professionals state that SCNT must be considered an experimental technology at this stage (Loi et al., 2007). However, as the technique develops rapidly, the feasibility that SCNT can potentially become more applicable in the production of offspring from cryopreserved cells for genetic research purpose will be demonstrated.

Prospective directions & future solutions

The overall goal of this study is to contribute to basic knowledge in the development of endangered marbled cat and flat-headed cat embryos and the feasibility of producing their offspring using ig-NT technology. This has revealed the possibility of investigating their chronology. However, several hurdles have been placed in front of the ultimate goal of cloned offspring production. Accordingly, the way to enhance the potential application of this technology is first to get a better understanding of the fundamental biology of undifferentiated cell types, reprogramming, gene expression and epigenetic modification.

Secondly, further advances in SCNT technology will provide the opportunity to generate identical individuals and could potentially aid in preserving endangered species. Several efforts, such as generating embryonic stem cells and using the pluripotent cell as a donor nucleus, aim to increase the efficiency of ig-NT with regards to post-natal survival rates (Gómez et al., 2006a). In the case of inter-species/generic transfer, the genetic background of the surrogate mother and the embryos may be important. Obviously, the surrogate mother must be selected according to the phylogenetic distance and the pregnancy length, which must be close between the two species. The surplus generic or sub-specific hybrid females produced by artificial inseminated wild cat semen in female domestic cats may provide a source of conspecific recipients for embryo transfer (Swanson et al., 2006). Another challenge is the use of inner cell mass (ICM)-trophoblastic vesicle exchange by dissecting the ICM of the endangered produced by NT and then transferring to the ICM-removed trophoblastic vesicle of phylogenetically related domestic animals produced by IVF before transfer to recipients (Loi et al., 2007). This is to maximize pregnancy acceptance and possibly obtain normal live inter-species /generic NT offspring in the near future.

Lastly, much appreciable progress have been made in the development of assisted reproductive technologies for generating *in vitro* derived wild felid embryos. It may not be cost-effective to pursue a one-directional effort in advancing SCNT technologies. Future research and application should focus on 1) increasing fundamental research including understanding basic reproduction such as the estrus cycle, seasonality, structural anatomy and gamete physiology and 2) combining other ARTs including artificial insemination, IVF and intracytoplasmic sperm injection, in conjunction with gamete/embryo cryobanking. These combined efforts can make a useful and sustainable contribution to conserving genetic resources in endangered felids.

In conclusion

This study demonstrates the principal success in establishing ig-NT/ET in marbled cats and flat-headed cats in Thailand. Although the production of cloned offspring has not been yet achieved, several limiting factors related to results from this study have been discussed. A basic and in-depth understanding of underlying principles of NT needs to be studied more fully to improve its efficiency. The potential

use of SCNT as a tool for endangered species preservation is promising. Therefore, this study will allow further research to elucidate such challenges of ig-NT in endangered felids and demonstrate positive results for the future of cloning technology in wildlife conservation.

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APPENDICES

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APPENDIX A

All media were prepared using MilliQ water, otherwise indicate. The pH of all media was adjusted 7.2-7.4; the osmolarity were 300-310 mOsm for the fusion medium and 260-270 mOsm for embryo culture medium. All media were sterilized by filtration immediately after preparation then aliquot and stored at -20°C.

Stock solution

FSH	1 mg pFSH-P1/ml 0.9% NaCl
Oetradial	0.1 mg/ml ethanol
Hyaluronidase	0.5% (w/v) in M199 with hepes
Cycloheximide	1 mg/ml MilliQ water
Cytochalasin B	1mg/ml DMSO
6-DMAP	1 mg/ml M199 with $NaHCO_3$
Hoechst 33342	1 mg/ml MilliQ water

Oocyte collection medium	
M199 with hepes	25 mM
glutamine	0.292 g/ml
pyruvate	0.026 g/ml
BSA	0.4% (v/v)
Penicillin-streptomycin	1% (v/v)

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Oocyte Maturation medium	
M199	
glutamine,	0.292 g/ml
pyruvate	0.026 g/ml
BSA	0.4% (v/v)
FSH stock	1% (v/v)
Oestradiol	1% (v/v)
Penicillin-streptomycin	1% (v/v)

Enucleation medium	
M199 With hepes	25 mM
FBS	10% (v/v)
Cytochalasin B	7.5 µg/ml
Hoechst 33342	7 µg/ml

Fusion medium	
Mannitol	0.3 M
$MgSO_4.7H_2O$	0.1 mM
$CaCl_2$.2H2O	0.1mM

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Activation medium	
M199	
FBS	10% (v/v)
Cytochalasin B	5 μg/ml
Cycloheximide	10 μg/ml

Synthetic oviductal fluid plus amino acids medium (SOFaa)	
NaCl	6.294 g/l
KCl	0.534 g/l
KH_2PO_4	0.162 g/l
$CaCl_2H_2O$	0.251 g/l
$MgCl_26H2O$	0.1 g/l
$NaHCO_3$	2.106 g/l
Na lactate	282 μl/l
Na pyruvate	0.033 g/l
Phenol red	0.0013 g/l
Glutamine	0.146 g/l
Essential acids (0.5X BME)	1% (v/v)
Non essential amino acids (MEM)	1 % (v/v)

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APPENDIX B

List of publications and conferences

1. Thongphakdee, A., Numchaisrika, P., Rungsiwiwut, R., Omsongkram, S., Chatdarong, K. and Techakumphu, M. 2004. *In vitro* development of marbled cat (*Pardofelis marmorata*) cloned embryos. **J Thai Vet Med Assoc** 55: 31-42.
2. Thongphakdee, A., Numchaisrika, P., Omsongkram, S., Chatdarong, S., Kamolnorranath, S., Dumnui, S. and Techakumphum, M. 2005. *In vitro* development of marbled cat embryos derived from interspecies somatic cell nuclear transfer. **Reprod Dom Anim** 41: 219-226.
3. 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: 142-147
4. Thongphakdee, A., Numchaisrika, P., Rungsiwiwat, R., Kamolnorranath, S., Chatdarong, K and Techakumphu, M. 2007. Inter-generic marbled cat and flat headed cat cloned blastocysts generated from domestic cat and rabbit oocytes. **Reprod Fert Dev Suppl** 19: 163.
5. Rungsiwiwut, R., Thongphakdee, A., Numchaisrika, P., Sirivaidayapong, S. and Techakumphu, M. 2005. The effect of culture media and culture time on the *in vitro* maturation of domestic cat oocytes. **Thai J Vet Med** 35: 39-45.
6. Numchaisrika, P., Rungsiwiwut, R., Thongphakdee, A. and Techakumphu, M. 2005. A preliminary study of the *in vitro* development of Asian elephant cloned embryos, reconstructed using a rabbit recipient oocyte**. Reprod Fert Dev Suppl** 17: 179-180.
7. Numchaisrika, P., Thongphakdee, A., Rungsiwiwat, R., Pruksananon, K., Virutamasen, P. and Techakumphu, M. 2007. The development of intra- and interspecies cloned embryo derived from rabbit oocytes; The effect of donor cell sources. **Reprod Fert Dev Suppl** 19: 153

8 Rungsiwiwat, R., Thongphakdee, A., Numchaisrika, P., Virutamasen, P. and Techakumphu, M. 2007. Mouse cloning by using laser assisted zona opening and elcectro-fusion technique. **Reprod Fert Dev Suppl** 19: 158

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