EXPRESSION OF GENES CRUCIAL FOR PREIMPLANTATION OF FELINE EMBRYOS DEVELOPMENT AFTER NUCLEAR TRANSFER

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นายสุเมธ อิ่มสุนทรรักษา

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ปัจจุบันสัตว์ตระกูลแมวป่าถึง 36 ชนิด กำลังอยู่ในสภาวะใกล้สูญพันธุ์ ซึ่งเทคโนโลยีการช่วยการเจริญพันธุ์พื้นฐาน ไม่สามารถเพิ่มจำนวนสัตว์ในกลุ่มใกล้สูญพันธุ์ได้ เนื่องจากขาดแคลนตัวสัตว์ที่จะใช้นำมาเข้ากระบวนการผสมพันธุ์ ดังนั้นเทคโนโลยีการโคลนนิ่งข้ามสายพันธุ์ จึงเป็นอีกทางเลือกหนึ่งที่สามารถแก้ปัญหาดังกล่าวได้ การศึกษาครั้งนี้แบ่งออกเป็น 2 การทดลอง การทดลองแรก เป็นการศึกษาการเจริญเติบโตของตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่งในหลอดทดลอง จากผลการทดลองพบว่า น้ำยาที่นำมาใช้เลี้ยงไข่ระหว่างกระบวนการทำโคลนนิ่งมีผลต่ออัตราการเจริญของตัวอ่อนแมวบ้านโคลนนิ่ง โดยไข่ที่เลี้ยงในน้ำยา Emcare ให้อัตราการเจริญสู่ระยะบลาสโตซีสสูงกว่าไข่ที่เลี้ยงในน้ำยา 199H (33.3% และ 18.1% ตามลำดับ) การเลี้ยงตัวอ่อนแมวบ้านโคลนนิ่งแบบ ไม่เลี้ยงร่วม หรือเลี้ยงร่วมกับเซลล์เยื่อบุท่อนำไข่ของแมวบ้าน ให้อัตราการเจริญของตัวอ่อนไม่แตกต่างกัน (44.4% และ 38.0% ตามลำดับ) นอกจากนี้ยังพบว่าตัวอ่อนแมวลายหินอ่อนโคลนนิ่งข้ามสายพันธุ์ด้วยไข่แมวบ้านหยุดการเจริญเติบโตที่ระยะมอรูล่า ทั้งการเลี้ยงแบบไม่เลี้ยงร่วม หรือเลี้ยงร่วมกับเซลล์เยื่อบุท่อนำไข่ของแมวบ้าน (28.6% และ 32.6% ตามลำดับ) จากการใช้ไข่ของแมวบ้านและโค เป็นไซโตพลาสผู้รับสำหรับการทำโคลนนิ่งข้ามสายพันธุ์ในสัตว์ตระกูลแมวพบว่า ตัวอ่อนแมวบ้านโคลนนิ่งและตัวอ่อนโคโคลนนิ่ง มีอัตราการเจริญสู่ระยะบลาสโตซีสสูงกว่า ตัวอ่อนแมวลายหินอ่อนโคลนนิ่งข้ามสายพันธุ์ด้วยไข่โค (34.4% กับ 29.6% และ 8.6% ตามลำดับ) และยังพบว่าตัวอ่อนแมวบ้านโคลนนิ่งข้ามสายพันธุ์ด้วยไข่โคหยุดการเจริญเติบโตที่ระยะมอรูล่า (15.2%) จากการทดลองแรกสามารถสรุปได้ว่า น้ำยาที่ใช้เลี้ยงไข่ระหว่างกระบวนการทำโคลนนิ่งมีผลต่อการเจริญสู่ระยะบลาสโตซีส ของตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่ง อย่างไรก็ดีการเลี้ยงตัวอ่อนร่วมกับเซลล์เยื่อบุท่อนำไข่ไม่สามารถช่วยสนับสนุนให้การเจริญเติบโตของตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่ง อีกทั้งยังพบว่าไข่ของแมวบ้านและโคสามารถช่วยสนับสนุนการเจริญเติบโตของตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่งข้ามสายพันธุ์ได้ถึงในระยะแรกของการพัฒนาตัวอ่อนเท่านั้น การทดลองที่สองเป็นศึกษาการแสดงออกของยีนที่เชื่อว่ามีผลต่อกระบวนการ nuclear reprogramming (Oct4 DNMT1 DNMT3a DNMT3b HAT1 และ HDAC1) ในตัวอ่อนแมวบ้านโคลนนิ่ง ตัวอ่อนแมวดาว และแมวลายหินอ่อนโคลนนิ่งข้ามสายพันธุ์ด้วยไข่แมวบ้าน จากการทดลองพบว่า การแสดงออกของยีน Oct4 และ HAT1 ในตัวอ่อนแมวบ้าน

โคลนนิ่ง และแมวดาวโคลนนิ่งข้ามสายพันธุ์ มีรูปแบบการแสดงออกคล้ายกับตัวอ่อนแมวบ้านที่เกิดจากการปฏิสนธิในหลอดแก้ว (IVF) โดยในระยะแรกของการเจริญเติบโตมีการแสดงออกของ Oct4 และ HAT1 mRNA ต่ำ จนกระทั่งถึงระยะ 8 เซลล์ และค่อยๆแสดงออกเพิ่มขึ้นอย่างต่อเนื่องจนกระทั่งแสดงออกสูงที่สุดในระยะบลาสโตซีส ในทางตรงกันข้ามพบว่าตัวอ่อนแมวลายหินอ่อนโคลนนิ่งข้ามสายพันธุ์มีการแสดงออกของยีน Oct4 และ HAT1 ต่ำตลอดระยะของการเจริญเติบโตจนถึงระยะมอรูล่า และไม่พบการเจริญสู่ระยะบลาสโตซีสในตัวอ่อนแมวลายหินอ่อนโคลนนิ่งข้ามสายพันธุ์ การศึกษาการแสดงออกของยีน HDAC1 ในตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่ง พบว่ามีความแปรปวนสูง และมีรูปแบบการแสดงที่ต่างไปกับตัวอ่อน IVF ส่วนระดับการแสดงออกของยีน DNMT1 มีรูปแบบการแสดงออกที่ลดลงอย่างต่อเนื่องตั้งแต่ระยะ 2 เซลล์ จนถึงระยะบลาสโตซีส ซึ่งตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่งมีการแสดงออกที่สูงกว่าตัวอ่อน IVF ตั้งแต่ระยะมอรูล่าจนถึงระยะบลาสโตซีส จากการศึกษายังพบว่าตัวอ่อน IVF มีการแสดงออกของ DNMT3a ต่ำที่สุดที่ระยะ 4 เซลล์ แล้วค่อยๆ เพิ่มสูงขึ้นจนถึงระยะบลาสโตซีส ซึ่งต่างกับตัวอ่อนสัตว์ตระกูลแมวโคลนนิ่งที่ในระยะ 4 เซลล์ ยังมีการแสดงออกสูงกว่าตัวอ่อน IVF จากนั้นค่อยๆ ลดลงจนถึงระยะมอรูล่าและแสดงออกเพิ่มขึ้นอย่างรวดเร็วในระยะ บลาสโตซีส รูปแบบการแสดงออกของยีน DNMT3b ในตัวอ่อนสัตว์ตระกูลแมว ทั้ง IVF และโคลนนิ่ง มีความคล้ายคลึงกัน โดยมีการแสดงออกต่ำมากในระยะ 4 เซลล์จนถึงระยะมอรูล่า จากนั้นมีการแสดงออกเพิ่มขึ้นอย่างรวดเร็วในระยะบลาสโตซีส ซึ่งจากการทดลองนี้แสดงให้เห็นว่า การแสดงออกของยีน Oct4 ในตัวอ่อนสัตว์ตระกูลแมวมีผลต่อการเจริญเติบโตของตัวอ่อน โดยเฉพาะอย่างยิ่งการเจริญสู่ระยะบลาสโตซีส นอกจากนี้ยังพบว่า ตัวอ่อนสัตว์ตระกูลแมวที่เกิดจากการทำโคลนนิ่งมีการแสดงออกที่ผิดปกติของยีนที่เกี่ยวกับ การเติมหมู่เมทิลบนสายดีเอ็นเอ (DNA methylation) และการเติมหมู่อะซิติลบนโปรตีนฮีสโตน (histone acetylation) ซึ่งแสดงให้เห็นว่าอาจเกิดกระบวนการ nuclear reprogramming ที่ไม่สมบูรณ์ ของเซลล์ต้นแบบในการทำโคลนนิ่งในสัตว์ตระกูลแมว จากการศึกษาครั้งนี้ทำให้เข้าใจถึงรูปแบบของการแสดงออกของยีนที่เกี่ยวกับ reprogramming ในสัตว์ตระกูลแมว ซึ่งจะสามารถนำความรู้ที่ได้ไปใช้ในการปรับปรุง และเพิ่มประสิทธิภาพในการทำโคลนนิ่งสัตว์ตระกูลแมวใกล้สูญพันธุ์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SUMETH IMSOONTHORNRUKSA : EXPRESSION OF GENES CRUCIAL FOR PREIMPLANTATION OF FELINE EMBRYOS DEVELOPMENT AFTER NUCLEAR TRANSFER. THESIS ADVISOR : ASST. PROF. RANGSUN PARNPAI, Ph.D., 126 PP.

INTERSPECIES CLONING/FELINE EMBRYO/GENE EXPRESSION /NUCLEAR REPROGRAMMING

There are, in fact, 36 of 37 species of felid family classified as being threatened or endangered. The conventional assisted reproduction technique (ART) is inappropriate for producing endangered felid species, due to the lack of animals for breeding program. Interspecies cloning is a novel technology that may serve as a valuable model for study *in vitro* and *in vivo* development of cloned felid embryos. Two experiments were carried out in this research.

The first experiment aimed to investigate the *in vitro* development of cloned felid embryos. The results of this experiment indicated that the manipulation medium had an effect on the development rate of cloned domestic cat embryos. The Emcare holding medium gave significantly greater blastocyst formation than that of 199H (37.3 vs 21.9%, respectively). There was no significant difference between the developments to blastocyst stage of non co-culture (44.4%) and co-culture (38.0%) of the cloned domestic cat embryos with domestic cat oviductal epithelium cells. Moreover, the non co-culture and co-culture systems could not support the development of interspecies cloned marbled cat embryo beyond morula stage (28.6 vs 32.6%, respectively). The development to blastocyst stage of intraspecies of cloned

domestic cat (39.8%) and bovine (30.9%) was higher than that of interspecies cloned marbled cat with bovine oocytes (9.2%). The interspecies cloned domestic cat with bovine oocytes arrested development at morula stage (16.6%). These results demonstrated that the manipulation medium during the cloning process involved in the increased development to blastocyst stage of cloned felid embryos. However, the co - culture system of cloned felid embryos was not beneficial to the development. The domestic cat and bovine oocytes could support mitotic cleavage of felid nuclei only in the early stage of development.

In the second experiment, the transcription levels of genes believed to be crucial for nuclear reprogramming (Oct4, DNMT1, DNMT3a, DNMT3b, HAT1 and HDAC1) were investigated in cloned felid species, domestic, leopard and marbled cats. The results showed that transcription levels of Oct4 and HAT1 were low at an early development stage and dramatically increased at 8-cell to blastocyst stage in cloned domestic and leopard cats and IVF derived embryos. In contrast, transcription levels of Oct4 and HAT1 in interspecies cloned marbled cat embryos were low throughout the development up to the morula stage. Moreover, the interspecies cloned marbled cat embryos with low transcription level of Oct4 and HAT1 could not develop into blastocyst. The development was arrested at the morula stage. The transcription levels of HDAC1 of cloned felid embryos had altered an expression pattern compared to IVF derived embryos. The DNMT1 transcript levels dramatically decreased throughout development of felid embryos. Cloned felid embryos showed higher transcript of the DNMT1 gene than IVF derived embryos. For mRNA level of DNMT3a, it was found that IVF derived embryos rarely had any transcript at 4- to 8-cell stages and increased at morula up to blastocyst stage whereas the transcription

levels decreased in an early development and increased again at the blastocyst stage of cloned felid embryos. The level of DNMT3b mRNA decreased from 2-cell to morula stages but became remarkably high at blastocyst stage. The results of the second experiment clearly demonstrated that Oct4 mRNA transcription levels in felid embryos had some effect on the *in vitro* development efficiency particularly at the blastocyst stage. The cloned felid embryos showed aberrant level of genes involved in DNA methylation and histone hypoacetylation. The results suggested that incomplete nuclear reprogramming of felid donor nuclei occur in cloned felid embryos. This research indicated that genome reprogramming in the felid species was abnormal which may not be enough for the establishment of embryonic totipotence. The nuclear transfer technique of felid species needs improvement, especially in the genome reprogramming to improve the efficiency of cloned felid embryos for the conservation of endangered felid species.

School of Biotechonology

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LIST OF ABBREVIATIONS

BSA	=	bovine serum albumin
CHX	=	cyclohexymide
CB	=	cytochalasin B
CD	=	cytochalasin D
CT	=	cycle threshold
°C	=	degree celsius
∅	=	diameter
DNMT	=	DNA methyltransferase
DEOC	=	domestic cat epithelium oviductal cell
DC	=	direct current
eCG	=	equine chlorionic gonadotropin
FBS	=	fetal bovine serum
HAT1	=	histone acetyltransferase 1
HDAC 1	=	histone deacetylase 1
ICM	=	inner cell mass
IU	=	international unit
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
kg	=	kilogram
µg	=	microgram

LIST OF ABBREVIATIONS (Continued)

µl	=	microliter
µsec	=	microsecond
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
M	=	molar
mSOFaa	=	modified synthetic oviduct fluid with amino acids
HEPES	=	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
NT	=	nuclear transfer
Oct4	=	octamer binding transcription factor
PA	=	parthenogentic activation
h	=	hour
hCG	=	human chlorionic gonadotropin
rpm	=	round per minute
SCNT	=	somatic cell nuclear transfer
SEM	=	standard error means
199H	=	tissue culture medium 199 supplement with HEPES buffer
TE	=	trophectoderm
V	=	volts

CHAPTER I

INTRODUCTION

1.1 Background

In 1997, the birth of the first cloned sheep, Dolly, which was not created from the fertilization of an oocyte and a sperm but created by the transfer of a nucleus from somatic cell (mammary gland) into a matured oocyte devoid of its own nuclear DNA by the process of nuclear transfer (NT) or cloning (Wilmut et al., 1997) surprise the whole world. Recently, somatic cell nuclear transfer (SCNT) had been successfully achieved in various species such as cattle (Cibelli et al., 1998; Kato et al., 1998), goat (Baguisi et al., 1999), mice (Wakayama et al., 1998), pig (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), rabbit (Chesne et al., 2002), cat (Shin et al., 2002), mule (Woods et al., 2003), horse (Galli et al., 2003) rat (Zhou et al., 2003), dog (Lee et al., 2005), ferret (Li et al., 2006) and buffalo (Shi et al., 2007)

NT technique can be divided into two types, intraspecies and interspecies NT. Intraspecies NT, the nucleus of a somatic cell is taken from one species and placed into an enucleated egg of the same species. While, Interspecies NT is different from intraspecies NT because the nucleus of a somatic cell form one species is transferred to an enucleated egg from a different species. Thus, interspecies NT has the potential to conserve genes from critically endangered wild life species which few or no oocytes available.

The survival of most species in the felid family are considered to be under threated or endangered. In fact, 36 of 37 species of this family are risk of extinction (Convention on International Trade in Endangered Species, CITES). The declining population could be attributed to poaching and human destruction of natural ecosystem. In Thailand, marbled cat (*Pardofelis marmorata*) is a wild cat that is also risk of extinction, the real population has not yet been reported but it is considered to be endangered. The size of marbled cat is a bit larger than a big domestic cat (*Felis catus*). The coat color ranges from brownish and grey with pattern of irregular dark marbled blotches and spots, outlined with black fur. However, in term of reproductive characteristic, the marbled cat is similar to domestic cats. The marbled cat can give birth to one to four kittens. It takes about 81 days gestation period. The kitten birth weight is about 100-110 g. Marbled cat had been heavily persecuted and CITES holds the marbled cat on appendix I.

Recently, the feasibility of producing viable domestic cat offspring by NT was demonstrated (Shin et al., 2002). In term of endangered felids, NT has a potentially valuable technique for assuring the continuation of species with dwindling number. Although interspecies NT had been reported in several species such as gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), korean tiger (Hwang et al., 2000), leopard cat (Lorthongpanich et al., 2003), African wild cat (Gomez et al., 2004), banteng (Sansinena et al., 2005) and marbled cat (Thongphagdee et al., 2005). However, there has not been reported on the successful of embryo transfer to full term development of marbled cat interspecies NT. Up to now, the number of marbled cat is very few leading to risk of extinction. In addition, the reproductive physiology of marbled cat is not well understood. The natural breeding is a difficult technique to reproduce the

marbled cat. Moreover, marbled cat are now absence form zoos in Thailand (unpublished data) and the population in nature has not been reported. However, the assisted reproductive technology, NT, can be used for conserve and increase the number of marbled cat. Using of domestic cat oocytes as a recipient cytoplast would be greatly beneficial in marbled cats cloning research, that is because the abundant of domestic cats. Interspecies NT might become an alternative method of produce cloned embryos, especially in some species that whose oocyte are difficult to obtained. Therefore, it might be applied for saving highly endangered species. Domestic cat oocytes cytoplasm had been successfully used as xenogenic oocyte to produce interspecies NT embryo of African wild cat (Gomez et al., 2003; 2004).

Successful NT is measured initially by the efficiency of blastocyst development and viable offspring. However, the relatively successful development of cloned embryos to blastocyst stage does not reflected in their implantation and subsequent development competence following transfer to recipient animal (Gurdon and Colman, 1999). While the NT technique is a powerful tool for cloning animals, its efficiency in terms of the numbers of live offspring born is low because of the high rate of fetal loss after transfer (Daniels et al., 2000). This fetal loss is due to a high frequency of embryonic, fetal, and neonatal abnormalities (Young et al., 1998; Niemann et al., 2002). The low efficiency and abnormal development of cloned animal are mainly due to incomplete reprogramming and abnormal gene expression. The expression of several important genes have been assessed in cloned embryos (Daniels et al., 2000; Wrenzycki et al., 2001). In a study in which global gene expression were analyzed by microarray in cloned embryos derived from stem cells and somatic cell, 4% of more the 10,000 genes differ in expression from the controls

(Humpherys et al., 2002). Epigenetic modification or nuclear reprogramming defects described in cloned embryo include errors in X inactivation (Eggan et al., 2000; Xue et al., 2002), imprinting (Humpherys et al., 2001; Inoue et al., 2002), DNA methylation (Dean et al., 2001; Humpherys et al., 2001; Kang et al., 2001), histone acetylation and methylation (Santos et al., 2003) and widespread alterations in gene expression (Humpherys et al., 2002) including the failure to activate Oct4 and related key pluripotency gene (Boiani et al., 2002; Bortvin et al., 2003). There have been studies on specific gene expression in cloned embryos (Daniels et al., 2000; 2001; Niemann et al., 2002) but the molecular mechanism of reprogramming in cloned embryos are still unclear. To date, there had been no study on specific gene expression profiles of felid family NT embryos. These profiles might explain the developmental failures.

1.2 Research objectives

1.2.1 To study the potential of domestic cat oocyte as recipient cytoplast for felids cloning.

1.2.2 To analyze the transcriptions level of genes that involve in nuclear reprogramming in preimplantation cloned felid embryos by using real-time quantitative PCR.

1.3 Research hypotheses

1.3.1 Domestic cat oocytes could be used as recipient cytoplasts to support the development of cloned felid embryos. After cloned embryos transfer, domestic cat can be pregnant and healthy kittens born.

1.3.2 Interspecies NT can be use as a tool for the endangered felid species conservation.

1.3.3 Some gene expression profile of felids cloned embryos when compare with *in vitro* fertilized embryo of domestic cat can be used to assess embryo viability prior to transfer to recipient animal.

1.4 Scope of the study

1.4.1 The potential of domestic cat to be use as recipient cytoplast to produce the cloned marbled cat embryos was investigated. In addition, the manipulation medium, *in vitro* culture systems (coculture and non-coculture with DEOC) and type of recipient cytoplasts were conducted to study the *in vitro* development of cloned felid embryos.

1.4.2 Transcription levels of pluripotent (Oct4), histone acetylation (HAT1, HDAC1) and DNA methylation (DNMTs) gene in intra- and interspecies cloned felid embryos were investigated. During culture, individual 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stage embryos were harvested for mRNA extraction and gene expression analysis using real-time quantitative PCR. These results were compared with those of *in vitro* produced embryos (IVF) for each stage of embryo development.

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CHAPTER II

LITERATURE REVIEW

2.1 Somatic cell Nuclear Transfer (SCNT)

The birth of “Dolly” has demonstrated the successful production of cloned animal via transplantation of somatic cell into enucleated oocytes (Wilmut et al., 1997). Dolly has progressed from being a novel technology to widely used technique of generating identical individual and a model for understanding the cellular and molecules aspects of nuclear reprogramming. Successful cloning by SCNT have been achieved in mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999), bovine (Cibelli et al.,1999; Kato et al. 1999), pig (Polejaeva et al. 2000), cat (Shin et al., 2002), rabbit (Chesne et al., 2002), horse (Galli et al., 2003), mule (Woods et al. 2003), dog (Lee et al., 2006), ferret (Li et al., 2006) and buffalo (Shi et al., 2007).

However, the technique of SCNT is not only a valuable tool for producing animal with the same genetic traits, it is also a prospective alternative to save endangered animal species. In endangered species, the lack of oocytes and recipients precludes the use of traditional SCNT. An approach that transfers the nuclei of one species to an oocyte of another species is termed interspecies cloning. This may be the only alternative to produce these endangered species. Previous study have shown that of domestic species such as cattle, sheep, rabbit and cat can be used for dedifferentiation of somatic cell nuclei from rat, pig, monkey, sheep (Dominko et al., 1999), gaur (Lanza et al., 2000), buffalo (Kitiyanant et al., 2001), giant panda (Chen

et al., 2002), banteng (Sansinena et al., 2005), African wildcat (Gomez et al., 2003, 2004), leopard cat (Lorthongpanich et al., 2004) and marbled cat (Thongphakdee et al., 2006). They could support the development of the cloned embryos to blastocyst stage. In addition, successful production of the interspecies cloning such as gaur, mouflon and African wide cat (Lanza et al., 2000; Loi et al., 2001; Gomez et al., 2004) have demonstrated that the interspecies cloning can considered to be an effective method to conserve highly endangered species.

For felid species, “CC” is the world’s first cloned domestic cat using adult somatic cell as donor nuclei (Shin et al., 2002). Thereafter, interest in the studies to use the SCNT technique to produced cloned embryos for preservation and commercial purposes (Table 2.1 and 2.2) have increase. However, SCNT in domestic cat has been a powerful technique to understand the basic knowledge in efforts to increase the efficiency of embryo production. To date there have been several reports describing the source (Skrzyszowaka et al., 2003; Kitiyanant et al., 2003; Yin et al., 2005) and modification (Gomez et al., 2003; Yin et al., 2007) of donor cell and the nuclear transfer procedure (Gomez et al., 2003; Wen et al., 2003) the effects on the cloned domestic cat embryos development. SCNT in cats not only provides the opportunity to genetically duplicate a deceased pet, but also presents the prospect of preserving endangered felids. Clearly, natural breeding should be the preferred method for propagation and genetic management within a population. On the other hand, when populations or subpopulations are at risk of extinction, SCNT may be a valuable approach for species restoration. Most wild felids are threatened, and the feasibility of using this technology to preserve endangered felid was demonstrated with the birth of African wildcat (*Felis silvestris lybica*) cloned kittens (Gomez et al.,

2004a). Since then, many studied in the interspecies cloned of felid species including korean tiger (Hwang et al., 2004), leopard cat (Lorthongpanich et al., 2004; Yin et al., 2006), rusty spotted cat (Gomez et al., 2004b), black-footed cat (Gomez et al., 2006) and marbled cat (Thongphakdee et al., 2006) have been reported. However, felids cloning are difficult and so far low numbers of live cloned offspring have been reported. (Shin et al., 2002; Gomez et al., 2004; Yin et al., 2005; 2007). This may be directly related to the insufficientcy reprogramming of the donor cell nuclei when transplantation into enucleated oocytes.

However, the efficiency of producing SCNT animal is very low. Less than 1% of cloned embryos gave rise to live born offspring (Table 2.3). The defects are due to severe developmental problems including a high rate of abortion during early gestation and increase prenatal death (Han et al., 2003). It is unclear whether the development failures of cloned embryos are due to the incomplete nuclear reprogramming or the SCNT procedure itself. The procedures of SCNT that involves a series of complex methods including donor cell preparation, oocyte preparation, encleation, cell injection, fusion, activation, embryo culture and embryo transfer. If some parts of these step are suboptimal, these will influenced the production of cloned embryo and live offspring. Many laboratories worldwide have been studies to generated information about nuclear reprogramming and how to increase the efficiency to produce cloned embryos and animal. Further advance in SCNT technology will provide the opportunity to generating identical individuals for research purposes and can potential aim for preserving endangered species.

Table 2.1 *In vitro* development of domestic cat cloned embryos reconstructed with different donor cells.

Cell type of donor nucleus	Donor nucleus synchronization method	No. of fused/injected (%)	No. of embryo developed to morula (%)	blastocyst (%)	Reference
Cumulus	Serum-starved 5d	82/140 (59)	35 (43)	3 (4)	Gomez et al., 2002
Fetal fibroblasts	Cycling	115/193 (60)	37 (32)	6 (5)	Skrzyszowska et al., 2002
Cumulus	Cycling	65/143 (45)	20 (31)	5 (8)	“
Fetal fibroblasts	Cycling	80/134 (60)	38 (48)	6 (8)	Kitiyanant et al., 2003
Adult fibroblasts	Cycling	124/192 (65)	32 (26)	6 (5)	“
Cumulus	Cycling	116/175 (66)	44 (38)	3 (3)	“
Muscle	Cycling	76/178 (43)	32 (42)	8 (11)	Wen et al., 2003
Adult fibroblasts	Serum-starved 5d	75/95 (79)	6 (8)	1 (1)	Gomez et al. 2003
	Contact inhibited	105/152 (69)	11 (11)	3 (3)	“
	Roscovitine	95/105 (91)	15 (16)	3 (3)	“

Table 2.2 *In vitro* development of interspecies cloned embryos reconstructed by fusion of somatic cells of non-domestic felids with enucleated domestic cat oocytes

Species	No. of fused/injected (%)	No. of cleaved (%)	No. of blastocysts (%)	Reference
Korean tiger (*Panthera tigris altaica*)	N/A[a] (54)	N/A (65)	N/A (9)	Hwang et al., 2004
African wildcat (*Felis silvestris lybica*)	425/484 (88)	357/425 (84)	101/357 (28)	Gomez et al., 2003
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)	Gomez et al., 2004b
Black-footed cat (*Felis nigripes*)	170/170 (100)	144/170 (84.7)	3/144 (2.1)	Gomez et al., 2006
Marbled cat (*Pardofelis marmorata*)	25/63 (40)	14/25 (56)	0/14 (0)	Thongphakdee et al., 2006

[a] N/A: number not available.

Tabled 2.3 Efficiency of SCNT

Species	Fused	Transfered	Live clones/recipient	Efficiency (%)[a]	References
Sheep	227	29	1/13	0.4	Wilmut et al., 1997
Cattle	68932	3435	148/935	0.2	Cibelli et al.,1999
Goat	138	47	1/15	0.7	Baguisi et al., 1999
Pig	188	110	¼	0.5	Polejaeva et al. 2000
Mouse	463	274	3/25	0.6	Wakayama et al., 1998
Cat	ND[b]	87	1/8	<1.1	Shin et al., 2002
Rabbit	612	371	4/27	0.7	Chesne et al., 2002

[a] Efficiency (%): live born cloned/fused embryos x 100.

[b] ND: not described.

2.2 DNA methylation

A process termed DNA methylation is the modification of DNA which involves in the the addition of methyl groups to the carbon 5 of the cytosine residues at cytosine-guanine (CpG) dinucleotide. DNA methylation plays a role in the control of imprinted gene expression, X chromosome silencing and believed to protect the genome against parasitic elements such as transposons, retrotransposones and viruses (Bestor et al., 2000). However, while cell specific methylation configurations are relatively stable in somatic cells, dynamic variations in DNA methylation patterns occurs in mammalian preimplantation embryos (Beaujean et al., 2004). DNA methylation is mediated by DNA methyltransferase. Four active DNA methyltransferase, DNMT1, DNMT2, DNMT3a and DNMT3b, have been reported in mammals. These four enzymes contain highly conserved DNA methyltransferase motif (Kumar et al., 1994). DNA methyltransferase catalyzed the methyl transfer from S-adenosyl-L-methionine (AdoMet) to the cytosine in CpG dinucleotide (Figure 2.1).

DNMT1 is the most abundant DNA methyltransferase in mammalian cells (Robertson et al., 1999). It has a marked preference for hemimethylated DNA (5-to 30-fold) and therefore, it can considered to be main the responsible for maintenance of established methylation pattern after DNA replication (Figure 2.2) (Chen and Li, 2006). There are two from of DNMT1. In oocyte DNMT1o is expressed. And DNMT1s is expressed in early embryos and somatic cell.

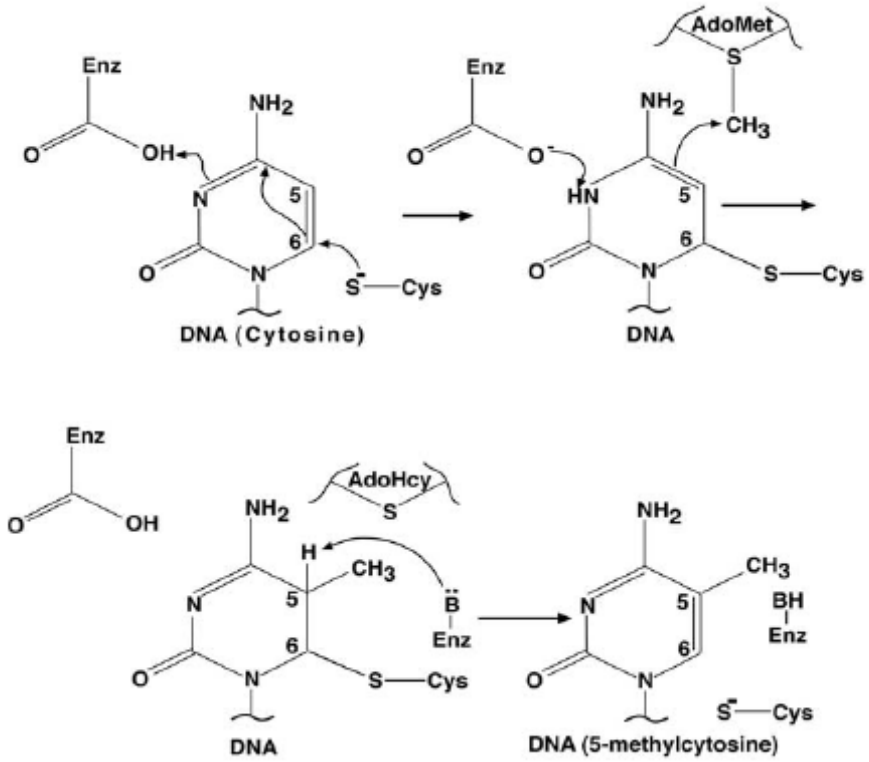

Figure 2.1 Mechanism of methyl group transfer catalyzed by DNA (cytosine-5) Methyltransferases (Pradhan et al., 2003).

DNMT2 is not essential for *in vitro* DNA methyltransferase (Okano et al., 1998). However, a recent finding suggests that DNMT2 may be catalytically active *in vivo* (Liu et al., 2003).

DNMT3 member are implicated in *de novo* methylation in germ cell and developing embryos. DNMT3a has a preference for unmethylated DNA, whereas DNMT3b can methylate either hemimethylated or unmethylated DNA (Figure 2.2) (Okano et al., 1999). It has been proposed that *de novo* methyltransferase may participate in the maintenance of methylation based on the findings that DNMT3a and

3b may play roles in the restoring methylation at sites missed by DNMT1 during replication in ES cell (Liang et al., 2002)

However, a recent report demonstrated that deletion of DNMT1or DNMT3a and 3b together led to embryonic lethality and DNA methylation machinery is not composed of stand alone enzyme, rather a complex network of different protein working in concert (Okano et al., 1999).

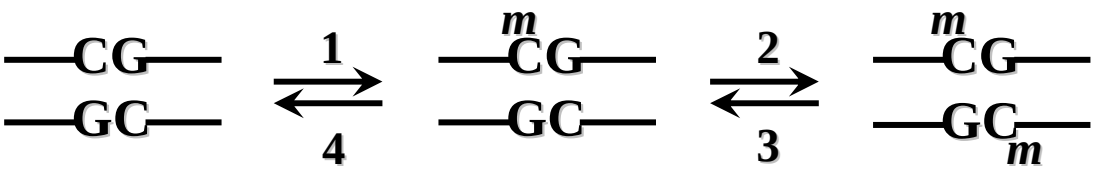

Figure 2.2 Methylation of cytosine residues in the mammalian genome. After replication, daughter strands of fully methylated DNA are hemimethylated (reaction 3) and the original pattern of DNA methylation is maintained by the DNA methyltransferase (reaction 2), which preferentially methylates the cytosine residues at hemimethylated CpG sites. Further replication without methylation of the hemimethylated DNA results in fully unmethylated DNA (reaction 4). *De novo* methylation (reaction 1) is also considered to be mediated by the DNA methyltransferase, although the efficiency of *de novo* methylation is low.

DNA methylation can be thought to have a twofold biological significance, gene regulation and structural fidelity (Bestor et al., 2000; Robertson and Wolffe, 2000). DNA methylation has been firmly established as playing a critical part in transcriptional repression. This has been described in the context of development and

establishes the allele-specific expression status in many imprinted loci through differential DNA methylation of parental alleles (differential methylated regions, DMRs) (Li et al., 1993). DNA methylation has been implicated in genome defence associated with the silencing of parasitic retrotransposons (Yoder et al., 1997) and to a function in the maintenance of the structural integrity of chromosomes and prevention of chromosomal rearrangements (Chen et al., 1998). The DNA methylation pattern shows global changes during early mouse development (Dean et al., 2003). Upon fertilization a majority of the sperm-derived genomic DNA is rapidly demethylated before the onset of DNA replication by an uncharacterized active mechanism (Mayer et al., 2000; Santos et al., 2002). In contrast, oocyte-derived DNA is passively demethylated only after DNA replication initiates, by the nuclear exclusion of DNMT1. The global level of DNA methylation remains at the lowest level in the morula and blastocyst stages until implantation, when sudden genome-wide *de novo* methylation occurs by DNMT3a and DNMT3b. The genome-wide demethylation and remethylation in early embryos seems to be conserved across species as observed in cow, rat and pig, although their timing with respect to developmental stages is slightly different (Dean et al., 2001). Successfully cloned embryos have to follow these methylation dynamics to erase the tissue-specific DNA methylation pattern and establish a new embryo-specific DNA methylation pattern on numerous genes.

2.3 Histone acetylation

The basic unit of chromatin is the core nucleosome in which 146 bases pair of DNA are wrapped around the histone octamer, that consist of two molecules each of core histone H2A, H2B, H3 and H4 (Luger et al. 1997) as show in Figure 2.3. The

modification of histone include the addition of various chemical groups such as methyl, phosphate, acetyl, ribosyl and ubiquitin groups (Sterner and Berger, 2000). One of the best characterized transcriptional modifications of histone is the acetylation of lysine residues (Figure 2.3).

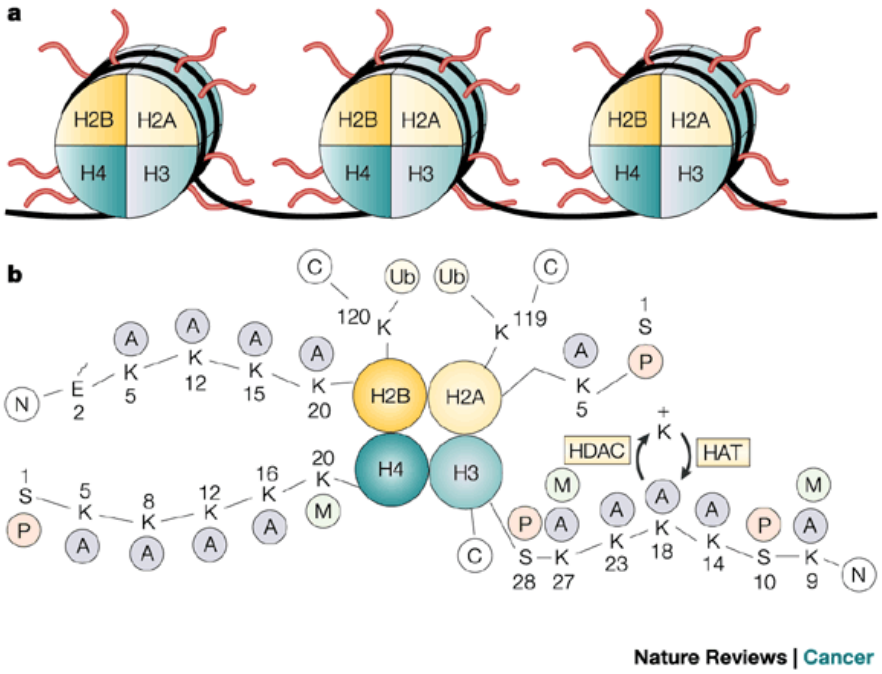

Figure 2.3. The core proteins of nucleosomes are designated H2A, H2B, H3 and H4. Each histone is present in two copies, so the DNA (black) wraps around an octamer of histones - the core nucleosome (a). The amino-terminal tails of core histones (b). Lysines (K) in the amino-terminal tails of histones H2A, H2B, H3 and H4 are potential acetylation/deacetylation sites for histone acetyltransferases and histone deacetylases (Marks et al., 2001).

The modification mediated by the histone acetyltransferase (HATs) has been associated with transcriptional activity (Wolffe et al., 1997). Acetylation neutralizes

the positive charge and thus increases the hydrophobicity of the histone, leading to a reduce affinity of acetylated N- terminal domains of histone to DNA thus opening up the chromatin structure (Figure 2.4). HATs catalyzes the transfer of acetyl moiety from acetyl coenzyme A to aminoterminal tails of lysine residue of histone (Figure 2.5)

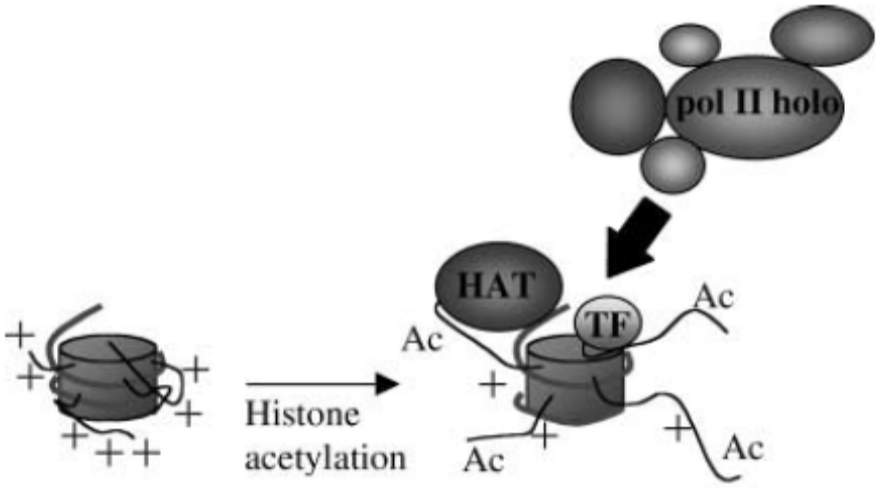

Figure 2.4 Acetylation of a core nucleosome by a histone acetyltransferase leads to a loss of positive charges and a better accessibility of the associated DNA for transcription factors (*TF*) or other DNA binding proteins. Thereby, the transcription factor can bind and recruit the RNA-polymerase II holoenzyme (*pol II holo*) and enable transcription (Hasan and Hottiger, 2002).

Acetylation is a reversible process and the histone deacetylase (HADCs) is responsible for the opposite reaction (Figure 2.5) which often results in transcriptional repression (Pazin and Kadonaga, 1997). A specific combination of these histone modifications on a given gene provides a recognition site for interacting molecules and thus contributes to regulating the gene activity (Strahl and Allis, 2000; Jenuwein

and Allis, 2001). Bovine oocytes and early embryos express several histone acetylases and deacetylases with some variability in the transcript levels depending on the developmental stages (McGraw et al., 2003). In mouse oocytes, histone H3 and H4 are globally deacetylated on several lysines at the metaphase II of the second meiosis, which was reproduced in somatic nuclei transferred into the same stage of oocytes (Kim et al., 2003). This genome wide decrease of histone acetylation may contribute to the erasure of the previous gene expression patterns specific to the donor cell differentiation.

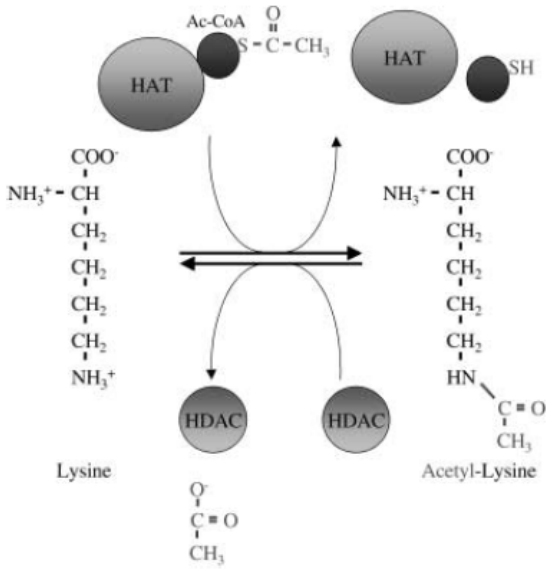

Figure 2.5 Schematic diagram of the acetylation of a lysine residue (left) to an acetyl-lysine residue (right). The reaction is catalyzed by a histone acetyl transferase and its cofactor acetyl-coenzyme A (Ac-CoA). The deacetylation reaction is catalyzed by the activity of a histone deacetylase (Hasan and Hottiger, 2002).

2.4 Nuclear Reprogramming

In mammalian development, two major reprogramming events occur. Both involve epigenetic modification. The first occurs during germ cell development and the second during preimplantation development (Reik et al., 2001). During SCNT, the donor nucleus must undergo nuclear reprogramming, which is likely to involve epigenetic alteration (Dean et al., 2001). Incomplete nuclear reprogramming, coupled with the inherent inadequacies of *in vitro* culture, may account for the poor performance of SCNT. The nuclear reprogramming mechanisms of SCNT involve two steps. The first is dedifferentiation of the differentiated donor somatic cell to a totipotent embryonic stage, refer to the erasure of the donor cell epigenetic pattern after SCNT and the reestablishment of embryonic epigenetic characteristic and gene expression in the cloned embryos. The second step is redifferentiation of cloned embryos to differentiation somatic cell type during later development, refer to as redifferentiation of cloned embryos from a totipotent status to various differentiated stage for tissue generation or organogenesis during postimplantation development (Yang et al., 2007).

Another factor in the nuclear programming equation is played by methylation of DNA. During normal early embryonic development in mammal the global pattern genomic DNA methylation undergoes marked changes (Figure 2.6). During mouse and bovine pre-implantation development, the maternal genomes are also demethylated. Reduction in global methylation was first observed at the 2-cell stage and proceeds through the next cell division, suggesting that the maternal genome is somehow protected from the active demethylation activity and instead undergoes passive demethylation (Mayer et al., 2000). Both mouse and bovine genomes are

hypomethylate by the 8- to 16-cell stage. In the mouse, the genome wide *de novo* methylation begins in the inner cell mass (ICM) of the blastocyst stage embryo. In bovine embryos this process commences at 8- to 16-cell stage before blastocyst formation. In cloned embryos precocious *de novo* methylation takes place at the 4-cell stage and the trophectoderm (TE) hypermethylated and morula of cloned embryos show the methylation patterns more closely approximated those of donor somatic nucleus than those of normal fertilized 16-cell embryos.

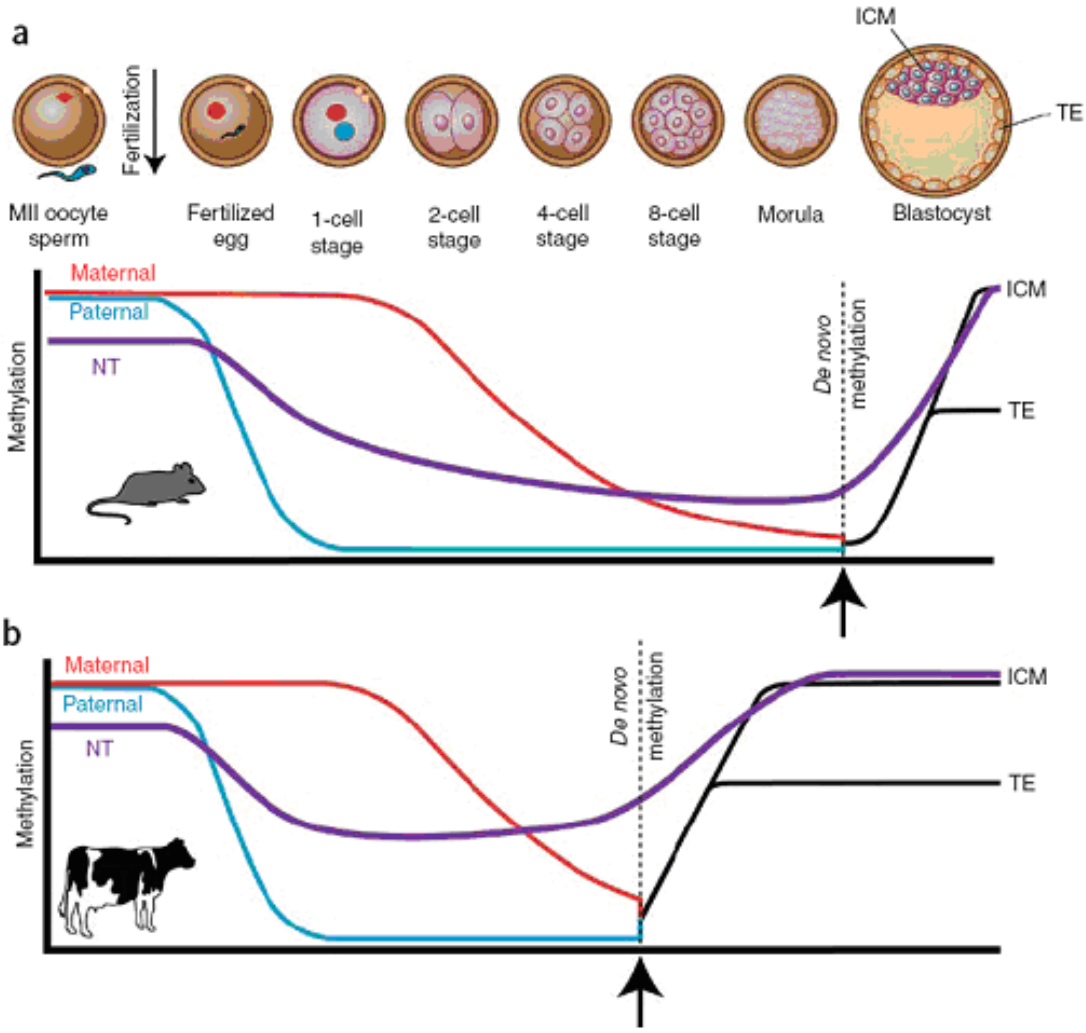

Figure 2.6 Methylation dynamics during normal early embryonic development and in clones (Yang et al., 2007).

2.5 Gene expression in cloned embryos

In mammal, the early development stage of preimplatation embryos rely on maternal RNA and protein, until the embryonic genome becomes activated (EGA) and control the regulation of development. EGA occurs at 2-cell stage in mouse (Flach et al., 1982), 4- to 8-cell stage in pig and human (Braude et al., 1988; Hyttel et al., 2000) and 8- to 16-cell stage in bovine (Memii and First, 1998). EGA is very important for a large number of genes that require activation before the embryo can undergo further development. After SCNT, the nuclei of differentiated somatic cell must undergo reprogramming to reestablish an embryonic gene expression profile for development following the EGA stage. The successful reprogramming of a somatic cell after SCNT would results in an embryo with the same profile of gene transcription of embryos derived for *in vivo* or *in vitro* fertilization.

One of most widely characterized genes associated with pluripotency is a gene encoding Oct4 (Scholer et al., 1990). Oct4 is a transcription factor belonging to the POU (Pit-Oct-Unc) family that regulates the expression of target gene by binding to the octamer motif ATGCAAAT within the promoter or enhancer regions (Herr and Cleary., 1995). A gene that encodes a POU domain transcription factor and that has an essential role in the control of developmental pluripotency, form morphologically normal but developmentally deficient blastocysts (Niwa et al., 2000). In mice, Oct4 association with the toti/pluripotent cell type has been established (Pesce and Scholer, 2001; Pan et al., 2002). During the murine preimplantation period, a residual maternal Oct4 expression is found in unfertilized oocytes and in the subsequent cleavage stage (Palmieri et al., 1994). Embryonic Oct4 expression is initiated at the 4- to 8-cell stage and is concomitant with an abundant expression in the nuclei of all blastomers. The

majority of cloned animal derived by SCNT from somatic cell nuclei develop to blastocyst stage but die after implantation, that lack an Oct4, which play an essential role in control of development pluripotency, develop to the blastocyst stage and also die after implantation, because they lack pluripotent embryonic cells (Bortvin et al., 2003). Gene expression analyses in cloned mouse embryos shows incomplete reactivation of the pluripotency gene Oct4 and also ten Oct4 related gene (Boiani et al., 2002; Brotvin et al., 2003). Oct4 is expressed in the mouse ICM and in the ICM and TE of pig and bovine embryos (Nichols et al., 1998). Thus, Oct4 activation has been used as a marker for reprogramming in many approaches that use ES cells or oocytes as a reprogramming environment.

Aberrant gene expression is frequently observed in cloned embryos which probably results in abnormal development and contributes to early loss of cloned fetuse (Daniels et al., 2000; Rideout et al., 2001; Boiani et al., 2002). Daniels and colleagues (2000) reported that the transcription of some implantation related gene such as IL-6, FGF4 and FGFr2 were delayed and aberrant in some cloned embryos. Over expression of IGF2 and similarly imprinted genes have been found in cloned embryos (Han et al., 2003)

Although no study has been able to measure the epigenetic status of cloned embryos nor follow their development after transfer to recipients. Several studies have shown aberrant methylation pattern in cloned embryos when compare with *in vivo* or *in vitro* fertilized control (Dean et al., 2001; Han et al., 2003). The few studies that had focused on the acetylation of histone in cloned embryos have found aberrancies (Santos et al., 2003; Enright et al., 2005; Suteevun et al., 2006). Kishigami and colleagues (2006) demonstrated that the HDAC inhibitor trichostatin A (TSA)

increases cloning efficiency this suggest that hypoacetylation may be one of the limiting factor for the development of clone embryos (Rybouchkin et al., 2006).

However, SCNT is a powerful technique for generating identical individual and preservation of endangered animal. It is unclear whether the development failure of cloned embryos are due to the incomplete nuclear reprogramming or SCNT procedures it self. Especially the application of SCNT in domestic cat and endangered wild cat was demonstrated that the low number success of offspring cloned kitten and suggest that the reprogramming of somatic cell in cat embryos produce by SCNT is incomplete. Various strategies had been used to modify the SCNT procedure in effort to increase efficiency of cloned embryos and animal production. To date, there had been no reported on the effect of manipulation medium, culture system and the use of bovine oocyte as recipient cytoplast on *in vitro* development of cloned domestic cat and wild cat. This is the first investigate to modify the SCNT procedure to increase the *in vitro* production efficiency of cloned felid embryos. And this is the first detailed analysis of specific genes transcription that involve in the nuclear reprogramming in reconstructed oocytes after intra-and interspecies SCNT compared with natural producing embryos (IVF) of felids family. In present day, there had been no study on specific genes expression profiles in cloned felid embryos. The results from this study should explain the developmental failures of cloned felid embryos. However, this study was conducted with the experiment on intra and interspecies NT between three felid species belonging to three different felid genuses, leopard cat (*Prionailurus bengalensis*) marbled cat (*Pardofelis marmorata*) and domestic cat (*Felis catus*). In addition, the identification of those genes which express during preimplantation embryo development was determined by real-time quantitative PCR. Eventhough

leopard, marbled and domestic cats are different species and genus but after using a domestic oocytes as recipient cytoplast in interspecies SCNT, satisfy results could be obtained and expression of gene crucial for preimplantation embryo development can be analyzed. The identification of genes whose expression profiles are frequently abnormal in cloned embryos will provide marker for the diagnosis of viability prior to embryo transfer and therefore, potentially navigate the time and money-consuming transfer of nonviable embryos to recipient animals.

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CHAPTER III

REPROGRAMMING OF MARBLED CAT (*Pardofelis marmorata*) NUCLEI AFTER RECONSTRUCTED WITH ENUCLEATED DOMESTIC CAT AND BOVINE OOCYTES

3.1 Abstract

Nuclear transfer (NT) of domestic cat offer many benefits as a model for conservation of endangered felid species. The aim of this study was to investigate *in vitro* development of reconstructed felid embryos. Experiment 1 was conducted to examine the effect of manipulation medium on *in vitro* development of reconstructed domestic cat embryos. The blastocyst formation rate of NT with Emcare holding medium (33.3%) was significantly greater than that of 199H (18.1%). Experiment 2 was carried out to investigate the effect of culture system on development of intraspecies cloned domestic cat and interspecies cloned marbled cat embryos. There was no significant difference between the developments to blastocyst stage of non co-culture and co-culture the cloned domestic cat embryos with DOEC (44.4 vs 38.0%). These non co-culture and co-culture systems could not support the development of interspecies cloned marbled cat embryos beyond morula stage (28.6 vs 32.6%). Experiment 3, the dedifferentiate of domestic and marbled cats nuclei after reconstructed within domestic cat and bovine cytoplast were investigate.

The development to blastocyst stage of intraspecies cloned domestic cat (34.4%) and bovine (29.6%) were higher than interspecies cloned marbled cat with bovine cytoplast (8.6%). The interspecies cloned domestic cat with bovine cytoplast arrested develop at morula stage. These results demonstrated that the manipulation medium during NT process involve in the increase development to blastocyst stage of cloned felid embryos. Alternatively, the co-culture system of cloned felid embryos was not beneficial to the development. The domestic cat and bovine cytoplasts could support the mitotic cleavage of felid nuclei. This study was beneficial to using interspecies cloning for propagate endangered felid species.

3.2 Introduction

"CC" is the world's first cloned domestic cat using adult somatic cell as donor nuclei (Shin et al., 2002). To date, several groups have successfully cloned kittens (Gomez et al., 2004; Yin et al., 2005; 2007). There have been many efforts currently to study the conservation of endangered felid species. In term of endangered felids, nuclear transfer (NT) has been used to produce cloned embryos and has been suggested for the preservation and increase numbers of the endangered felid species such as African wildcat (Gomez et al., 2003; 2004), leopard cat (Lorthongpanich et al., 2004; Yin et at., 2005) and marbled cat (Thongphakdee et al., 2006).

Marbled cat (*Pardofelis marmorata*) is a wildcat that has been considered risk of extinction in Thailand. The remaining population of marbled cat has not yet been reported. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has listed the marbled cat in Appendix I, international commerce prohibited. Interspecies NT is considered as a high potential technique to

conserve the number of endangered species. Since endangered species have limited supply of recipient oocytes, the oocyte cytoplasm of domestic species such as cattle, sheep, rabbit and cat have been used for dedifferentiation of somatic cell nuclei from rat, pig, monkey, sheep (Dominko et al., 1999), gaur (Lanza et al., 2000), buffalo (Kitiyanant et al., 2001), giant panda (Chen et al., 2002), banteng (Sansinena et al., 2005), African wildcat (Gomez et al., 2003, 2004), leopard cat (Lorthongpanich et al., 2004; Yin et at., 2005) and marbled cat (Thongphakdee et al., 2006). Gomez and colleagues (2003; 2004) have suggested that domestic cat oocytes can be used for wildcat preservation by interspecies NT technique. This suggestion was proved with the cloned kitten offspring of African wildcat. Previous report of Thongphakdee and colleagues (2006) demonstrated that rabbit oocyte could support the development of interspecies cloned marbled cat embryos to blastcysts stage but domestic cat oocyte does not. Due to the abundant of cattle oocyte and cattle *in vitro* embryo production system is well understood. Thus, it seems to be more possible to use the cattle oocyte as the recipient cytoplast for endangered felid cloning and to date, there have been no report on the feline cloning using cattle oocyte as recipient cytoplast.

However, the factors involved in the success of NT are very complex. Many protocols have modified and utilized in NT processes. Although several media for the preservation of oocytes, such as Tissue Culture Medium 199 (TCM 199) with and without HEPES buffer, Tyrode's medium with HEPES (TALP- HEPES) have been used to manipulate the oocytes in NT process of felid species (Gomez et al., 2003; Yin et al., 2005). To date, there has been no report on the effect of manipulation medium in the process of felid species NT on *in vitro* development.

Therefore, the objectives of this study were to investigate the effect of manipulation medium on *in vitro* development of cloned domestic cat embryos during NT procedure and to compare the *in vitro* development of cloned marbled cat embryos using domestic cat and bovine oocytes as recipient cytoplast.

3.3 Materials and methods

All chemicals used in this study were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) unless otherwise noted.

3.3.1 Preparation of recipient cytoplast

3.3.1.1 Domestic cat recipient cytoplast

Female domestic cats (age 9 to 36 months) for the oocytes donor was cared for using procedures that exceeded the standards established by Laboratory Animal Care of Suranaree University of Technology. Healthy female cats were selected for superstimulated. The cats were treated with 200 IU equine chorionic gonadotropin (eCG, Intervet) for 5 days. Then, ovaries were removed from the reproductive tract (Figure 3.1A and B). The superstimulated cats were anesthetized by intramuscularly injected of 0.0002 mg/kg Atrophine and 0.5 mg/kg Xylazine followed by 20 mg/kg Ketamine hydrochloride 10 minutes after the first injection. After surgical collected the ovaries, the surgical area was examined thoroughly for sign of bleeding. If no sign of bleeding the operative site was closed in layers and post-operative care that exceeded the standards established by Laboratory Animal Care of Suranaree University of Technology were used. The ovaries were placed into a ∅ 60 mm culture dish (Nunc.) containing modified Dulbecco's phosphate buffer saline (mDPBS) supplement with 0.1% polyvinylpyrrolidone (PVP, P-0950) and sliced with

a 21 gauge needle to release cumulus oocyte complexes (COCs) (Figure 3.1C). Only COCs exhibiting uniform, dark pigmented cytoplasm and intact cumulus cells investments were used for further culture (Figure 3.1D). The COCs were collected and matured in *in vitro* maturation (IVM) medium consisting of TCM 199 (M-5017) supplemented with 0.36 mM sodium pyruvate (P-5280), 2.2 mM calcium lactate (L-4388), 2mM L-glutamine (G-5763), 1.13 mM cystein (C-8152), 0.3 mg/ml fatty acid free bovine serum albumin (BSA, A-6003), 0.5 IU/ml eCG, 1 IU/ml human chorionic gonadotropin (hCG, Intervet) in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 24 h.

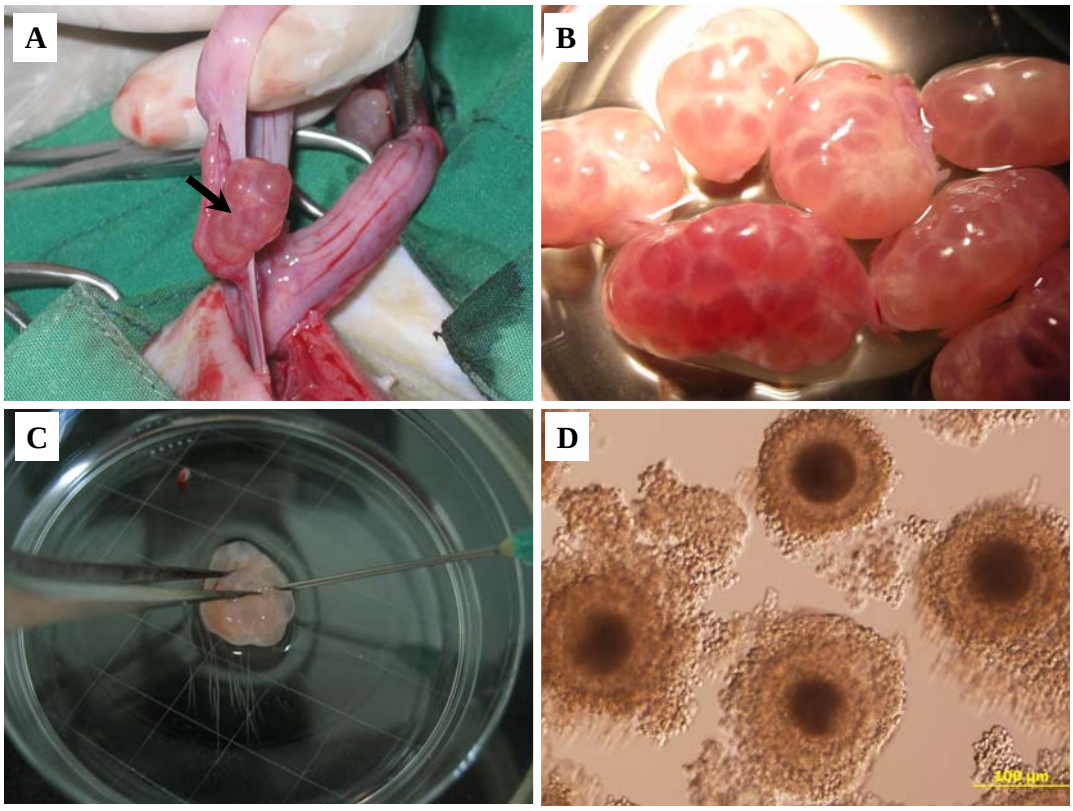

Figure 3.1 Domestic cat ovaries (arrow) (A) and (B) were removed from superstimulated with hormone. Ovary was sliced with needle to release COCs (C). Morphology of domestic cat COCs before *in vitro* matureation (D).

3.3.1.2 Cattle recipient cytoplast

Abattoir-derived cattle ovaries were transported to the laboratory within 4 h after slaughter. The COCs were collected by aspiration from follicles (∅2 to 6 mm) using 18-gauge needle attached to 10 ml syringe. The collected oocytes were matured in TCM199 supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 0.02 AU/ml follicle stimulating hormone (FSH, Antrin), 50 IU/ml hCG and 1 μg/ml estradiol-17β (E-8875) in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 21 h.

3.3.2 Preparation of donor cell

Ear skin of domestic cat, marbled cat (Figure 3.2A) and bovine were used as fibroblast donor. The ear skin biopsies were cut into small pieces (1 mm x 1 mm) then placed into ∅ 60 mm culture dishes and covered with glass slide. Then alpha-modified minimum essential medium (αMEM, M-7145) supplemented with 10% FBS culture medium was added and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. After 8-10 days of culture, the monolayer outgrown with fibroblastic like cell morphology (Figure 3.2B) were passaged for 1 to 2 times to increase the cell number. The donor cells were frozen in culture medium supplemented with 10% (v/v) dimethyl sulfoxide (DMSO, Merck) and stored in liquid nitrogen for further use as donor cells.

The frozen fibroblasts were thawed at 37°C and transferred into centrifuge tube containing culture medium and incubated for 5 min. After centrifugation at 3,000 rpm for 5 min, the supernatant was removed and resuspended with culture medium and cultured on ∅ 35 mm culture dish in a humidified atmosphere of 5% CO_2 in air at 37°C for 2 to 3 days prior to nuclear transfer (Figure 3.2C, D). At subconfluence, the

fibroblasts were trypsinized using trypsin(T-4799) / EDTA (100935V, BDH) to separate into single cells and use as donor cells (Figure 3.6A).

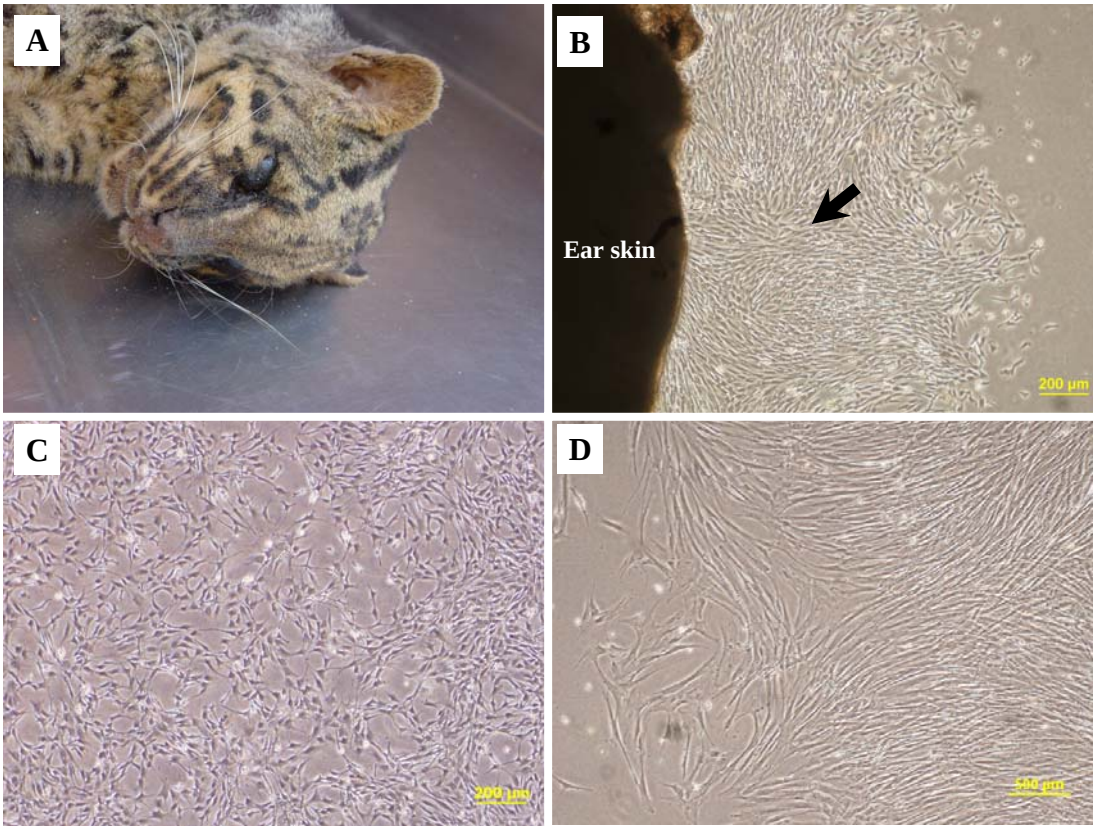

Figure 3.2 Primary cell culture of felid family. Ear skin was collected from a natural death marbled cat (A). Fibroblasts (arrow) outgrown from the marble cat ear skin after 10 days culture (B). Fibroblasts monolayer of domestic cat (C) and bovine (D) (magnification 100x).

3.3.3 Nuclear transfer and embryo culture

3.3.3.1 Oocyte enucleation

After cat and cattle oocytes maturation (Figure 3.3A), the expanded cumulus cells were removed by repeated pipetting in 0.2% hyarulonidase (S-3506). The Oocytes that had extruded first polar body (MII oocyte) (Figure 3.3B) were

selected for nuclear transfer. The MII oocytes were placed into Emcare holding medium (ICP bio) supplemented with 5 µg/ml cytochalasin B (CB, C-6762). The nucleus were removed (enucleated) by using sharp micropipette with Narishige micromanipulators to cut the zona pellucida above the first polar body and squeezed out the first polar body and metaphase plate in a small volume of surrounding cytoplasm (Figure 3.4). Successful enucleation of each oocyte was confirmed by 5 µg/ml Hoechst 33342 (C-2261) staining of the corresponding karyoplast that was squeezed out and observed under ultraviolet light using a fluorescent microscope (Figure 3.5).

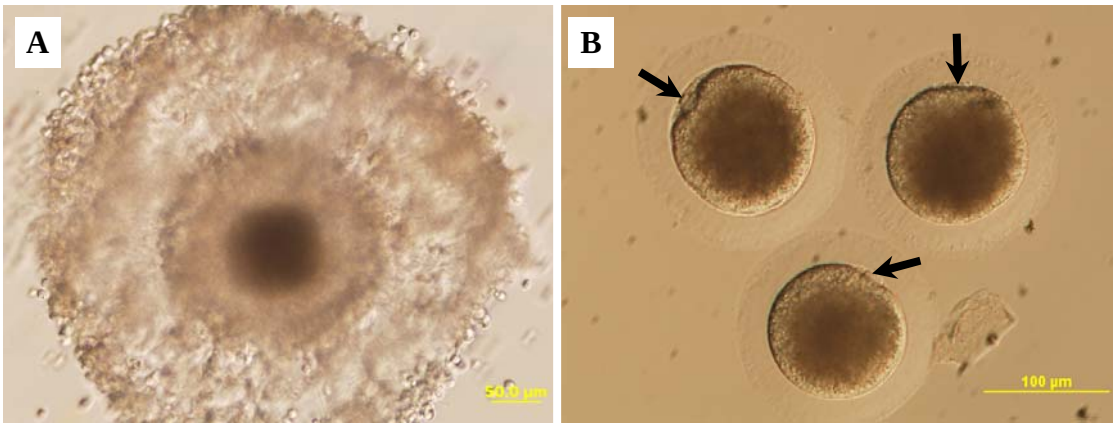

Figure 3.3 Domestic cat oocyte after *in vitro* maturation 24 h (A). After cumulus cells removal, the first polar body (arrows) were extrudes from the matured oocytes (B) (magnification 200x).

3.3.3.2 NT using domestic cat as recipient cytoplast

Single domestic cat or marbled cat fibroblast (Figure 3.6B) was inserted into the perivitelline space of enucleated domestic cat oocyte (Figure 3.7). Then, the cytoplasm/cell couplets were equilibrated in fusion medium containing 0.3 M mannital (M-9647) and 0.1 mM magnesium chloride (M-2393) and transferred to

fusion drop which fusion electrodes were already attached to the micromanipulator. Each couple was placed between both tips of fusion electrode, then double DC pulse of 30 V, 30 μsec. were delivered by electro fusion machine (Voltrain EP-1, Cryologic) (Figure 3.8). After 45 min, the fused oocytes were visually evaluated by confirming the presence or absence of donor cell in the perivitelline space. The fused couplets were then activated with 7% ethanol for 5 min followed by incubation in holding medium supplemented with 10 μg/ml cyclohexymide (CHX, C-6798) and 1.25 μg/ml cytochalasin D (CD, C-8273) in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 5 h. After activation, the reconstructed embryos were cultured as previously described by Gomez et al. (2003). Briefly, cloned embryos were cultured in Tyrode's medium (Ty I-medium) supplemented with 1% MEM non-essential amino acids (NEAA, M-7145), 0.3 mg/ml BSA, 0.36 mM sodium pyruvate, 2.2 mM calcium lactate and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C. On day 2 after culture, the 8-cell stage embryos were selected and cultured in Ty I- medium without BSA supplemented with 2% MEM essential amino acid (EAA, B-6766) and 10% FBS (Ty II- medium) in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38°C for 6 days. Half of the medium was daily changed and the development of embryos was daily observes time.

3.3.3.3 NT using cattle oocyte a recipient cytoplast

NT and embryo culture method were conducted according to Laowtammathron et al. (2005). Briefly, single domestic cat, marbled cat or cattle fibroblast was inserted into the perivitelline space of enucleated cattle oocytes. The cytoplasm/cell were equilibrated in Zimmermann fusion medium and transferred to fusion drop which fusion electrodes were already attached to the micromanipulator.

Each couplet was placed between both tips of fusion electrode then cell fusion was induced with double DC pulse of 24 V for 15 μsec generated by a fusion machine (SUT F-1, Suranaree University of Technology). The number of couplets successfully fused was recorded at 1 h after the electro-stimulation. The fused couplets were activated by the same procedures as previously described in cat oocyte except using culture temperature of 38.5°C and cultured in a humidified atmosphere of 5% CO_2 in air. The reconstructed embryos were cultured in mSOFaa medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 2 days. Thereafter, embryos at 8-cell stage were selected and co-cultured with cattle oviductal epithelium cells in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 6 days.

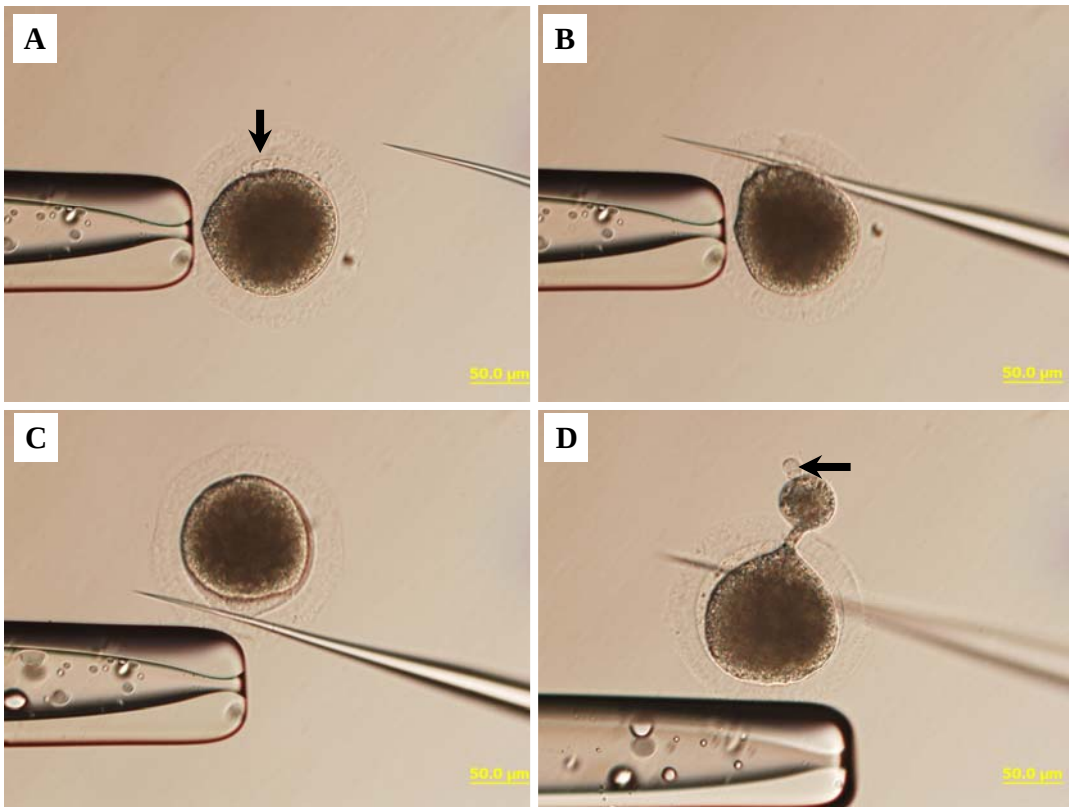

Figure 3.4 Enucleation procedure of domestic cat oocyte. Arrow indicated the first polar body (magnification 200x).

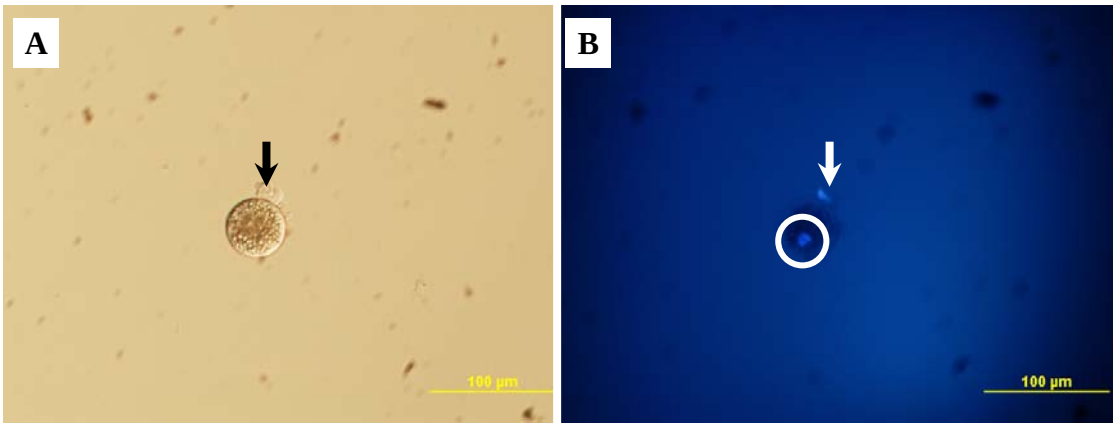

Figure 3.5 The squeezed out cytoplasm of MII oocyte after stained with Hoechst 33342. Bright field (A) and fluorescent image (B) of the first polar body (arrow). The successful enucleation shows metaphase spindle removal (circle) (magnification 200x).

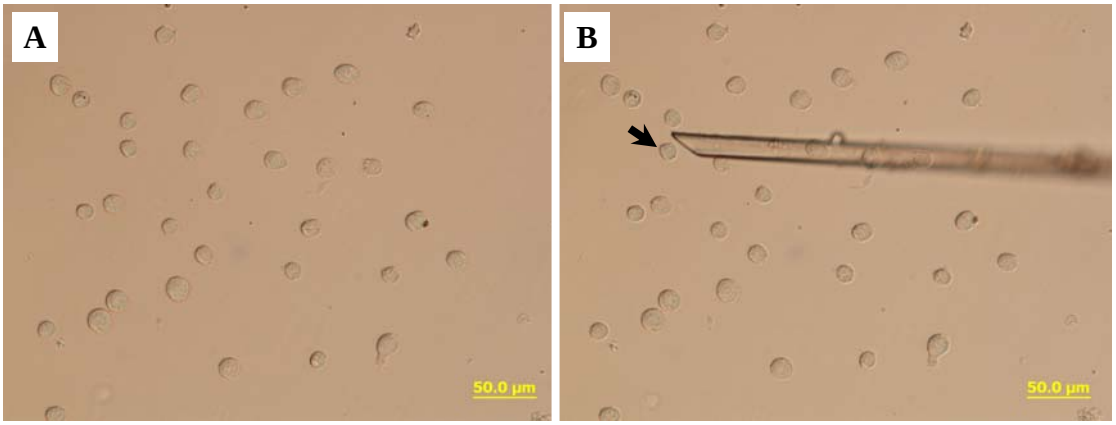

Figure 3.6 The donor cells after trypsinized and separated into single cell (A) and the cells diameter 14 to 16 µm (B; arrow) were chosen as donor cell (magnification 200x).

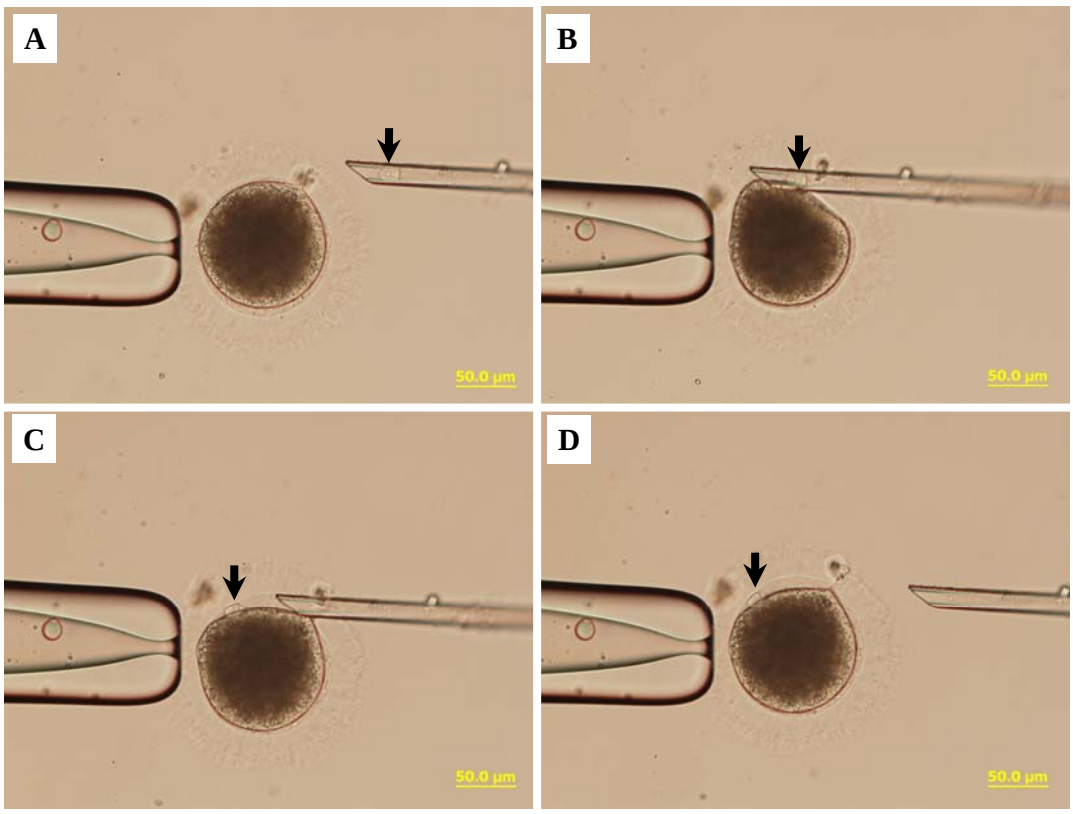

Figure 3.7 The donor cell was inserted into the perivitelline space of enucleated oocytes. Arrow was indicated the donor cell (magnification 200x).

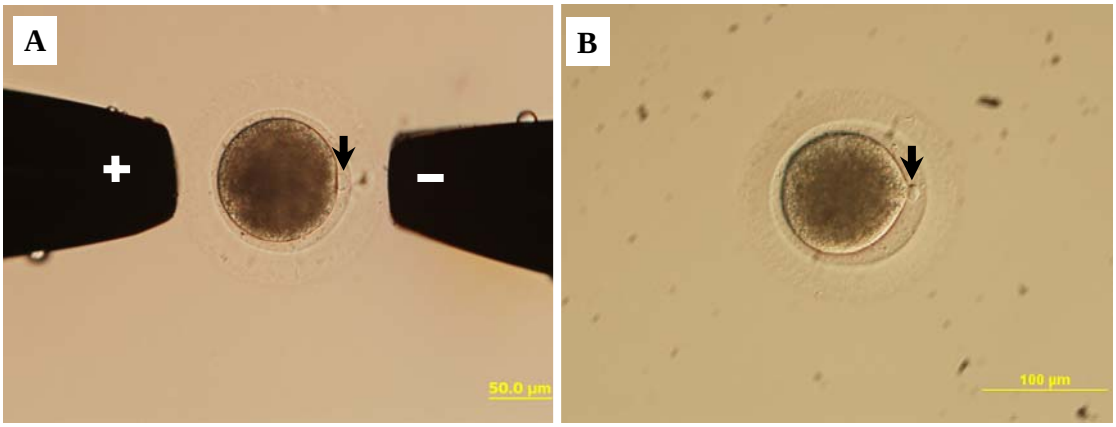

Figure 3.8 The cytoplasm/cell couplet during placed between both tips of fusion electrode (A) and 15 min after fusion the donor cell began to fuse into cytoplast (B). Arrow indicated the donor cell (magnification 200x).

3.3.4 Parthenogenetic activation (PA)

The cat MII oocytes were activated with 7% ethanol for 5 minutes followed by incubation in holding medium supplemented with 10 μg/ml CHX and 1.25 μg/ml CD in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 5 h. Thereafter, the embryos culture was does as described above.

3.3.5 Embryonic cell count

Blastocysts stage embryos from every production system were stained with Hoeschst 33342 and the cell nuclei were counted.

3.3.6 Experimental design

Experiment 1 was carried out to investigate the effect of NT manipulation medium, HEPES-buffer TCM-199 supplement with 10% FBS (199H) or Emcare holding medium during denude, enucleation, injection, activation and holding steps on *in vitro* development of cloned domestic cat embryos. Fusion, cleavage and blastocysts developmental rates and total cell number were observed and counted. The better manipulation medium was used for further experiment.

Experiment 2 was carried out to investigate the effect of culture system on the development of cloned domestic and marbled cat embryos using cat oocyte as recipient cytoplast, co-culture with domestic cat oviductal epithelium cells (DOEC) or non co-culture as *in vitro* culture system. The reconstructed domestic and marbled cat embryos were cultured in Ty I- medium for 2 days. Thereafter, the embryos at the 8-cell stage were selected and co-culture and non co-culture with DOEC in Ty II- medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 6 days. The developmental rate of cloned marbled cat of each culture system was compared with cloned domestic cat.

Experiment 3 was designed to investigate the effect of recipient cytoplast on dedifferentiation of felid nuclei after using cat or cattle oocyte to produce interspecies cloned embryos. The fibroblast of marbled cat or domestic cat was transfer into enucleated domestic cat or cattle oocyte followed by fusion, activation and cultured under different culture system depending on the oocyte. The fusion, cleavage and *in vitro* development rate were evaluated.

3.3.7 Statistical analysis

Experiments were repeated at least four times in each treatment group. Data were analyzed by ANOVA using Statistical Analysis Systems (SAS). A probability value of $P<0.05$ was considered to be significantly different.

3.4 Results

3.4.1 The effect of NT manipulation medium on *in vitro* development of cloned domestic cat embryos.

Effect of 199H and Emcare holding medium on *in vitro* development the cloned domestic cat embryos were investigated. The result indicated that the fusion rate was significant different between oocyte in 199H and Emcare holding medium (63.7% and 73.3 %, respectively). The cleavage and 8-cell development rates of cloned domestic cat embryos cultured in 199H (82.9% and 72.7%, respectively) were not different from the embryos cultured in Emcare holding medium (89.2% and 66.7%, respectively). These similar results could be found in PA embryos cultured in those two different manipulation media as well (88.4% and 69.6%, respectively for 199H group and 92.1% and 82.9%, respectively for Emcare holding medium group). However, the cloned and PA embryos cultured in Emcare holding medium could

develop to blastocyst stage with a significantly higher value (33.3% and 26.3%, respectively) than these embryos in 199H group (18.1% and 15.9%, respectively) (Table 3.1). The total cell number of cloned blastocysts in the 199H was not different from the embryos from Emcare holding medium (521 ± 9.2 and 537 ± 7.6, respectively). However, the cloned blastocysts from either 199H or Emcare holding medium had higher cell number than the PA embryos (227 ± 23.6 and 218 ± 30.4, respectively). The results can be concluded that Emcare holding medium could better support the *in vitro* development of cloned domestic cat than 199H since they have higher blastocyst rate from Emcare holding medium group (Table 3.1). Therefore, the Emcare holding medium was designed to be used as a manipulation medium for further experiments.

Table 3.1 Developmental rate of cloned domestic cat and parthenogentic activation (PA) embryos derived from different manipulation medium.

Manipulation media	Experiment	Fused (%)	Cleavage (%)	8-cell (%)	Morula (%)	Blastocyst (%)	Mean cell (no. ± S.E.M.) of blastoyst
199H	NT	88/138[a] (63.7)	73/88[a] (82.9)	64/88[a] (72.7)	32/88[b] (36.4)	16/88[b] (18.1)	521 ± 9.2
	PA		61/69[a] (88.4)	48/69[a] (69.6)	35/69[b] (50.7)	11/69[b] (15.9)	227 ± 23.6
Emcare	NT	84/114[b] (73.7)	75/84[a] (89.2)	56/84[a] (66.7)	42/84[b] (50.0)	28/84[a] (33.3)	537 ± 7.6
	PA		70/76[a] (92.1)	63/76[a] (82.9)	47/76[a] (61.8)	20/76[a] (26.3)	218 ± 30.4

Values with different superscripts within each column are significantly different ($P<0.05$)

3.4.2 The effect of co-culture and non co-culture system on *in vitro* development of cloned cat embryo.

The cloned domestic and marbled cat embryos were first cultured in non co-culture system from after activation to 8-cell stage embryos were separated into two groups for either co-culture or non co-culture with DOEC. The results indicated that the marbled cat donor cells had similar capacity to fuse into domestic cat cytoplasm as domestic cat donor cells (75.1% and 82.7%, respectively). They were not significant different of the cleavage (84.9% and 89.3%, respectively) and 8-cell stage development rates (60.5% and 53.3%, respectively) between cloned domestic cat and marbled cat embryos (Table 3.2). The development to morula stage of cloned domestic cat embryos were not significant different between non co-culture and co-culture system (59.3% and 48.0%, respectively). The development to morula stage of domestic cat from both non co-culture and co-culture systems were significant higher than cloned marbled cat embryos from non co-culture and co-culture system (28.6% and 32.6%, respectively). The blastocyst rate of cloned domestic cat embryos was not significant different between non co-culture and co-culture system (44.4% and 38.0%, respectively). In contrast, cloned marbled cat could not development beyond morula stage (Table 3.2). The mean cell number from ten stained of cloned domestic cat blastocysts produced from non co-culture and co-culture system were 519 ± 7.6 and 518 ± 30.4 cells, respectively (Table 3.2). These data showed that co-culturing the cloned domestic cat embryos with DOEC could not enhance the blastocyst rate and also could not support the development of interspecies cloned marbled cat embryos beyond morula stage.

3.4.3 The effect of recipient cytoplast on dedifferentiation of felid nuclei after using cat or cattle enucleated oocytes as recipient cytoplast to produce interspecies cloned embryos.

The results of developmental rate of intraspecies and interspecies cloned felid and bovine embryos are showed in Table 3.3. The fusion rate of domestic cat fibroblasts into domestic cat recipient cytoplasts was significant lower than those domestic cat fibroblasts fused into bovine recipient cytoplasts (76.8% and 84.6%, respectively). However, there were no significant different of the fusion rates after using bovine enucleated oocyte as recipient cytoplast for different donor nuclear species such as bovine (81.7%), domestic cat (84.6%) and marbled cat fibroblasts (82.3%). The cleavage rate after using domestic and marbled cat fibroblast fused into domestic cat recipient cytoplast (86.5% and 86.3%, respectively) was lower than fused into bovine recipient cytoplast (90.9% and 93.5%, respectively) but the data were not significant different (Table 3.3). The development to 8-cell stage of domestic and marbled cat cloned embryos reconstructed with domestic cat recipient cytoplast (67.7% and 61.1%, respectively) was significant lower than that fused into bovine recipient cytoplast (76.8% and 81.7%, respectively). The development to morula stage among the interspecies cloned embryos was not significant different. The development to morula stage of interspecies cloned embryos was significant lower than the intraspecies cloned embryos. The percentage of blastocyst derivation of intraspecies cloned domestic cat and cloned bovine embryos were 34.4% and 29.6%, respectively (Figure 3.9) but blastocyst formation of interspecies cloned marbled cat derived from bovine recipient cytoplast was only 8.6% (Fig. 3.10A) while marbled cat fibroblasts reconstructed with domestic cat oocytes could not develop beyond morula stage.

Table 3.2 *In vitro* development of cloned domestic and marbled cat embryos from non co-culture and co-culture with domestic cat oviductal cell.

Donor nuclei	Fused (%)	Cleavage (%)	8-cell (%)	Treatment	Morula (%)	Blastocyst (%)	Mean cell (no.± S.E.M.) of blastoyst
Domestic cat	172/208 [a] (82.7)	146/172[a] (84.9)	104/172[a] (60.5)	non co-culture	32/54[a] (59.3)	24/54[a] (44.4)	519 ± 7.6
				co-culture	24/50[a] (48.0)	19/50[a] (38.0)	518 ± 30.4
Marbled cat	178/237 [a] (75.1)	159/178[a] (89.3)	95/178[a] (53.3)	non co-culture	14/49[b] (28.6)	0/49[b]	NA
				co-culture	15/46[b] (32.6)	0/46[b]	NA

Values with different superscripts within each column are significantly different ($P<0.05$)

NA is not available.

The interspecies domestic cat fibroblasts reconstructed with bovine oocytes could not produce blastocyst formation. The total embryonic cell number of intraspecies cloned domestic cat and bovine blastocysts was 536 ± 13.9 cells and 127 ± 1.8 cells, respectively (Table 3.3, Fig. 3.9B, D). However, the cell number of the blastocysts derived from interspecies cloned marbled cat using bovine enucleated oocyte as recipient cytoplast was only 16 ± 0.4 cells (Fig. 3.10B), which was much lower than the intraspecies cloned blastocysts. Eventhought, the blastocyst cell number was low but blastocyst like morphology was observed similar to the blastocoel formation from this group. Therefore, we called them as blastocyst like embryos (Fig. 3.10A). The result of this experiment concluded that the domestic cat and marbled cat fibroblasts could dedifferentiate after reconstructed with either domestic cat or bovine enucleated oocytes but those two recipient cytoplasts could not support the development of those embryos beyond morula stage.

Table 3.3 Effect of recipient cytoplast on *in vitro* development of domestic cat, marbled cat and bovine cloned embryos.

Embryo types	Fused (%)	Cleavage (%)	8-cell (%)	Morula (%)	Blastocyst (%)	Mean cell (no.± S.E.M.) of blastoyst
Domestic cat fibroblast +domestic cat oocyte	96/125[b] (76.8)	83/96[b] (86.5)	65/96[b] (67.7)	50/96[a] (52.1)	33/96[a] (34.4)	536 ± 13.9
Marbled cat fibroblast +domestic cat oocyte	95/128[b] (74.2)	82/95[b] (86.3)	58/95[b] (61.1)	21/95[c] (22.1)	0/95[b]	NA
Bovine fibroblast +bovine oocyte	98/120[a] (81.7)	94/98[a] (95.9)	76/98[a] (77.6)	44/98[b] (44.9)	29/98[a] (29.6)	127 ± 1.8
Domestic cat fibroblast +bovine oocyte	99/117[a] (84.6)	90/99[a,b] (90.9)	76/99[a] (76.8)	15/99[c] (15.2)	0/99[b]	NA
Marbled cat fibroblast +bovine oocyte	93/113[a] (82.3)	87/93[a,b] (93.5)	76/93[a] (81.7)	11/93[c] (11.8)	8/93[b] (8.6)	16 ± 0.4

Values with different superscripts within each column are significantly different ($P<0.05$)

NA is not available.

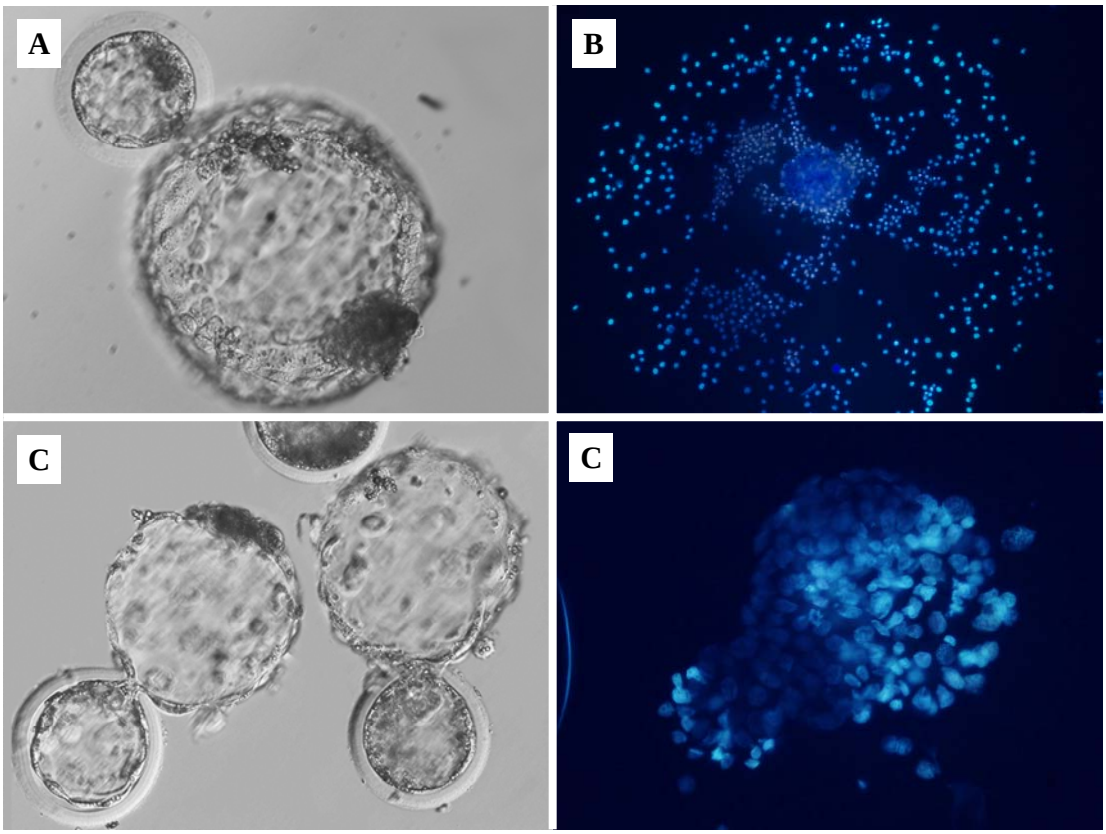

Figure 3.9 Morphology of the blastocysts derived from intraspecies cloned embryo stained with Hoechst 33342 for determining the embryonic cell number of domestic cat (A, B) and bovine embryo (C, D).

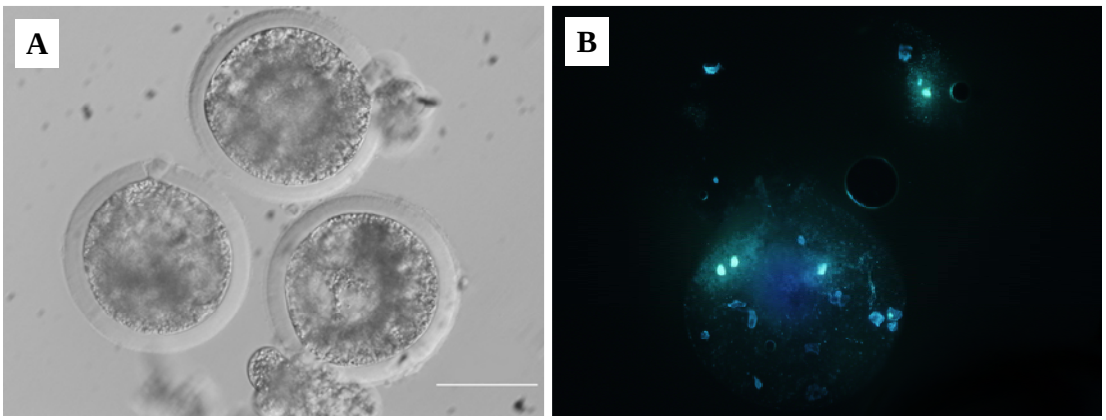

Figure 3.10 The blastocysts of interspecies cloned marbled cat using bovine recipient cytoplast (A) and the blastocyst stained with Hoechst 33342 for determining the embryonic cell number (B).

3.5 Discussion

NT in domestic cat has been a powerful technique to understand the basic knowledge in efforts to increase the efficiency of embryo production. To date there have been several reports describing the source (Skrzyszowaka et al., 2003; Kitiyanant et al., 2003; Yin et al., 2005) and modification (Gomez et al., 2003; Yin et al., 2007) of donor cell and the nuclear transfer procedure (Gomez et al., 2003; Wen et al., 2003) the effects on the development of cloned domestic cat embryos. However, the effect of the handing or holding medium for manipulate the cat oocyte during NT procedure on *in vitro* development has not yet been reported. This study, we demonstrated that the Emcare holding medium could promote the development to blastocyst stage of cloned domestic cat better than 199H. During normal NT procedure the oocytes were held in a culture medium buffered with zwitterionic buffer in a CO_2-free atmosphere. HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) is an organic zwitterionic buffer commonly used to maintain pH levels of basal medium in cell culture. HEPES are usually supplemented in TCM 199, Ham F-10 or Tyrode's medium to produce holding medium for manipulate the oocyte during NT process. The Emcare holding medium is a commercial media used in embryo transfer industry. The composition of this media is not provided by the manufacturers. However, they claim that it has optimized nutritional composition to enhance embryo development and morpholonopropane sulfonic acid (MOPS) an inert zwitterions buffer which provides a more stable microenvironment. A possible explanation for the Emcare holding medium that promote the high rate of embryo develop to blastocyst stage may be due to this buffer media is less toxic when compare to TCM 199

supplement with HEPES. Our result indicated that the manipulation medium does effect the *in vitro* development of cloned domestic cat embryos.

Then, we applied this NT procedure to study interspecies NT by fusion the ear fibroblasts of marbled cat with enucleated oocytes of domestic cat. The result showed that the cloned marbled cat embryos arrested at morula stage. Several studies have shown that embryo co-culture with somatic cell can enhance *in vitro* development (Tervit et al., 1994; Donnay 1997; Lee et al., 1997). Numerous cell lines have been used to prepare a monolayer of feed cell such as vero cell (Menezo et al., 1990; Pegoraro et al., 1998), buffalorat liver (Donnay at al., 1997) but the epithelial cells of the reproductive tract are probably the most physiologically appropriate cells for co-culturing (Xu et al., 2001). The favorable effect of co-culture system could be explained by secretion of beneficial embryotrophic factors (Bavister, 1992) or provide a direct cell to cell contract surface with the embryos which may mimic the natural environment (Joo et al., 2001). However, the result from this study demonstrated that cloned domestic cat embryos developed up to blastocyst stage and the total cell number were not significantly different between embryo co-culture with DOEC and non co-culture system. In addition, co-culture does not improve the development to blastocyst stage in cloned marbled cat, which suggested that the beneficial effect of co-culture with DOEC is not additive. A possible reason for this discrepancy is the fact that, TCM 199 supplement with 10% FBS used as a medium for culture DOEC at 38.5°C, 5% CO_2 in air. After culture the DOEC swelled up with actively movement of cilia. Then swelled DOEC were chosen for co-culture with 8-cell stage of cloned embryos in Tyrode's medium at 38°C, 5% CO_2, 5% O_2 and 90% N_2. We found that after DOEC co-culture with the cloned embryos, the DOEC cells were withered and

no movement was observed. Thus, we can conclude that the medium and environment used in this co-culture study was not properly support the DOEC growth which the beneficial effect from this co-culture may be lost. Previously, there was a report indicating that the domestic cat oocyte could not support development of cloned marbled cat embryos to blastocyst stage (Thongphakdee et al., 2006). Although, we tried to improve the culture system without success. We tried to find another mammalian oocyte to be used as recipient cytoplast to study interspecies cloning of marbled cat. Previous study has been shown that bovine oocyte could support development of interspecies cloning from various mammalian species (Dominko et al., 1999). In this study we also demonstrated that bovine oocytes are able to dedifferentiate felid species (domestic and marbled cats) somatic nuclei and support development of the interspecies cloned embryos. Although, the cloned domestic cat embryos with bovine oocytes arrest at morula stage but cloned marbled cat with bovine oocytes were able to develop to reach blastocyst stage. However, the total cell number of those blastocyst embryos was very low (16±0.4) in comparison with intraspecies cloned domestic cat blastocysts (536±13.9) and intraspecies cloned bovine blastocyst (127±1.8). Perhaps the low cell number of cloned marbled cat blastocysts in bovine oocytes may be due to blastomere fragmentation. We then called them blastocyst like embryos. Previously, cloned domestic cat that have morphology and the total cell number similar to our study have been succeed to produce cloned kittens by transfer the reconstructed embryos into oviduct of recipient (Yin et al., 2007). From their study indicated though *in vitro* culture produce the low quality of blastocysts but *in vivo* culture may support development of embryos until implantation and birth of kittens. Currently, the bovine oocytes have been used as

recipient cytoplast for several interspecies cloning such as gaur (Lanza et al., 2000), buffalo (Kitiyanant et al., 2001), banteng (Sansinena et al., 2005), dog (Murakami et al., 2005) and human (Illmensee et al., 2006). The bovine oocytes have been shown to achieve success in blastocyst development of several species. Alternatively, not every species have been able to develop to reach blastocyst stage when reconstructed with bovine oocytes. There have been reported that the cloned equine (Zhou et al., 2007), mouse (Park et al., 2004) embryos derived from fibroblasts arrest development at 8-cell stage which is similar to this study. In addition, the rabbit oocytes are capable to dedifferentiate somatic cell from several species especially interspecies domestic cat cloning (Wen et al., 2003). In the case of marbled cat cells reconstructed with domestic cat oocytes could not develop to blastocyst stage but rabbit oocytes able to support the development to blastocyst stage (Thongphakdee et al., 2006). These results indicated that interaction between nucleus of donor cell and many factor from cytoplast may involve in nucleus-cytoplatmic compatibility and reprogramming of donor nucleus. Oocyte factors stored in the cytoplasm and the exchange of these cytoplast factors may be species specific and the kind of factors may play role in dedifferentiated and reprogramming of cell nuclei (Parther and First, 1990; Chen et al., 2007). *In vitro* embryo developments are inhibited at specific stage depending on species. There is a morula to blastocyst block for embryo reconstructed with domestic cat oocytes and 8- cell block for reconstructed with bovine oocytes. Although the maternal zygotic transition (MZT) has the key role to *in vitro* development of cloned embryos and we do not know the time of transition is cytoplast specific or donor nuclei specific in the interspecies cloned embryos. Our results demonstrated that the early development of cloned marbled cat was influenced by recipient cytoplasts or

recipient cytoplast specific, while the development to blastocyst was donor nucleus specific. However, most reconstructed embryos had undergone the first cleavage, none of them development past morula stage. Our result suggested that some essential factor might be different from the recipient cytoplast and also its unknown whether the culture method for interspecies reconstructed embryos should matched the culture medium for the donor nuclei or for the recipient cytoplast.

3.6 Conclusion

This study demonstrated that the manipulation medium have an effect on *in vitro* development of cloned domestic cat embryos, Emcare holding medium was able to increase the blastocyst rate of reconstructed domestic cat and increase the efficiency of NT. The cloned marbled cat development arrested at morula stage when reconstructed with domestic cat oocytes. The co-culture system could not support the *in vitro* development of cloned felid species. On the other hand the bovine oocytes were able to dedifferentiate both domestic and marbled cats' karyoplast. The interspecies cloned domestic cat with these oocytes could not develop beyond morula stage and the interspecies cloned marbled cat showed blastocyst like morphology. However, further experiment are needed to study the *in vivo* development of cloned marbled cat embryos to establish the potential of interspecies cloning embryos to produce viable cloned kitten.

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CHAPTER IV

TRANSCRIPTION LEVEL OF GENES CRUCIAL FOR NUCLEAR REPROGRAMMING OF CLONED FELID EMBRYOS

4.1 Abstract

Abnormalities in nuclear and epigenetic reprogramming are usually founded in cloned embryos including felid species. However, genome reprogramming is still a mystery. This is the first investigation on the transcription levels of Oct4, DNMT1, DNMT3a, DNMT3b, HAT1 and HDAC1 genes that are believed to be crucial for nuclear reprogramming in cloned felid species. Total mRNA was extracted from 1-cell to blastocyst stage of domestic cat metaphase II oocytes, *in vitro* fertilized (IVF) and cloned felid embryos. The quantitative real-time PCR was used to detect the transcription level. The levels of Oct4 and HAT1 were low at an early developmental stage and dramatically increased at 8-cell to blastocyst stage in cloned domestic and leopard cats and in IVF derived embryos. However, the cloned marbled cat showed low Oct4 and HAT1 transcript levels throughout development. No blastocyst formation was observed in these embryos. The transcription level of HDAC1 of cloned felid embryos had altered expression patterns compared to IVF derived embryos. The DNMT1 transcript levels of felid embryos were dramatically decreased throughout development while cloned felid embryos showed a higher transcription of DNMT1 than IVF derived embryos. The DNMT3a mRNA of IVF

derived embryos started very low at 2-cell to 4-cell and dramatizing increased at morula and blastocyst stages. In contrast, in cloned felid embryos' DNMT3a, the transcription level dramatically decreased in the early development stage and increased at blastocyst stage. The level of DNMT3b mRNA started to decrease from a high transcription at blastocyst stage until no transcription existed at 2-cell to morula stages. The results demonstrated that transcription levels of Oct4 in felid embryos might have some effect on their *in vitro* development efficiency, particularly at blastocyst stage. The cloned felid embryos showed aberrances in transcription level of DNA methylation and histone acetylation genes. This suggested the occurrence of incompletes nuclear reprogramming of the felids' donor nuclei. These studies indicated that genome reprogramming in the felid species is not enough for reestablishment of embryonic totipotency. Improvement of cloning techniques is needed to efficiently produce the cloned embryos vital for the conservation of endangered felid species.

4.2 Introduction

The successful birth of the cloned sheep, Dolly, astonished scientists (Wilmut et al., 1997). It demonstrated that nuclear programming is a process that can turn differentiated somatic nuclei back to a totipotent stage. Animal cloning has now been performed in many different animal species. The successful production of a gaur (Lanza et al., 2000) and mouflon (Loi et al., 2001) demonstrated that interspecies cloning might be applied to save highly endangered animal species. Interspecies cloning involves the transfer of a cell nucleus of one species into an enucleated oocyte of another species. Interspecies cloning can become an alternative method of

producing cloned embryos, especially in species whose oocytes are difficult to obtain and is a powerful technique for the preservation of endangered animals, such as buffalo (Kitiyanant et al., 2001), giant panda (Chen et al., 2002), banteng (Sansinena et al., 2005), African wildcat (Gomez et al., 2003; 2004), leopard cat (Lorthongpanich et al., 2004; Yin et at., 2006) and marbled cat (Thongphakdee et al., 2006).

However, until now the successful production of animal clones from somatic cells of several species has been quite low. Less than one percent of cloned embryos develop into live cloned offspring (Han et al., 2003). After cloning, the donor nucleus requires epigenetic reprogramming to a totipotent ground stage (Humpherys et al., 2002), and the nuclei of differentiated cells must undergo reprogramming to establish an embryonic profile of gene expression for proper development. Unsuccessful gene expression is frequently observed in cloned embryos, which probably resulted in abnormal development and early loss of the cloned fetus (Daniels et al., 2000; Rideout et al., 2001; Boiani et al., 2002). Nuclear reprogramming is brought about by epigenetic mechanisms such as DNA methylation and histone acetylation, which control the gene expression, specifically transcription. Epigenesis is not dependent on the DNA base sequence (Surani et al., 2001). DNA methylation in mammals involves the addition of a methyl group to the cytosine residues within CpG dinucleotide by DNA methyltransferases (DNMTs). The DNA methylation plays a role in controlling imprinted gene expression and X chromosome silencing (Bestor, 2000). Recent findings revealed that aberrant DNA methylation occurs in cloned bovine and sheep embryos (Kang et al., 2001; Beaujean et al., 2004). The histone modifications are also thought to play certain key roles in regulating through modulation of the chromatin structure by histone acetyltransferases (HATs). This causes the acetylation and

neutralization of the positive charges on the aminoterminus of the histones, leading to chromatin decondensation and activation of gene transcription. Conversely, histones can be deacetylated by histone deacetylases (HDACs), which cause chromatin condensation and gene repression (Bird, 2002). Hyperacetylation in cloned cattle embryos have been observed (Santos et al., 2003; Enright et al., 2005). Both HDAC1 and HAT1 genes were detected in *in vitro* production of bovine embryos at 8-cell stage, with its highest expression at blastocyst stage (McGraw et al., 2003). Additionally, Octamer-binding transcription factor Oct4, the transcription factor essential for pre-implantation development, played an essential role in the establishment and maintenance of a pluripotent cell (Nichols et al., 1998). The aberrance of Oct4 expression in cloned embryos suggests that this crucial developmental gene is not appropriately reprogrammed and that it may serve as a marker of nuclear reprogramming (Mitalipov et al., 2003).

In fact, 36 of 37 species of felid family are classified as threatened or endangered. There are several reports describing research focused on the interspecies cloning of endangered felids (Gomez et al., 2003; Lorthongpanich et al., 2004; Yin et al., 2005). However, felid cloning is difficult, and so far there has been a low number of live cloned offspring reported. (Shin et al., 2002; Gomez et al., 2004; Yin et al., 2005; 2007). This may be directly related to the insufficient reprogramming of the donor cell nuclei when transplanted into enucleated oocytes. The transcription level of genes that are involved in the nuclear reprogramming in felid embryos at different embryonic stages is still unknown. This study is the first detailed analysis of specific gene transcription levels involved in the nuclear reprogramming of *in vitro* and cloned

felid embryos. It has provided the foundation for conservation of endangered felid species.

To understand the nuclear reprogramming of felid embryos, the mRNA expression patterns of the Oct4, histone acetyltransferase (HAT1) histone deacetylase (HDAC1) and DNA methyltransferase (DNMT1, DNMT3a and DNMT3b), of IVF and cloned felid embryos that had received nuclei from domestic, leopard and marbled cat fibroblast cells were investigated. The real-time PCR approach was used to quantify the transcription levels of each gene at different felid pre-implantation embryonic stages (1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst).

4.3 Materials and methods

All chemicals used in this study were purchased from Sigma unless otherwise noted.

4.3.1 Preparation of animal and oocyte recovery

Random mature female domestic cats (9 to 36 months) were housed in facility and cared for using procedures that exceed the standards established by Laboratory Animal Care of Suranaree University of Technology. Healthy mature female cats were selected for superstimulated. Each cat was injected intramuscularly with 200 IU eCG five days apart. Then, ovaries were removed from the reproductive tract. The uperstimulated cats were anesthetized by intramuscularly injected of 0.0002 mg/kg Atrophine and 0.5 mg/kg Xylazine followed by 20 mg/kg Ketamine hydrochloride 10 minutes after the first injection. After the surgical to collect the ovaries the surgical area was examined thoroughly for sign of bleeding. If no sign of bleeding the operative site was closed in layers and post-operative care that exceeded

the standards established by Laboratory Animal Care of Suranaree University of Technology were used. The ovaries were placed into a ∅ 60 mm culture dish containing mDPBS supplement with 0.1% PVP and sliced with a 21 gauge needle to release COCs. The COCs were collected and matured in *in vitro* maturation (IVM) medium consisting of TCM 199 supplemented with 0.36 mM sodium pyruvate, 2.2 mM calcium lactate, 2mM L-glutamine, 1.13 mM cystein, 0.3 mg/ml BSA, 0.5 IU/ml eCG, 1 IU/ml hCG in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 24 h.

4.3.2 Donor cell preparation

Fibroblast cells were generated form the ears or abdominal skin of domestic, leopard and marbled cats after anesthesia or within 24 h after natural death. The donor skin were subjected to surgical biopsy using aseptic procedure and transported to the laboratory within 6 h in mDPBS. Tissues were cut into small pieces (1 mm x 1 mm) then placed into ∅ 60 mm culture dishes and covered with glass slide. Then αMEM supplemented with 10% FBS, culture medium was added and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. After 8-10 days of culture, the monolayer outgrown with fibroblastic like cell morphology were passaged for 1 to 2 times to increase the cell number. The donor cells were frozen in culture medium supplemented with 10% (v/v) DMSO and stored in liquid nitrogen for further use as donor cells.

The frozen fibroblasts were thawed at 37°C and transferred into centrifuge tube containing culture medium and incubated for 5 min. After centrifugation at 3,000 rpm for 5 min, the supernatant was removed and resuspended with culture medium and cultured on ∅ 35 mm culture dish in a humidified atmosphere of 5% CO_2 in air at

37°C for 2 to 3 days prior to nuclear transfer. At subconfluence, the fibroblasts were trypsinized using trypsin/ EDTA to separate into single cells and use as donor cells.

4.3.3 Nuclear transfer

Cumulus cells were removed from *in vitro* matured oocytes at 24 h post culture by gently pipetting in 0.2% hyarulonidase. The MII oocytes were selected for nuclear transfer. The MII oocytes were placed into Emcare holding medium supplemented with 5 μg/ml CB. Each nucleus was moved (enucleated) by using a sharp cutting micropipette. The zona pellucida above the first polar body was cut, the first polar body and metaphase plate were squeezed out with a small volume of surrounding cytoplasm with Narishige micromanipulators while viewing with inverted microscope. Successful enucleation was confirmed by 20 μg/ml Hoechst 33342 staining of the corresponding karyoplast that was squeezed and observed under ultraviolet light using a fluorescent microscope. A single domestic, leopard or marbled cats' fibroblast was inserted into the perivitelline space of the enucleated oocytes.

For fusion, the cytoplasm/cell couplets were equilibrated in fusion medium containing 0.3 M mannital and 0.1 mM magnesium chloride. The resulting couple was placed between both tips of fusion electrodes. Cell fusion was induced with double DC pulse of 30 V for 30 μsec delivered by electro fusion machine (Voltrain EP-1). The fused oocyte was visually evaluated by confirming the presence or absence of donor cell in the perivitelline space 45 min after fusion. The reconstructed oocytes were activated with 7% ethanol for 5 min followed by incubation in Emcare holding medium supplemented with 10 μg/ml CHX and 1.25 μg/ml CD in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 5 h.

4.3.4 *In vitro* fertilization (IVF)

Testes were collected from adult male cats following castration (Figure 4.1A). They were kept in mDPBS and maintained at room temperature before spermatozoa collection. The epididymides were removed from the testes and repeatedly sliced with a 21 gauge needle to release spermatozoa in a ∅ 90 mm petri dish containing mDPBS. The released sperm were centrifuged at 2,100 rpm for 7 min. After supernatant removal, the sperm pellet were overlaid with 1 ml Tyrode's medium containing 0.6 mg/ml BSA supplemented with 15 mM $NaHCO_3$, 0.36 mM sodium pyruvate, 2.2 mM calcium lactate and 2 mM L-glutamine (IVF medium). The sperm were allowed to swim up for 30 min at 38°C under 5% CO_2 in air. The swim up layer was carefully recovered and small aliquots were used to evaluate sperm concentration and sperm motility (Figure 4.9B). For IVF, oocytes were co-incubated with 4×10^5 sperm/ml in 50 µl droplets of IVF medium in a humidified atmosphere of 5% CO_2 in air at 38°C.

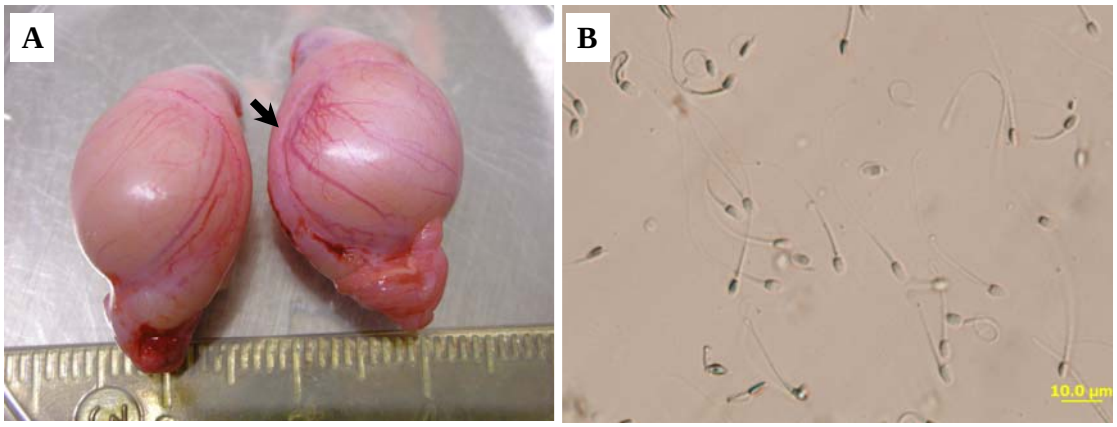

Figure 4.1 Testes of male domestic cat after colleted from castration (A) and spermatozoa (B) were collected from epididymises (arrow) (magnification 200x).

4.3.5 *In vitro* embryos culture

The reconstructed embryos and 20 h post insemination oocytes were cultured in Ty I-medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 2 days. Then 8-cell stage embryos were selected and cultured in Ty II-medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38°C for 6 days. Half of the medium was daily changed and the development of embryos was daily observed.

4.3.6 Embryo staining and cell count

The zona pellucidae of day 8 blastocysts were removed by 0.5% protease and washed in mDPBS supplement with 0.1% PVP. The zona-free embryos were incubated in 100 μl of 1:2 rabbit anti-domestic cat fibroblast cells for 45 min. The excess antibodies were washed out in mDPBS+PVP several times before incubation in 100 μl of 1:10 guinea pig complement (S-1639) supplement with 100 μg/ml propidium iodide (P-4170) and 10 μg/ml Hoechst 33258 (B-2883) for 45 min The embryos were mounted on glass slides and covered with glycerol. The mounted embryos were then counted to determine the number of trophectoderm (TE), blue color from Hoechst 33258 and inner cell mass (ICM) cells, red color from propidium iodide under fluorescence microscope.

4.3.7 mRNA isolation and cDNA synthesis

The mRNA samples were prepared from individual cloned and IVF embryos during pre-implantation stage. The MII oocytes were collected 24 h after IVM. The 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stage embryos were collected at approximately 12, 24, 36, 48, 120, 192 h after culture, respectively. Oligo $(dT)_{25}$ nucleotides attached to magnetic beads (Dynabeads mRNA purification kit, Dynal) were used to isolate polyA RNA following the manufacturer's instructions. Briefly,

an embryo was resuspended in 100 µl lysis/binding buffer (100 mM Tris-HCl; pH 7.5, 500 mM LiCl, 10 mM EDTA with pH 8.0, 1% LiDS and 5 mM DTT) and vortexed at room temperature for 5 min. Then, 20 µl pre-washed Dynabeads oligo $(dT)_{25}$ was mixed with the lysate and annealed by rotating for 5 min at room temperature. The Dynal MPC magnetic particle concentrator was used to easily remove the supernatant. The hybridized mRNA and oligo $(dT)_{25}$ magnetic beads were washed twice with 100 µl washing buffer A (10 mM Tris-HCl; pH 7.5, 0.15 M LiCl, 1 mM EDTA and 1% LiDS) and twice with 100 µl washing buffer B (10 mM Tris-HCl; pH 7.5, 0.15 M LiCl, 1 mM EDTA). To denature and remove the secondary structures, the mRNA bound to magnetic beads were resuspended with 10 µl RNase-free water and heated at 65°C for 5 min then quenched rapidly on ice for 3 min. The first stand cDNA synthesis was done at 55°C for 1 h in a final volume of 20 µl consisting of 1× first stand buffer, 5 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 40U RNasin ribonuclease inhibitor (Invitrogen) and 200U Superscript III RNase H-RT (Invitrogen). Reactions were terminated by incubation at 70°C for 15 min. The final volume was increased to 30 µl with distilled water and all cDNA samples were stored at -20°C until further use.

4.3.8 Real-time PCR analysis of gene expression

The quantification of all gene transcripts was done by real-time PCR. All oocytes and embryos were analyzed at least four times for every stage. The primer for β-actin and Oct4 genes was designed from conserved sequences of human gene from GenBank and primer for DNMT1, DNMT3a, DNMT3b, HAT1 and HDAC1 were followed as previously report (Suteevun et al., 2006). The sequences of the primers are shown in Table 3.1. Real-time PCR was executed on Lightcycler apparatus using

SYBR green incorporation. The reaction was performed in capillaries with the final volume of 20 µl. Each capillary contained 3 µl cDNA from a single oocyte, or a cloned or IVF embryo in every stage of preimplantation development. The reaction mixture consisting of 5 µM each of sequence-specific primer, 1.25 mM $MgCl_2$ and 2 µl of the SYBR green mix containing dNTPs (FastStart DNA mater SYBR green I, LightCycler). The reaction conditions were template denaturation at 95°C for 10 min followed by 45 cycles of 95°C denaturation for 10 sec, annealing (temperature as show in Table 3.1) for 10 sec and 72°C extension for 15 sec. At the end of the PCR reaction, melting curve analysis was performed for each sample to verify that a single specific product was generated. A melting cycle consisted of 95°C for 3 sec, 65°C for 5 sec and of a step cycle starting at 65°C up to 95°C with a 0.2°C/sec transition rate.

To compare the relative level of gene expression in cloned and IVF embryos, real-time data were analyzed by using the comparative CT method, ΔΔCT (Livak and Schmittgen, 2001). This method calculates the quantity of starting material (DNA or cDNA) in fold change relative to a calibrator sample and this change is given by $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = (CT_{target:sample} - CT_{reference:sample}) - (CT_{target:calibrator} - CT_{reference:calibrator})$. The CT, threshold cycle represents the PCR cycle at which an increase in the fluorescence signal above baseline can be first detected. In our experiments, there were six genes of interest (Oct4, DNMT1, DNMT3a, DNMT3b, HAT1 and HDAC1) while β-actin was used as the reference gene. The cDNA derived from cloned or IVF embryos and MII oocytes represent the sample and calibrator respectively. The 1/CT values were used to examine the temporal pattern of gene expression throughout preimplantation development.

4.3.9 Statistic analysis

The fusion, embryo cleavage and development rate of the different embryo types were analyzed by ANOVA using Statistical Analysis System (SAS). *In vitro* embryo production experiments were repeated at least three times. Statistically significant differences in relative expression levels of target genes between each development stage were calculated by two-way ANOVA using the SAS with Randomized Complete Block Design (RCBD), proportional data were $\log_{10}(X+1)$ transformed before analysis. Relative quantification of target genes expression level is presented as fold-difference. Data are presented as mean±SEM. P value of less than 0.05 was considered to be significant.

Table 4.1 Primer sequences used for Real-time PCR

Gene	Accession number	Primer sequences 5'-3'	Product size (bp)	Annealing temperature (°C)	References
Oct4	DQ486513	F-ATCCTGGGGGTTCTATTTGG R- CTGGTTCGCTTTCTCTTTCG	200	56	This study
DNMT1	AY173048	F- GAGGGCTACCTGGCTAAAGTC R- CATTCGCTTCCCGACTGAAA	88	60	Suteevun et al., 2006
DNMT3a	AY271299	F- CGAGGTGTGTGAGGACTCCAT R- ACGTCCCCGACGTACATGA	93	62	Suteevun et al., 2006
DNMT3b	AY244713	F- AGCATGAGGGCAACATCAAAT R- CACCAATCACCAAGTCAAATG	98	62	Suteevun et al., 2006
HAT1	BT021536	F- CTTCAGACCTTTTTGATGTGGTTTATT R- GCGTAGCTCCATCCTTATTATACTTCTC	112	58	Suteevun et al., 2006

Table 4.1 Primer sequences used for Real-time PCR (Continued)

Gene	Accession number	Primer sequences 5'-3'	Product size (bp)	Annealing temperature (°C)	References
HDAC1	AY504948	F- GCACTGGGCTGGAACATCTC R- GGGATTGACGACGAGTCCTATG	98	58	Suteevun et al., 2006
β-actin	NM001101	F- GGACTTCGAGCAAGAGATGG R- AGCACTGTGTTGGCGTACAG	234	60	This study

4.4 Results

4.4.1 *In vitro* developmental of felid embryos following *in vitro* fertilization and somatic cell nuclear transfer

The developmental rate of IVF and cloned domestic, leopard and marbled cat embryos are shown in Table 4.2. There were not significant differences in the fusion rate of domestic, leopard and marbled cats fibroblasts with enucleated domestic cat oocytes (74.1, 74.9 and 73.1%, respectively). The cleavage rate of IVF embryos was significant lower than the cloned embryos (69.4 vs. 86.3-93.8%, respectively; $P<0.05$). The development to 8-cell stage of cloned leopard cat embryos was significantly higher than the IVF and cloned marbled cat embryos (81.5 vs. 58.5 and 58.1%, respectively; $P<0.05$), but there were not significant differences when compared with cloned domestic cat embryos (68.0%). The development to morula stage of cloned domestic cat embryos was significantly higher than the IVF, cloned leopard and marbled cat embryos (55.7 vs. 27.5, 30.1 and 23.9%, respectively; $P<0.05$). The percentage of development to blastocyst stage of cloned domestic cat embryos was also significantly higher than the IVF and cloned leopard cat embryos (35.2 vs. 19.1 and 21.2%, respectively; $P<0.05$; Figure 4.2A, B and 4.3A). However, no blastocyst formation was observed in cloned marbled cat embryos. All embryos arrested at the morula stage (Figure 4.4). The number of TE in cloned leopard cat blastocysts was significant lower than the IVF and cloned domestic cat blastocysts (338.4 ± 26.9 vs. 462.1 ± 25.5 and 425.1 ± 14.9, respectively; $P<0.05$; Figure 4.2C, D and 4.3B).

Table 4.2 Comparison the *in vitro* development between IVF and cloned felid species embryos after reconstructed with domestic cat oocytes.

Type of embryos	Fused (%)	Cleavage (%)	8-cell (%)	Morula (%)	Blastocyst (%)	No. of cell in blastocyst (mean ± S.E.M.)		
						ICM	TE	% ICM
IVF	N/A	134/193[b] (69.4)	113/193[b] (58.5)	53/193b (27.5)	37/193b (19.1)	159.3 ± 12.5[a]	462.1 ± 25.5[a]	34.5[a]
Cloned domestic cat	122/164[a] (74.4)	108/122[a] (88.5)	83/122[ab] (68.0)	68/122a (55.7)	43/122a (35.2)	126.2 ± 6.0[b]	425.1 ± 14.9[a]	29.6[b]
Cloned leopard cat	146/195[a] (74.9)	137/146[a] (93.8)	119/146[a] (81.5)	44/146b (30.1)	31/146b (21.2)	121.6 ± 7.0[b]	338.4 ± 26.9[b]	35.9[a]
Cloned marbled cat	117/160[a] (73.1)	101/117[a] (86.3)	68/117[b] (58.1)	28/117b (23.9)	0/117c (0.0)	N/A	N/A	N/A

Values with different superscripts within each column are significantly different ($P<0.05$).

N/A is not available.

The ICM cell numbers of IVF blastocysts were significantly higher than the cloned domestic and leopard cats blastocysts (159.3 ± 12.5 vs. 126.2 ± 6.0 and 121.6 ± 7.0, respectively; $P<0.05$) and the percentage of ICM in cloned domestic cat blastocysts was significantly lower than the IVF and cloned leopard cat blastocysts (24.0 vs. 34.5 and 35.9, respectively; $P<0.05$).

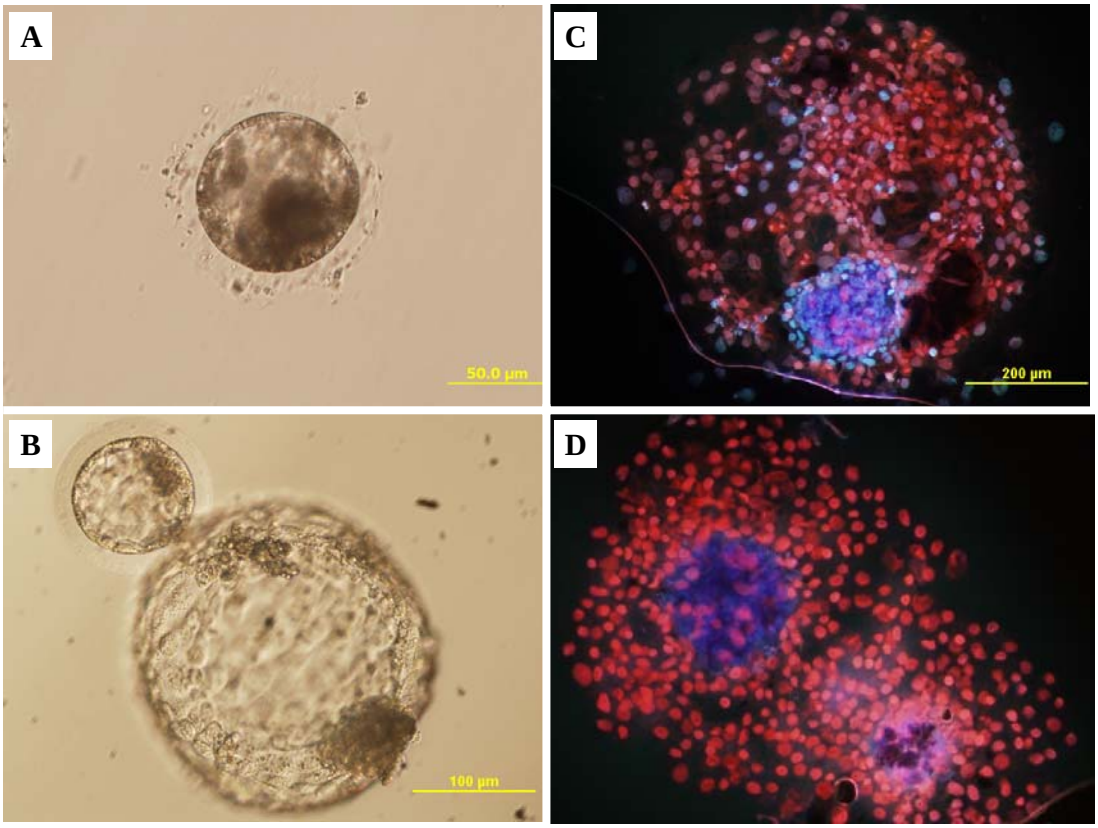

Figure 4.2 IVF derived blastocyst at 8 days (A) and hatching blastocyst of cloned domestic cat at 8 days after activation (B). Differential staining of IVF (C) and cloned domestic cat (D) blastocysts for count the number of TE (red) and ICM (blue) (magnification 200x).

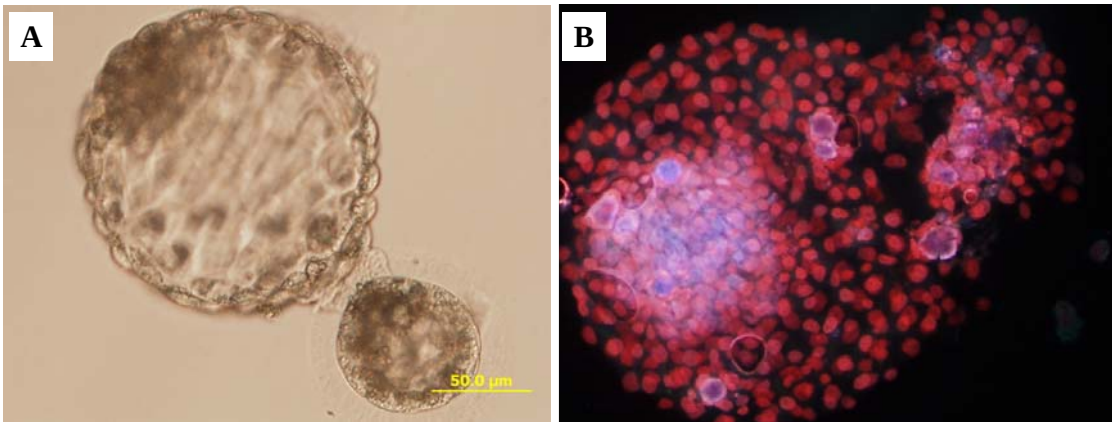

Figure 4.3 Hatching blastocyst of cloned leopard cat at 8 days after activation (A) and differential staining (B) of cloned leopard cat blastocyst for count the number of TE (red) and ICM (blue) (magnification 200x).

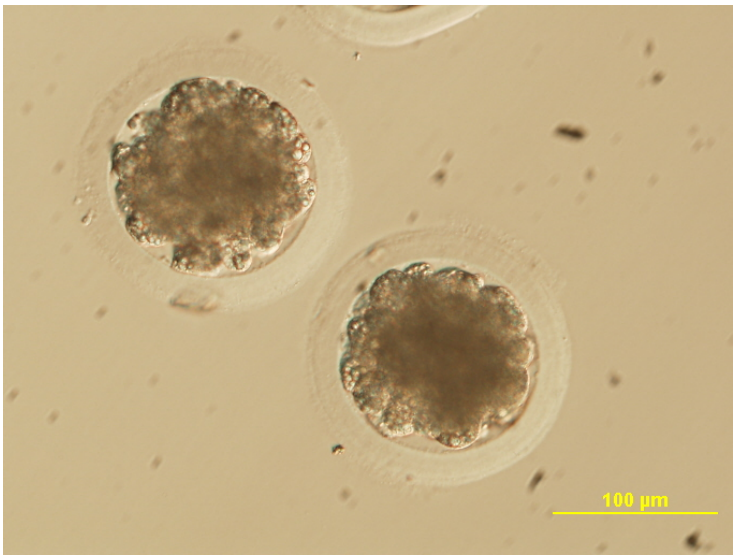

Figure 4.4 Morula stage of cloned marbled cat at 6 days after activation (magnification 200x).

4.4.2 Expression level of mRNA for pluripotent and chromatin remodeling genes

The Oct4, DNMT1, DNMT3a, DNMT3b, HAT1 and HDAC1 gene expression were studied in the preimplantation stages of cloned felid embryos. The cDNA from individual 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst were used in the real-time PCR. Real-time PCR analysis were performed for 6 genes in order to make relative quantitative of the mRNA levels in the preimplantation felid embryos development and to check for difference of the mRNA levels between IVF, cloned domestic, leopard and marbled cats embryos.

The expression of β-actin, reference gene displayed constant mRNA level (1/CT values) from the 1-cell up to blastocyst stage (Figure 4.5). The β-actin showed constant expression throughout pre-implantation development of felid embryos.

The expression of Oct4 mRNA were low at the 1-cell to 8-cell stage of IVF, cloned domestic and leopard cat embryos and increased in the morula and blastocyst stage. At morula stage the embryos derived from IVF showed significantly higher Oct4 transcript (6.4-fold; $P<0.05$) than cloned domestic (1.5-fold) and leopard cats (3.2-fold). However, there were no significant differences transcript levels at blastocyst stage in cloned domestic and leopard cats. For cloned marbled cat embryos, the mRNA level of Oct4 showed low expression throughout preimplantation development and had significantly lower transcription (0.2-fold; $P<0.05$) at morula stage when compare to the other embryos. The cloned marbled cat could not develop to blastocyst stage, thus the expression level of that transcription was not determined (Figure 4.6).

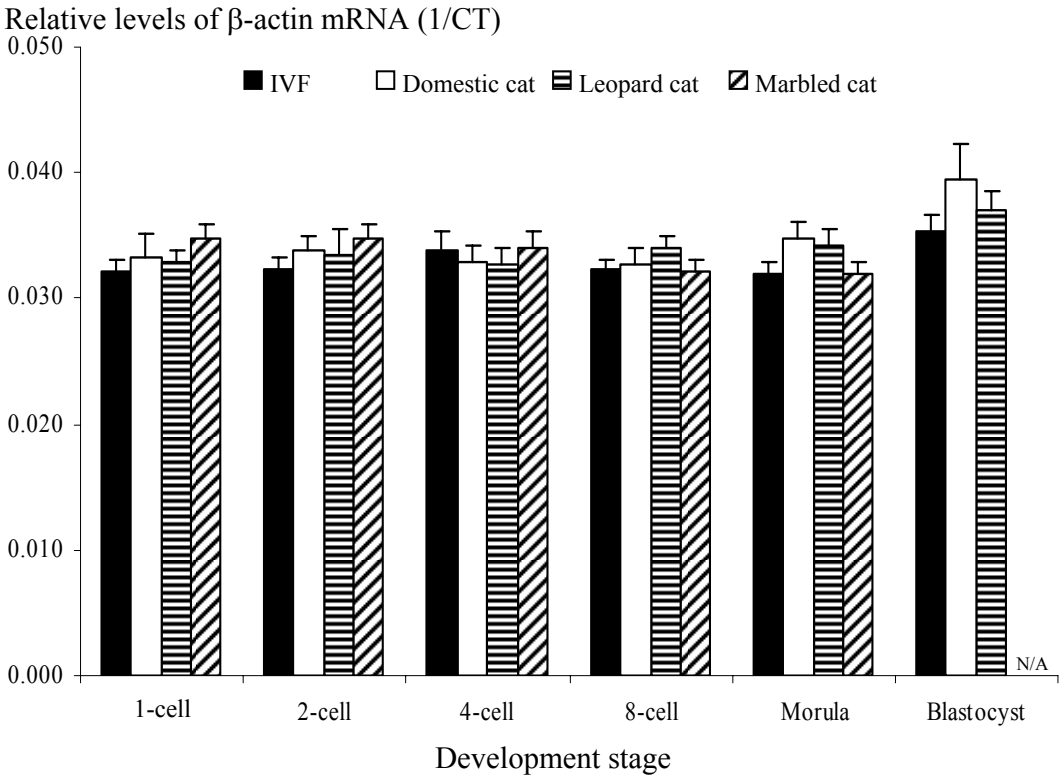

Figure 4.5 Relative expression levels of β-actin mRNA (1/CT) between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. N/A is not

The relative expression of passive DNA methylation gene, DNMT1 was similar in 1-cell stage for all four types of embryos. After the first cleavage, the cloned leopard and marbled cats embryos showed a higher expression of DNMT1 when compared to IVF embryos, but did not show any different expression when compared to cloned domestic cat embryos. DNMT1 expression seems to decrease after the 2-cell stage, especially in the morulae of cloned marbled cat where almost no transcript of DNMT1 was detected. In cloned domestic cat morulae and blastocysts, showed higher expression of DNMT1 (Figure 4.7).

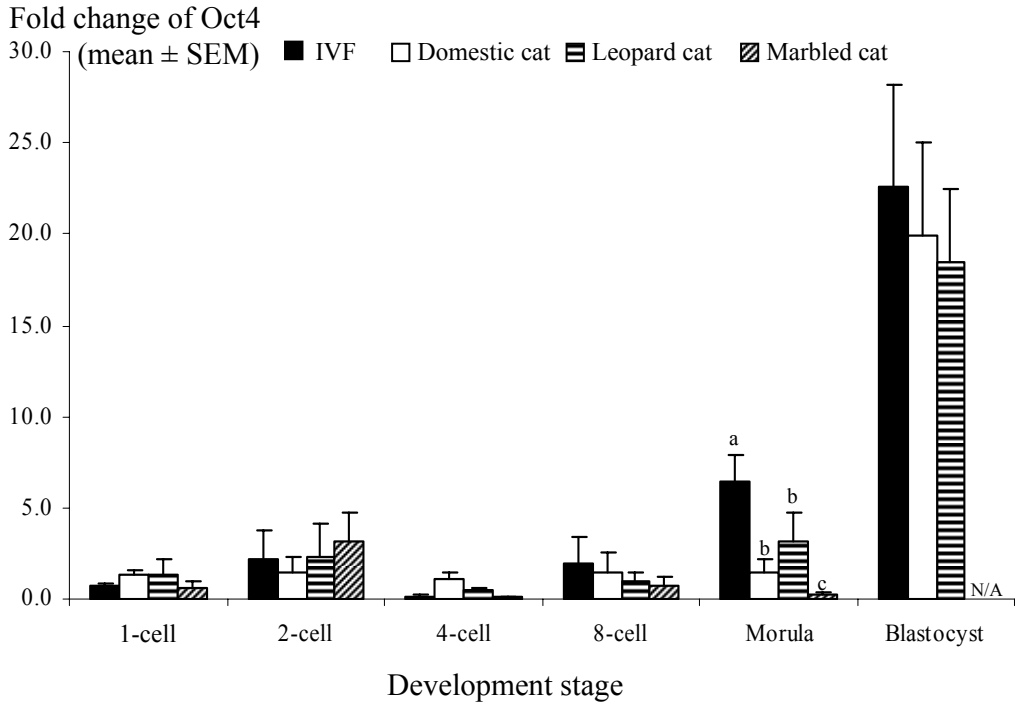

Figure 4.6 Comparison of the relative expression levels of Oct4 mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different ($P<0.05$). N/A is not available.

In contrast, the *de novo* DNA methylation genes, DNMT3a and DNMT3b, showed relatively higher expression levels (Figure 4.8 and 4.9). DNMT3a showed no significant difference in transcription level at 1-cell (2.0 to 2.6-fold) and 2-cell stage (1.8 to 2.5-fold) between IVF and cloned felid embryos. No transcription of DNMT3a was detected in IVF embryos at the 4-cell stage. However, 0.5 to 0.9-fold it was detected in all cloned felid embryos. In addition, the 8-cell stage of both IVF and cloned domestic cat embryos showed significantly lower expression of DNMT3a

(0.01 and 0.02-fold, respectively; $P<0.05$) than in cloned leopard and marbled cats embryos (0.4 and 0.7-fold, respectively). The morula stage of the cloned marbled cat had significantly lower expression of DNMT3a (0.01-fold; $P<0.05$) compared to the cloned domestic and leopard cats (0.3-fold) and the embryos derived from IVF had significantly highest expression (1.7-fold; $P<0.05$) when compared to other cloned felid embryos. Similarly, the expression level of DNMT3a in the IVF blastocysts was significantly higher (3.8-fold; $P<0.05$) in cloned domestic and leopard cats blastocysts (2.6 and 2.7-fold, respectively) (Figure 4.8).

The DNMT3b expression level in 1-cell of IVF and cloned felid embryos showed to appeared at similar level (0.1 to 0.4-fold) and the difference was not statistically significant. After the first cleavage, the embryos derived from IVF has significantly lower expression of DNMT3b (0.01-fold; $P<0.05$) compared to the cloned felid embryos (0.4 to 0.6-fold). In the embryos derived from IVF, the expression level of DNMT3b mRNA was not detected at 4-cell to morula stage. However, the 4-cell stage of the cloned marbled cat showed low expression levels of DNMT3b (0.06-fold). In the 4-cell to 8-cell stage of cloned domestic and leopard cats no DNMT3b was detected. In contrast, the morula stage of cloned domestic and leopard cats embryos showed a low expression level of DNMT3b (0.15 and 0.06-fold, respectively) but no significant difference from those IVF and cloned marbled cat (not detected). However, the high level of DNMT3b mRNA expression was found in the blastocysts derived from IVF and cloned domestic and leopard cats (Figure 4.9).

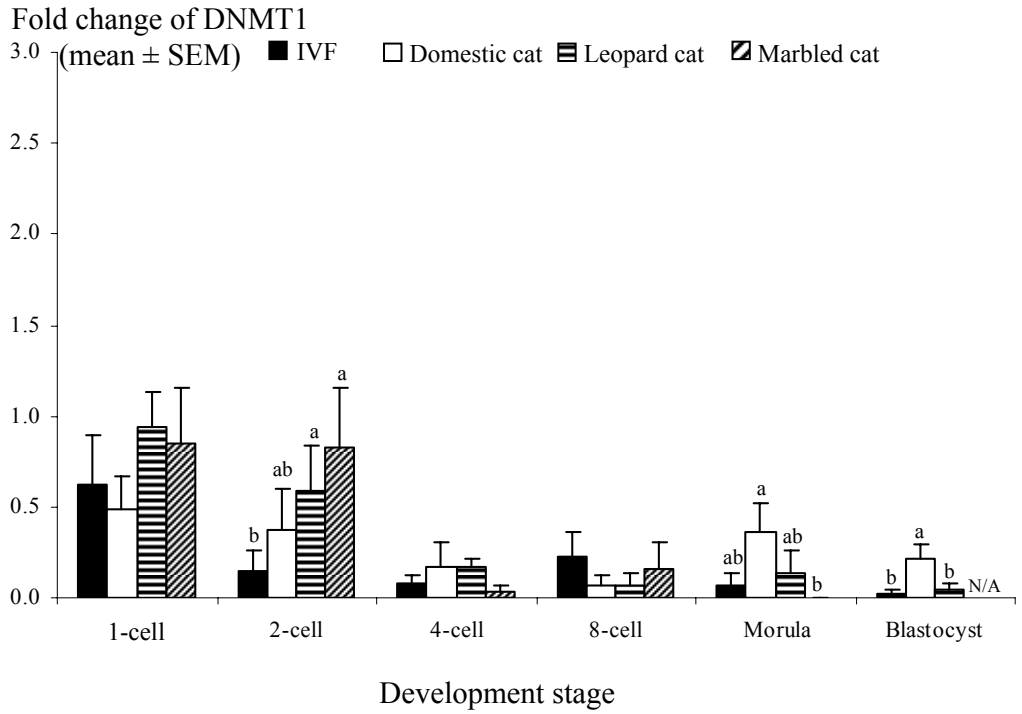

Figure 4.7 Comparison of the relative expression levels of DNMT1 mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different ($P<0.05$). N/A is not available.

The expression level of histone modification gene, HAT1 and HDAC1 are shown in the Figure 4.10 and 4.11. The HAT1 gene showed a low expression (0.17 to 0.31-fold) in the 1-cell stage of IVF and cloned felid embryos, which remained at a low level until 8-cell stage. However, the embryos derived from IVF increased in the HAT1 mRNA level from 8-cell stage and reached the highest level (6.03-fold) at blastocyst stage. In contrast, the 8-cell stage of cloned marbled cat showed high expression (2.47-fold), but lower expression in the morula stage (0.31-fold).

The cloned domestic and leopard cats embryos have significantly (P<0.05) lower transcription levels of HAT1 mRNA throughout preimplantation development compared to IVF embryos (Figure 4.10).

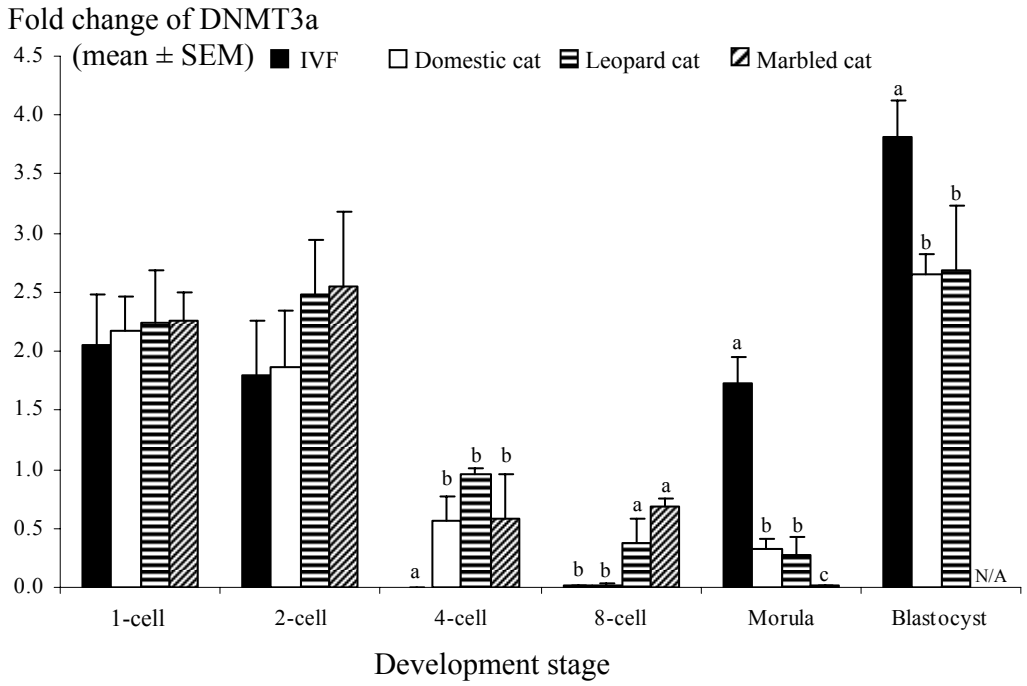

Figure 4.8 Comparison of the relative expression levels of DNMT3a mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different (P<0.05). N/A is not available.

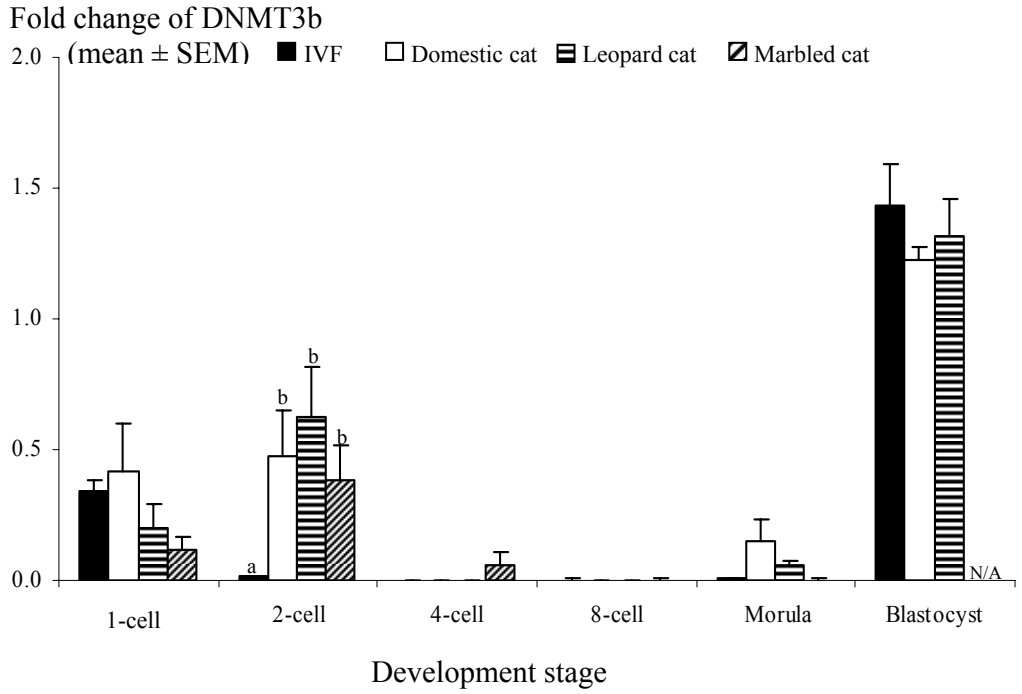

Figure 4.9 Comparison of the relative expression levels of DNMT3b mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different ($P<0.05$). N/A is not available.

As for the HDAC1 gene, there was not a significant difference of expression at the 1-cell stage for IVF and cloned felid embryos. The 2-cell stage of IVF embryos showed significantly lower expression of HDAC1 when compared to the cloned felid embryos. Thereafter, the 4-cell stage of cloned leopard cat embryos showed the highest expression (4.4-fold) compared to IVF, cloned domestic and marbled cats embryos (0.1, 0.2 and 0.6-fold, respectively).

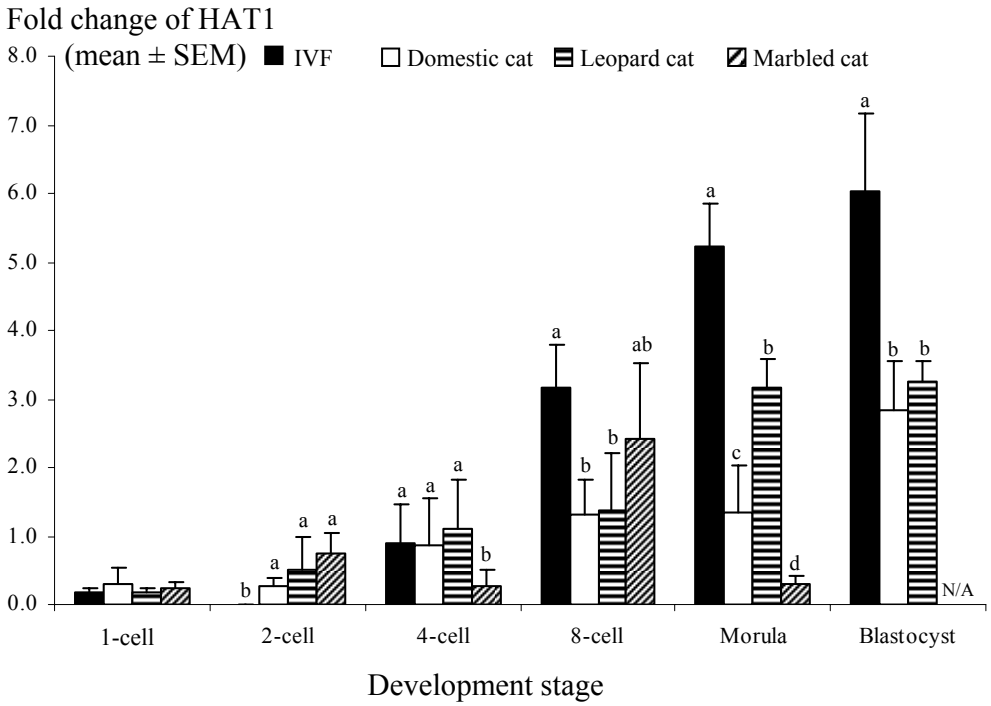

Figure 4.10 Comparison of the relative expression levels of HAT1 mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different ($P<0.05$). N/A is not available.

In contrast, the HDAC1 mRNA levels are similar in IVF and cloned felid embryos at the 8-cell stage (1.0 to 1.3-fold). For morula stage, cloned domestic and marbled cats embryos showed significantly lower expression (0.1-fold; $P<0.05$) than IVF (1.1-fold) and cloned leopard cat (0.7-fold) embryos. Additionally, the HDAC1 mRNA levels of IVF and cloned leopard cat blastocysts were significantly higher expressed (2.7 and 2.8-fold, respectively) than cloned domestic cat blastocysts (0.8-fold) (Figure 4.11).

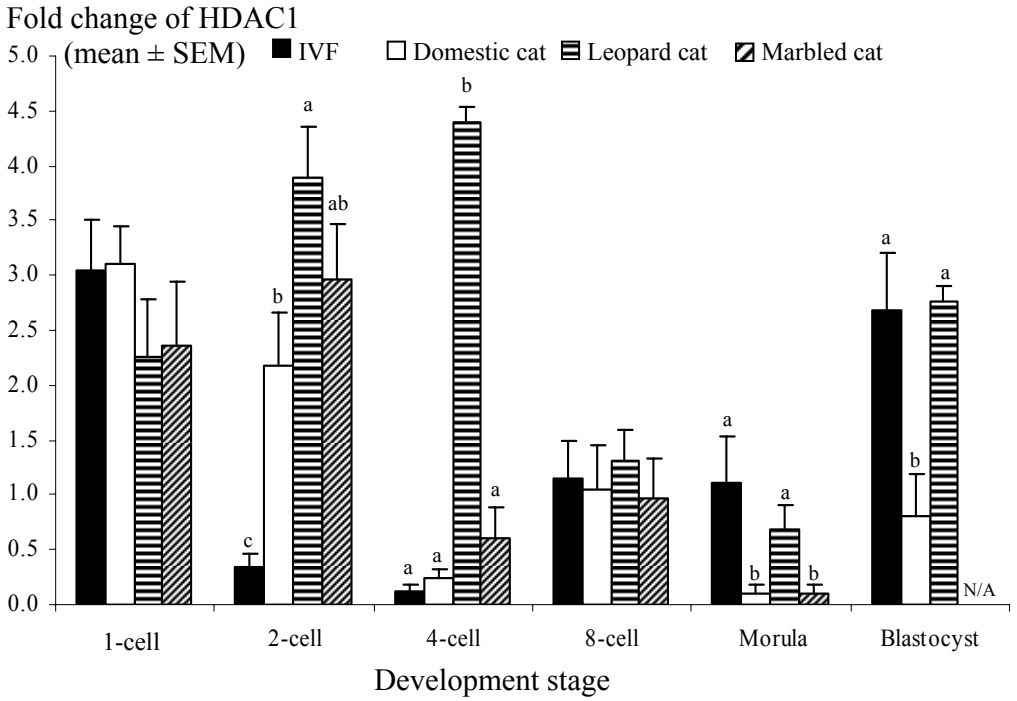

Figure 4.11 Comparison of the relative expression levels of HDAC1 mRNA between IVF, cloned domestic cat, cloned leopard cat and cloned marbled cat embryos at different stages of embryo development. The fold change is the difference between the embryos and the calibrator. Gene transcription levels of mature oocyte were used as the calibrator. Bar with difference superscripts within the same stage of development are significantly different ($P<0.05$). N/A is not available.

4.5 Discussion

The results of this study showed the developmental rate and cell number of cloned felid embryos generated from domestic, leopard and marbled cats' somatic cell reconstructed with domestic cat cytoplasts compared to IVF embryos of domestic cat. The fusion and cleavage rates of cloned felid embryos were similar to the earlier reports (Skrzyszowska et al., 2002; Kittiyanant et al., 2003; Gomez et al., 2003).

However, for the cloned domestic cat, the developmental rate to blastocyst stage was higher than previously reported (Skrzyszowska et al., 2002; Kittiyanant et al., 2003; Gomez et al., 2003; Yin et al., 2005) and also in the cloned leopard cat, it was higher than previous reported (Yin et al., 2006). One of the possible reasons for the contrasting results in embryo development may be due to the variations in the type of donor cells, embryo culture system and nuclear transfer procedures. Surprisingly, no blastocyst formation was observed in the cloned marbled cat. All cloned marbled cat embryos arrested at the morula stage, which this is similar to those previously reported (Thongphagdee et al., 2006). The number of TE and ICM cells at blastocyst stage is important for estimating the quality of embryos. The cell number of felid embryos in this study showed higher numbers in comparison with other previously reported (Skrzyszowska et al., 2002; Gomez et al., 2003; Yin et al., 2005; 2006). The reasons for the high cell number of felid embryos may be due to the modification of Tyrode's medium and the *in vitro* culture system used in this study (Pope et al., 2004). The modified Tyrode's medium and culture system are more suitable for the culture of felid embryos. Day 8 blastocysts of each felid embryo were used to determine the TE and ICM. The *in vitro* development investigation of cloned felid embryos, IVF embryos of domestic cat was used as control group to compare the development rate and cell number to indicate the success reprogramming of cloned embryos. Additional, the comparison of *in vitro* development between IVF and cloned felid embryos has not been reported. However, the mean proportion of IVF embryos development to the blastocyst stage in this study was lower than that reported by Gomez et al. (2003). Although, it is difficult to compare different studies, the difference in the frequency of development to blastocyst stage may be attributable to different genetic backgrounds

of donor cats (sperm and oocytes). The *in vitro* development investigation between cloned felid embryos and IVF derived domestic cats was used as a control to compare the development rate and cell number that indicates the successful reprogramming of cloned embryos. This result showed that the development and cell number of cloned embryos were similar to the IVF derived embryos. It may indicate that successful reprogramming of differentiated nuclei of somatic cell and domestic cat oocytes could support early development and be used as a recipient cytoplast to produce interspecies cloned embryos for the conservation of endangered felid species. However, since the cloned marbled cat could not develop to blastocyst stage, a possible explanation of that failure of development may be incomplete reprogramming of donor nuclei.

Thus, to analyzing the gene expression during cloned felids embryogenesis is a valuable tool for investigating the interaction between donor nuclei and recipient cytoplast and the molecular mechanism of nuclear reprogramming. This is the first research to investigate the transcription level of specific genes involve in nuclear reprogramming in felid embryos.

Quantitative analysis of gene expression often used housekeeping genes as an endogenous control against which the expression level of a target gene can be normalized. β-actin was used as a reference gene in earlier reports studies (Steuerwald et al., 2000; Li et al., 2005; Boonkusol et al., 2006), as well as in this study. The transcript expression of β-actin mRNA was shown to be constant during all of the developmental stages of the felid embryos, even though the number of cells from the oocyte to blastocyst stage increased by 300 to 500 cells in the blastocyst stage and the mRNA level of β-actin also increased in a proportional way. This is especially true after the timing of maternal zygotic transition (MZT). Once the embryonic genome is

activated, the transcript level should reflect the number of cells, whereas before the MZT, the maternal RNA pools are the only source of transcript. Thus, the housekeeping mRNA level should be higher in the oocyte and gradually degrade as the embryonic development progresses to the MZT (McGraw et al., 2003). However, this research indicated that β-actin is one of the best endogenous controls for preimplantation gene expression of felid embryos (Figure 4.13).

The Oct4 gene has been suggested to play an important role in controlling development of pluripotency events during embryogenesis (Bortvin et al., 2003). The results indicated that the Oct4 mRNA levels were low at 1-cell and higher at morula and blastocyst stage in IVF and cloned domestic and leopard cats. However, Oct4 mRNA levels of the cloned marbled cat was low at 1-cell to morula stage and unfortunately these cloned embryos could not develop to blastocyst stage. This observation was consistent with the report of Kurosaka et al., (2004), who found that during embryogenesis development the distribution of Oct4 transcript in bovine and mouse embryos was low at an early development stage and this was followed by increase of transcript at morula and blastocyst stage. However, the lack of Oct4 mRNA founded in the interspecies cloning of mice led to the failure of further development (Park et al., 2004). In the cloned marbled cat, we found that the low level of Oct4 transcription was detected throughout development, which may lead to the development arrest its progression to the blastocyst stage. This event suggests insufficient reprogramming of donor nuclei. This result demonstrated that cloned felid embryos showed low transcription levels of Oct4 mRNA compared to IVF derived embryos in every stages of development. The abnormalities in the expression pattern of Oct4 and Oct4 related genes were observed in cloned embryos (Humphreys et al.,

2002; Bortvin et al., 2003), which this study also agrees with these observations. However, Mitalipov et al., (2003) also demonstrated that the lack of Oct4 expression influenced the low blastocyst development in cloned embryos. They suggested that the aberrant pluripotentcy marker gene is not appropriately reprogrammed and may serve as a marker of nuclear reprogramming.

DNA methylation represents a major epigenetic modification of the genome. In this study, the transcription level of DNA methyltransferase genes (DNMTs) were also analyzed. The aberrant expression of DNMTs genes were founded during preimplantation of cloned felid embryos. In the cloned marbled cat embryo, no DNMTs gene expression was detected at morula stage, which has lead to its development failure. The morula and blastocyst stage of cloned embryos showed higher transcription of DNMT1. These embryos showed lower transcription of *de novo* DNA methyltransferase genes compared to IVF derived embryos. In bovine, somatic nuclei are resistant to the erasure of DNA methylation in early embryogenesis and cloned bovine have a tendency to preserve the DNA methylation patterns inherited from the donor cells (Bourc'his et al., 2001; Dean et al., 2001). Reestablishment of DNA methylation was also potentially deregulated by precocious *de novo* methylation in clones (Dean et al., 2001). Owing to the fact that the donor nucleus comes from a somatic cell, it is likely the mis-expression is due to improper reprogramming, or the improper regulation of DNA methyltransferase function is responsible for the abnormal patterns of genomic methylation and the inability of cloned embryos to properly recapitulate embryonic transcription. This abnormal methylation transition in cloned embryos could be due to the specific features of the somatic chromatin structure and/or defective regulation of DNMTs. Kang (2001) and

colleagues also demonstrated that the methylation pattern observed in cloned bovine embryos was dramatically different than that exhibited in both *in vitro* and *in vivo* produced control embryos. They also found that the DNA methylation level in clones can be higher or lower than the control embryos depending on the donor cell types, targeted DNA sequences, examined embryonic stages and detection methods. The abnormality of the DNA methylation level is also substantially variable among individual clones (Kang et al., 2001). It is clear that embryos produced by cloning exhibit a state of hypermethylation, as this phenomenon has been found in both cattle and mice (Kang et al., 2001; Dean et al., 2001; Bourc'his et al., 2001). There has been many studies to report the aberrant levels for the DNMTs mRNA in cloned embryos (Dean et al., 2001; Young et al., 2004; Suteevun et al., 2006).

Alteration of histone acetylation is also an important aspect of chromatin remodeling in cloning. This study presents the first investigation of histone acetylation in IVF and cloned embryos in felid species. In bovine oocytes, early embryos express several histone acetyltransferases and deacetylases with some variability in the transcription levels depending on the developmental stages (McGraw et al., 2003). In mouse oocytes, histone H3 and H4 are globally deacetylated on several lysines at the metaphase II of the second meiosis, which was reproduced in somatic nuclei transferred into the same stage of oocytes (Kim et al., 2003). This genome-wide decrease of histone acetylation may contribute to the erasure of the previous gene expression patterns specific to the donor cell differentiation. The result demonstrated that HAT1 mRNA transcription was low in cloned felid embryos at the 8-cell to blastocyst stage compared to IVF derived embryos and the level of HDAC1 mRNA was aberrant at an early development stage, 1-cell to 4-cell stage. This

suggests that hypoacetylation found in cloned felid embryos was inconsistent with the study of Suteevun et al., (2006), who measured the transcription of histone acetylation in cloned buffalo embryos. They found that hyperacetylation occurred in these embryos. To date there had been only a few studies that have focused on the acetylation of histone in cloned embryos and that found aberrancies (Santos et al., 2003; Enright et al., 2005; Suteevun et al., 2006). These results indicate variable levels of histone deacetylases and histone acetyltransferases transcripts throughout embryonic development and may indicate the ones that are involved in somatic reprogramming.

4.6 Conclusion

This study showed the transcription level of genes that involve in nuclear reprogramming. The development of cloned marbled cat embryos was arrested at morula stage and no blastocyst formation was observed in these embryos. However, the transcription level of pluripotent, DNA methylation and histone acetylation genes were found to be aberrant in the cloned marbled cat embryos which may lead to the failure of future development and incomplete reprogramming of marbled cat nuclei in the domestic cat cytoplast. Surprisingly, the cloned domestic and leopard cat after reconstructed with domestic cat cytoplast were able development to the blastocyst stage, the development rate and cell number were similar to the IVF derived embryos. Although cloned domestic and leopard cat embryos have normal developmental rate, the transcription level of pluripotent gene showed similar pattern to the IVF embryos but these embryo showed aberrant DNA methylation and hypoacetylation. These data provide new understanding to the transcription pattern of specific genes that are

crucial for nuclear reprogramming of cloned felid embryos. To determine the success of nuclear transfer, the abnormal nuclear and epigenetic reprogramming needed to be considered there condition may lead to developmental failure, implantation failure, fetal abnormality and poor postnatal heath.

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CHAPTER V

OVERALL CONCLUSIONS

Nuclear transfer (NT) of domestic cat can be use as a model for conservation of endangered felid species. However, NT has resulted in several abnormal of development such as high rate of abortion during early gestation and increase prenatal death. It is unclear whether the development failures of cloned embryos were due to the incomplete nuclear reprogramming or the NT procedure itself. This study was to base the understanding of the some factor that involve in increase the efficiency of *in vitro* production of clone felid embryos and also the transcription level of gene that are crucial for nuclear reprogramming. It has provided the basis knowledge for conservation of endangered felid species.

Manipulation medium, the Emcare holding medium and 199H used during manipulation of the domestic cat oocyte during NT process effected the cloned domestic cat embryo development to blastocyst stage. However, the co-culture system of cloned felid embryos was not beneficial to the development. The domestic cat and bovine cytoplasts could support mitotic cleavage of felid nuclei up to morula stage, but were not able to support the development up to blastocyst stage.

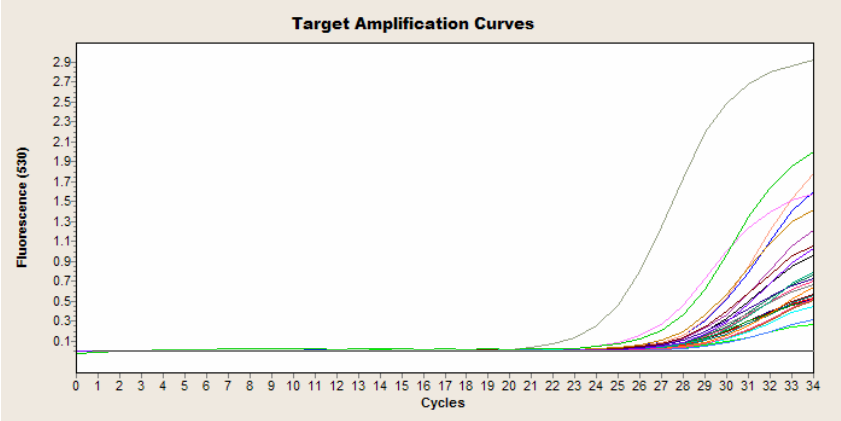
Transcription level of Oct4 in felid embryos might have some effect on the in vitro development efficiency particularly at blastocyst stage. The cloned felid embryos showed aberrant transcription level of DNA methylation and histone hypoacetylation genes. The results showed aberrant genes expression were found in

cloned felid embryos and suggested that incompletes nuclear reprogramming of felid donor nuclei occur in cloned felid embryos. This may lead to the developmental failure, implantation failure, fetal abnormality and poor postnatal heath in cloned felids species. Improvements of the cloning techniques are needed for the production efficiency of cloned embryos for the conservation of endangered felid species.

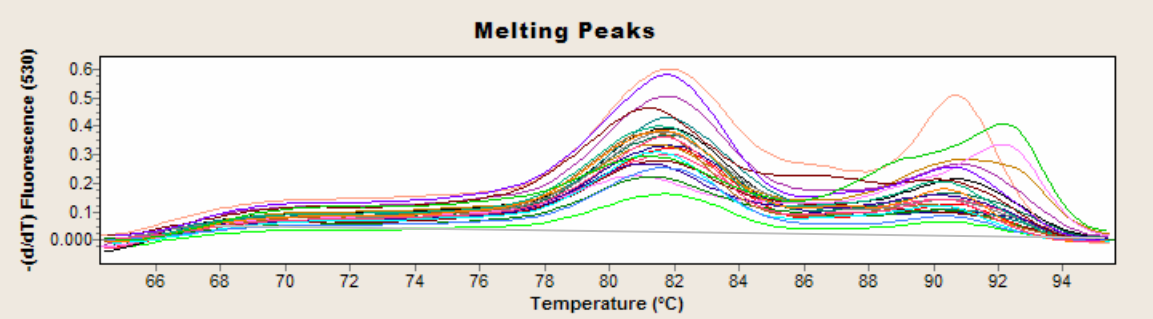
APPENDIX

Figure 1. A representative validation curve of real time PCR for β-actin gene.

(A) The amplification curve for the β-actin gene.



(B) The dissociation curve of PCR products of β-actin gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for β-actin primer validation, $E = 10^{(-1/\text{slope})}$.

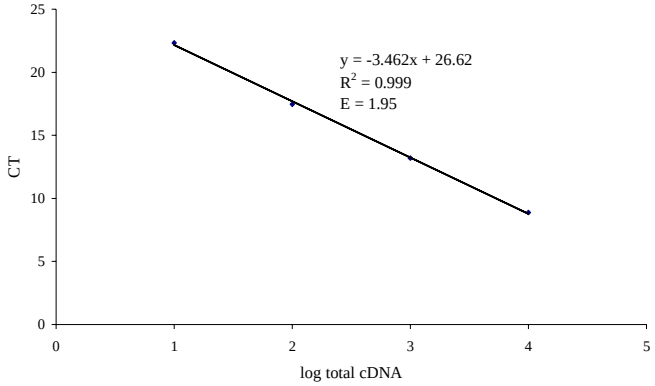
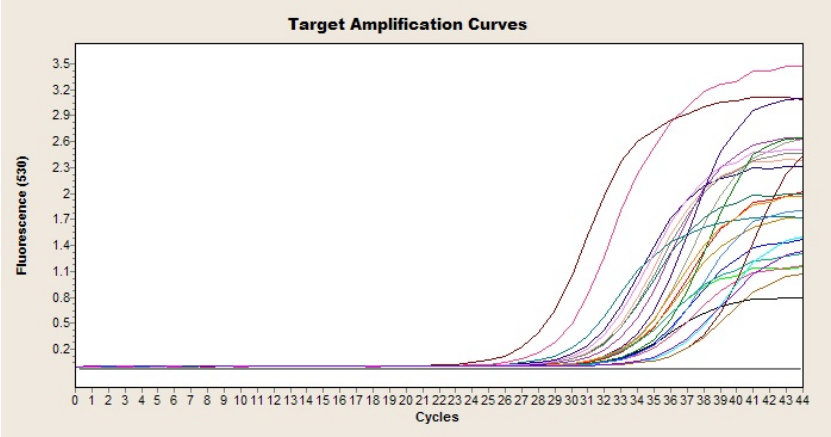
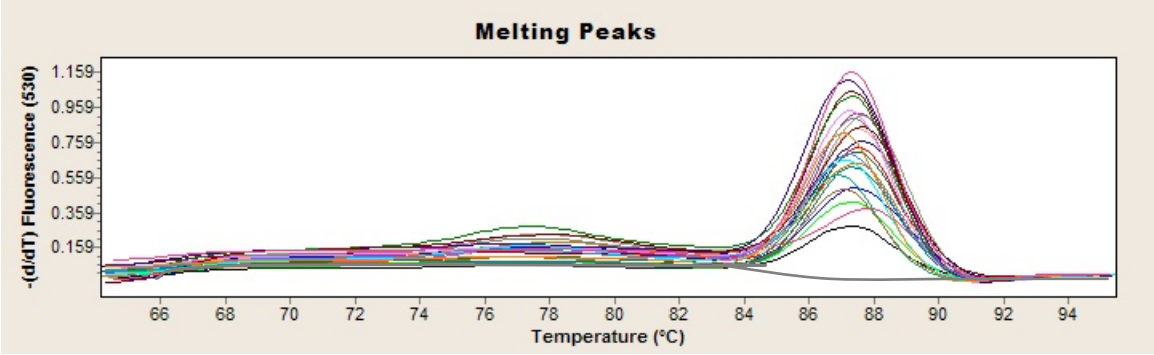


Figure 2. A representative validation curve of real time PCR for Oct4 gene.

(A) The amplification curve for the Oct4 gene.



(B) The dissociation curve of PCR products of Oct4 gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for Oct4 primer validation, $E = 10^{(-1/\text{slope})}$.

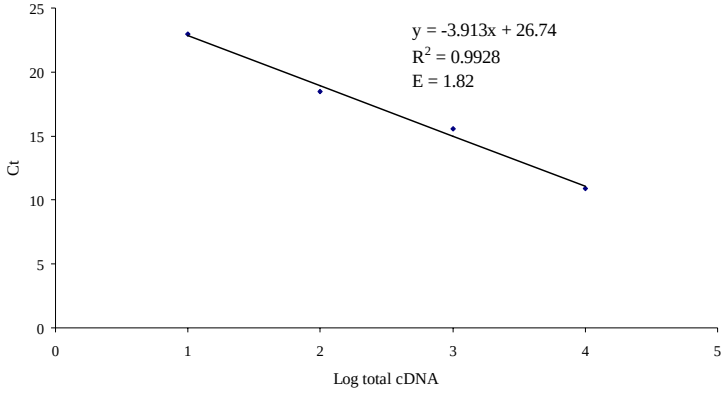
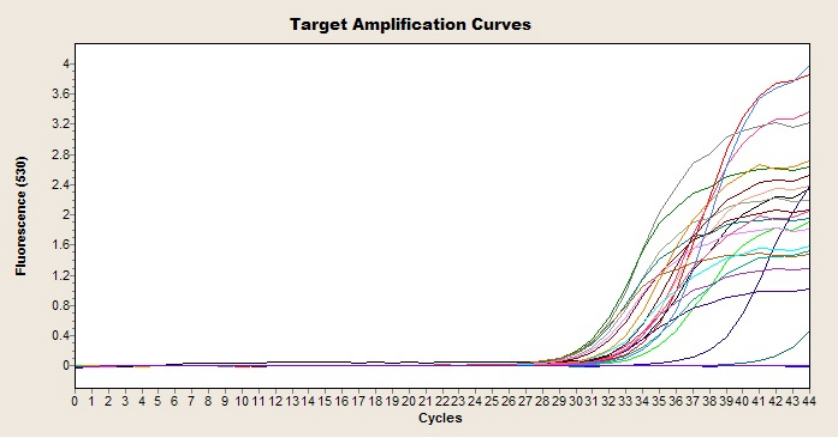
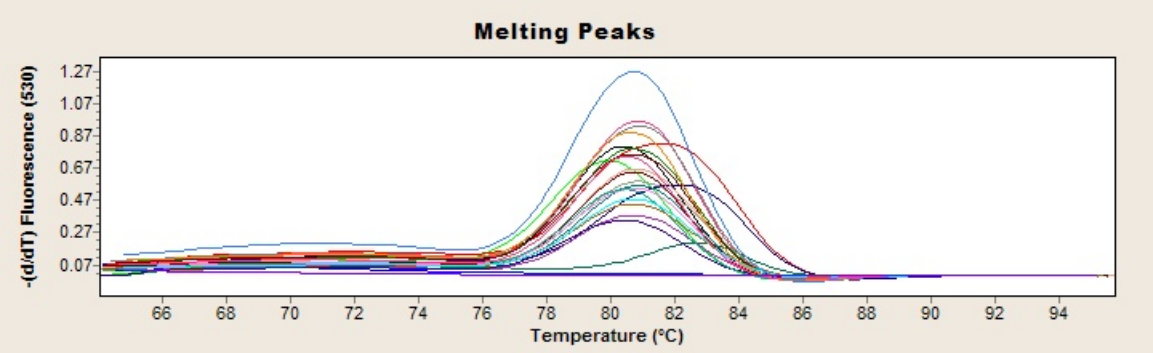


Figure 3. A representative validation curve of real time PCR for DNMT1 gene.

(A) The amplification curve for the DNMT1 gene.



(B) The dissociation curve of PCR products of DNMT1 gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for DNMT1 primer validation, $E = 10^{(-1/slope)}$.

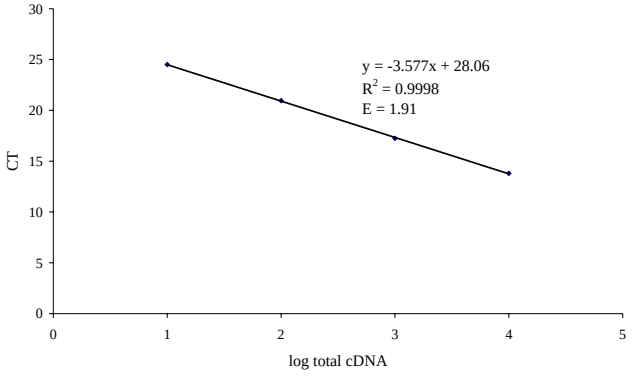
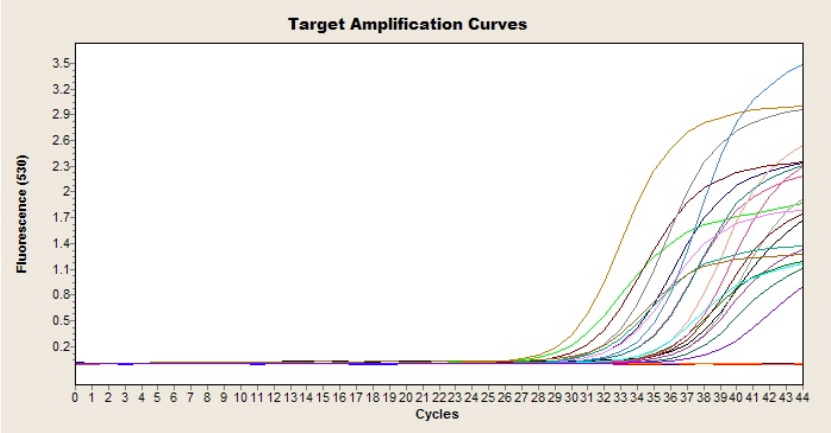
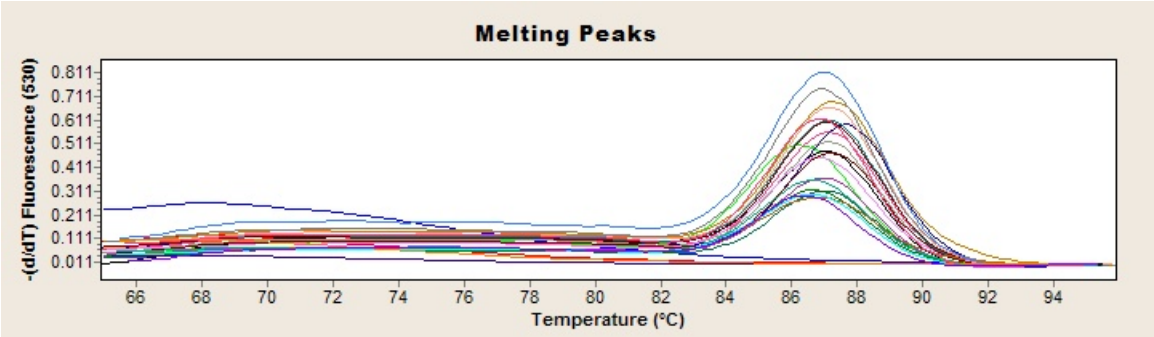


Figure 4. A representative validation curve of real time PCR for DNMT3a gene.

(A) The amplification curve for the DNMT3a gene.



(B) The dissociation curve of PCR products of DNMT3a gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for DNMT3a primer validation, $E = 10^{(-1/\text{slope})}$.

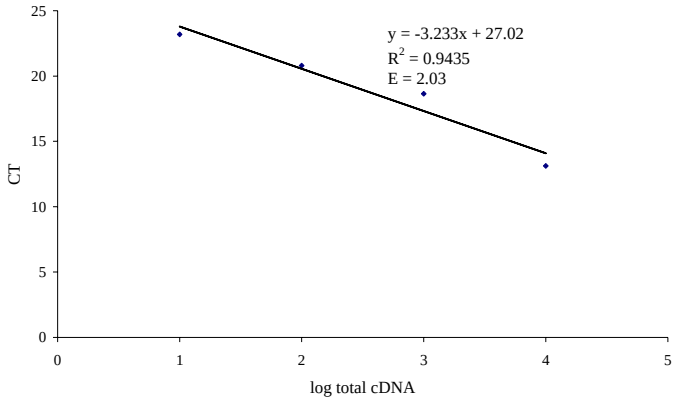
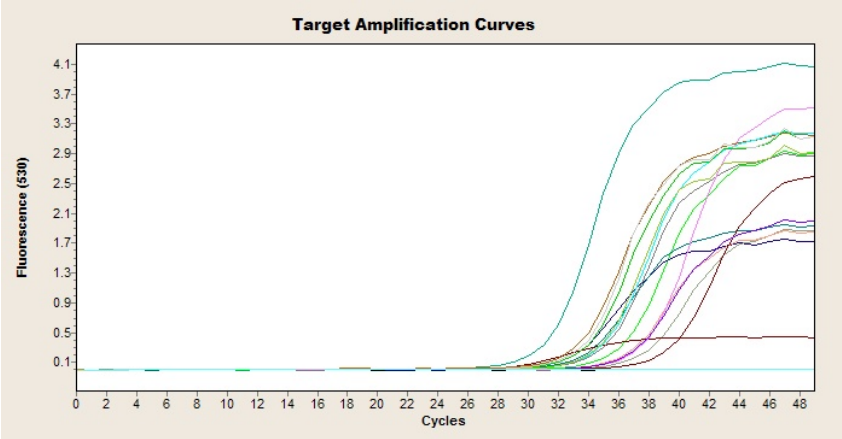
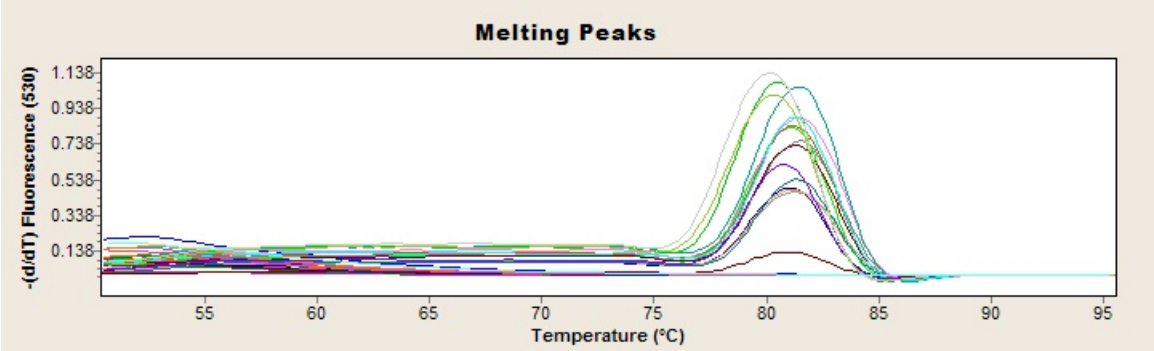


Figure 5. A representative validation curve of real time PCR for DNMT3b gene.

(A) The amplification curve for the DNMT3b gene.



(B) The dissociation curve of PCR products of DNMT3b gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for DNMT3b primer validation, $E = 10^{(-1/slope)}$.

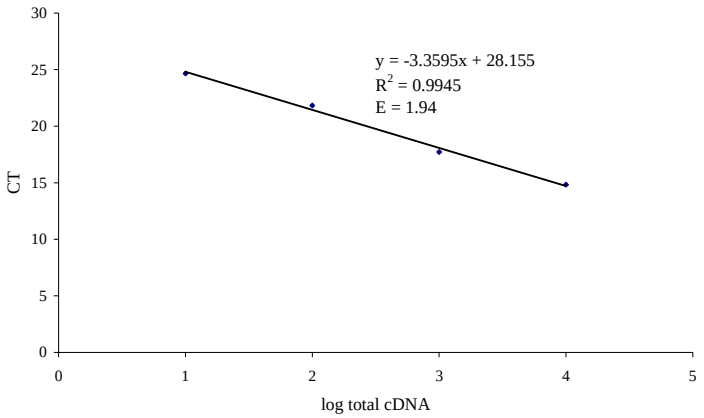
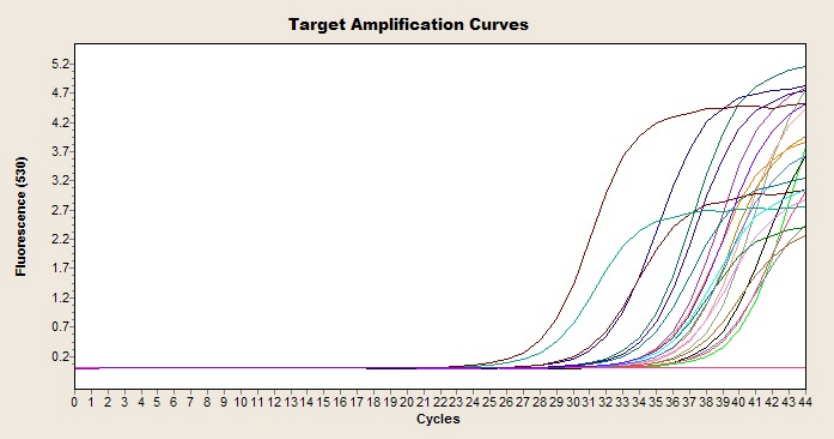
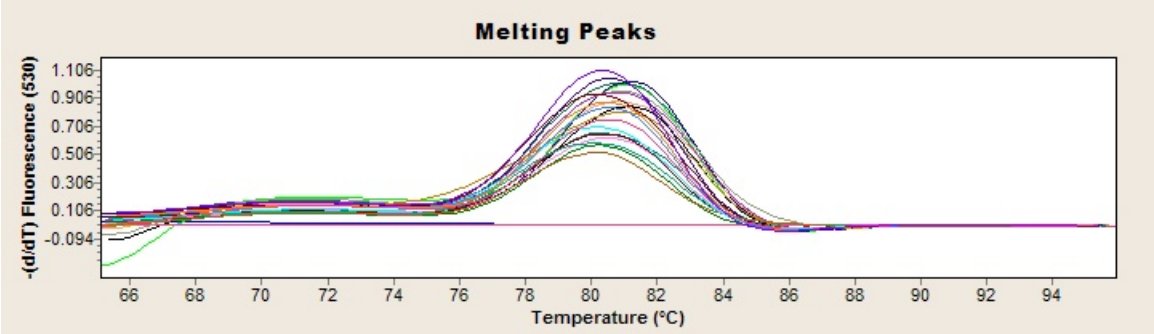


Figure 6. A representative validation curve of real time PCR for HAT1 gene.

(A) The amplification curve for the HAT1gene.



(B) The dissociation curve of PCR products of HAT1 gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for HAT1 primer validation.

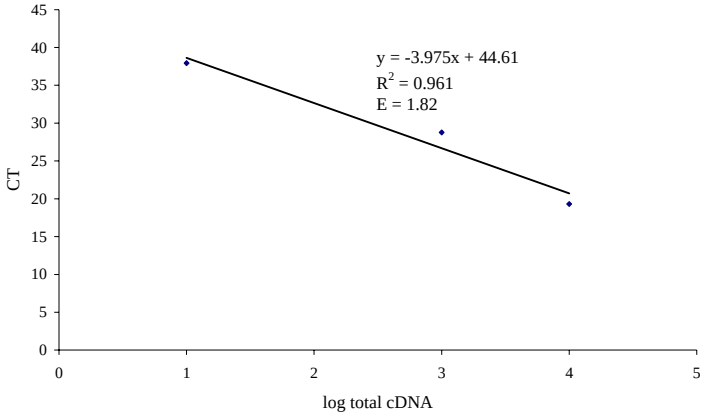
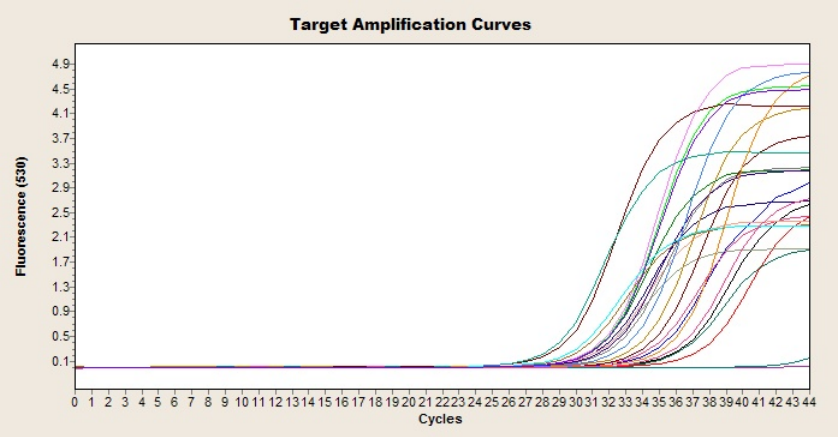
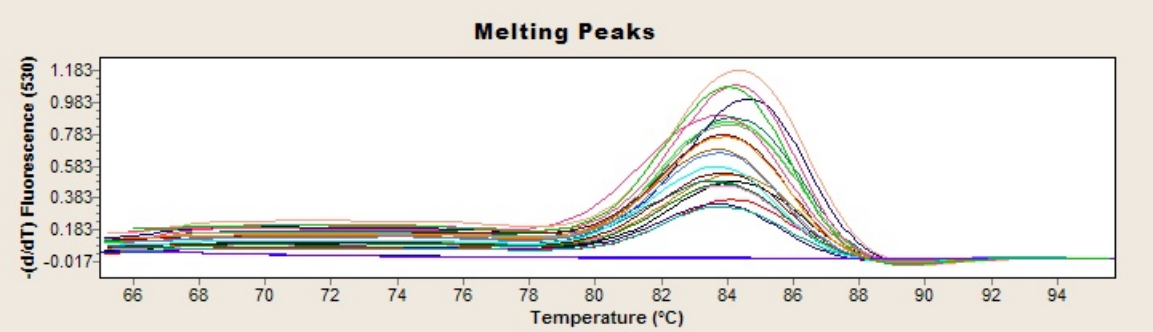


Figure 7. A representative validation curve of real time PCR for HDAC1

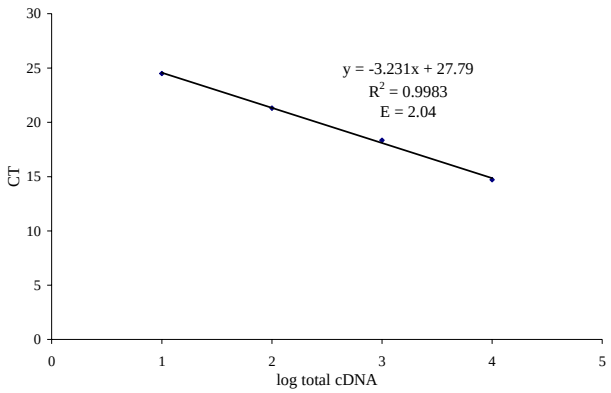
(A) The amplification curve for the HDAC1gene.



(B) The dissociation curve of PCR products of HDAC1 gene.



(C) The linear regression curve to determine the slope which corresponded to the amplification efficiency for HDAC1 primer validation, $E = 10^{(-1/\text{slope})}$.



Comparative C_T Method (Livak and Schmittgen, 2001)

Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. The $2^{-\Delta\Delta C}{}_T$ method may be used to calculate relative changes in gene expression determined from real time quantitative PCR experiments.

Derivation of the $2^{-\Delta\Delta C}{}_T$ method:

The equation that describes the exponential amplification of PCR is:

$$X_n = X_0 \times (E_X)^n \qquad (1)$$

where X_n is the number of target molecules at cycle n of the reaction, X_0 is the initial number of target molecules. E_X is the efficiency of target amplification, and n is the number of cycles. The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_0 \times (E_X)^{C_{T,X}} = K_X \qquad (2)$$

where X_T is the threshold number of target molecules, $C_{T,X}$ is the threshold cycle for target amplification, and K_X is a constant. A similar equation for the endogenous reference (internal control gene) reaction is

$$R_T = R_0 \times (E_R)^{C_{T,R}} = K_R \qquad (3)$$

Where R_T is the threshold number of reference molecules, R_0 is the initial number of reference molecules, E_R is the efficiency of reference amplification, $C_{T,R}$ is the threshold cycle for reference amplification, and K_R is a constant.

Dividing X_T by R_T gives the expression

$$\frac{X_T}{R_T} = \frac{X_0 \times (E_X)^{C_{T,X}}}{R_0 \times (E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K \qquad (4)$$

The exact values of X_T and R_T depend on a number of factors, including: reporter dye used in the probe, sequence context effects on the fluorescence properties of the probe, efficiency of probe cleavage, purity of the probe, setting of the fluorescence threshold. Therefore, the constant K does not have to be equal to one.

Assuming efficiencies of the target and the reference are the same:

$$E_X = E_R = E$$

$$\frac{X_0}{R_0} \times (E_X)^{C_{T,X} - C_{T,R}} = K \qquad (5)$$

$$X_N \times (E)^{\Delta C_T} = K \qquad (6)$$

where X_N is equal to the normalized amount of target (X_0 / R_0) and ΔC_T is equal to the difference in threshold cycles for target and reference ($C_{T,X} - C_{T,R}$).

Rearranging gives the expression

$$X_N = K \times (E)^{-\Delta C_T} \qquad (7)$$

The final step is to divide the X_N for any sample q by the X_N for the calibrator (cb):

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (E)^{-\Delta C_{T,q}} R_T}{K \times (E)^{-\Delta C_{T,cb}} R_T} = (E)^{-\Delta\Delta C_T} \qquad (8)$$

Here $\Delta\Delta C_T = - (\Delta C_{T,q} - \Delta C_{T,cb})$

The efficiency (corresponding PCR efficiency, E of one cycle in the exponential phase was calculated according to equation: $E = 10^{(-1/slope)}$) is close to two. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by:

$$\text{amount of target} = 2^{-\Delta\Delta C_T} \qquad (9)$$

BIOGRAPHY

Mr. Sumeth Imsoonthornruksa was born on June 11, 1981 in Bangkok. He obtains his Bachelor of Science degree in Biotechnology form the Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silapakorn University Sanamchandra Palace Campus, Nakorn Pathom, in 2003. After graduation, he worked as a research assistant in the Embryo Technology and Stem Cell Research Center, Suranaree University of Technology (SUT) with Dr. Rangsun Parnpai. During this time, he has chances to practice skills in the field of animal biotechnology including animal cloning, *in vitro* fertilization, embryos freezing and molecular biology techniques. In 2004, he decided to study master degree in the field of animal biotechnology. While study, he received research assistant scholarship form Dr. Rangsun Parnpai, Thailand Research Fund-Master Research Grants (Grant No. MRG-WII495S021) and SUT for financial support. His research topic was Expression of genes crucial for preimplantation of feline embryos development after nuclear transfer. The results from some part of this study have been presented as a poster presentation at (1) The 3rd Annual Conference of Asian Reproductive Biotechnology Society (ARBS) in Hanoi, Vietnam, November 29 - December 3, 2006. (2) The 33rd Annual Conference of the International Embryo Transfer Society, Kyoto, Japan, January 6-10, 2007. (3) The 4th Annual Conference of ARBS in Singapore, November 24-26, 2007.