THE EPIGENETIC PROFILES OF GAUR INTER-SPECIES SOMATIC CELL NUCLEAR TRANSFER EMBRYOS USING DIFFERENT DONOR CELL GENDER

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รูปแบบของอิพิเจเนติกของตัวอ่อนกระทิงโคลนนิ่งข้ามสปีชีย์โดยเซลล์ต้นแบบที่มีเพศแตกต่างกัน

นางสาวสุธาทิพย์ จิรันดร

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สุธาทิพย์ จิรันดร : รูปแบบของอิพิเจเนติกของตัวอ่อนกระทิงโคลนนิ่งข้ามสปีชีย์โดยเซลล์
ต้นแบบที่มีเพศแตกต่างกัน (THE EPIGENETIC PROFILES OF GAUR INTER -SPECIES
SOMATIC CELL NUCLEAR TRANSFER EMBRYOS USING DIFFERENT DONOR
CELL GENDEN) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 86 หน้า.

การย้ายฝากนิวเคลียสข้ามสายพันธุ์ (iSCNT) เป็นเทคนิคนี้ใช้สำหรับการผลิตตัวอ่อนโคลน
ข้ามสายพันธุ์เพื่อการอนุรักษ์สัตว์ใกล้สูญพันธุ์โดยใช้เซลล์ร่างกายเป็นเซลล์ต้นแบบและย้ายเข้าสู่ไข่
ที่ถูกกำจัดนิวเคลียสของสายพันธุ์ที่ใกล้ชิดหรือสายพันธุ์ที่ต่างออกไป อย่างไรก็ตามเทคนิค SCNT
ยังคงมีประสิทธิภาพต่ำมากซึ่งส่งผลทำให้การพัฒนาของลูกอ่อนในครรภ์เกิดความผิดปกติ โดยการ
แสดงออกของการเปลี่ยนแปลงที่ผิดปกติของอิพีเจเนติก (epigenetic) ในนิวเคลียสของเซลล์ร่างกาย
ดังนั้นการศึกษานี้ จึงได้ทำการตรวจสอบการเจริญของตัวอ่อนและการแสดงออกของยีน
pluripotency และ epigenetic modification ของตัวอ่อนที่ได้จาก SCNT และ iSCNT ที่ได้จากเซลล์
ต้นแบบที่มีเพศแตกต่างกันโดยใช้ตัวอ่อนที่ได้จาก IVF เป็นกลุ่มควบคุม โดยศึกษาการเจริญของตัว
อ่อนที่ได้จากเซลล์ไฟโบรบลาสเพศผู้และเพศเมียของโคและกระทิงย้ายเข้าสู่ไข่โคที่พร้อมปฏิสนธิ
ที่ได้ถูกกำจัดนิวเคลียส นอกจากนี้ทำการวิเคราะห์การแสดงออกของยีน pluripotent (*Oct4*) และยีน
ที่แสดงออกของรูปแบบ epigenetic (*Hat1, Hdac1, Dnmt1, Dnmt3a, Dnmt3b, Igf2* และ *Igf2r*) ใน
ตัวอ่อนเหล่านี้อีกทั้งทำการวิเคราะห์การแสดงออกของ Histone modifications (AcH4K5 and
HDAC1) ด้วยวิธี immunostaining ในการทดลองที่ 1 พบว่าอัตราการเจริญของตัวอ่อนโคลนนิ่งถึง
ระยะบลาสโตซิสในกลุ่มตัวอ่อนที่ได้จากเซลล์ต้นแบบโคเพศผู้และเพศเมีย (25% และ 23.1%
ตามลำดับ) และกลุ่มตัวอ่อนที่ได้จากเซลล์ต้นแบบกระทิงเพศผู้และเพศเมีย (27.5% และ 25.8%
ตามลำดับ) มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับตัวอ่อน IVF ที่เจริญถึง
ระยะบลาสโตซิส (P<0.05) นอกจากนี้จำนวนเซลล์ของ inner cell mass (ICM) และ trophectoderm
(TE) ที่ได้จากตัวอ่อนโคลนนิ่งทั้งสองกลุ่มมีน้อยกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับ
กลุ่มตัวอ่อนที่ได้จาก IVF   (P<0.05) ในขณะที่อัตราการเจริญของตัวอ่อนถึงระยะบลาสโตซิส
จำนวนเซลล์ของ ICM TE และเซลล์ทั้งหมดในกลุ่มตัวอ่อน SCNT และ iSCNT ที่ใช้เซลล์ต้นแบบที่
มีเพศแตกต่างกันไม่มีความแตกต่างกันทางสถิติ (P>0.05) การทดลองที่2 จากการศึกษาพบว่าระดับ
ของยีน *Oct4* และ *Hat1* มีการแสดงออกที่ลดลงอย่างมาก ในขณะที่การแสดงออกของยีนที่เกี่ยวข้อง
กับ *de novo* DNA methylation (*Dnmt3a* และยีน *DNMT3b*) มีการแสดงออกที่เพิ่มขึ้นอย่างมี
นัยสำคัญทางสถิติ (P<0.05) ในกลุ่มตัวอ่อน SCNT และ iSCNT เมื่อเปรียบเทียบกับกลุ่มตัวอ่อนที่
ได้จาก IVF นอกจากนี้ระดับการแสดงออกของยีนในกลุ่มตัวอ่อน SCNT และ iSCNT มีรูปแบบการ

แสดงออกที่เหมือนกันของยีน *Oct4, Hat1, Hdac1* และ *Dnmt3a* ที่ระยะบลาสโตซิส และสิ่งที่
น่าสนใจคือการแสดงออกของยีนในกลุ่ม imprintingคือ ยีน *Igf2* และ ยีน *Igf2r* นั้น มีการแสดงออก
ที่แตกต่างกันอย่างมีนัยสำคัญทางสถิติในกลุ่มตัวอ่อน SCNT และ iSCNT ที่ใช้เซลล์ต้นแบบที่มีเพศ
แตกต่างกัน (P<0.05) ในการทดลองที่ 3 พบว่าระดับการแสดงออกของ histone acetylation ของ
ระดับ AcH4K5 มีระดับต่ำในกลุ่มตัวอ่อน iSCNT ที่ใช้เซลล์ไฟโบรบลาสของกระทิงเพศผู้เป็นเซลล์
ต้นแบบเมื่อเปรียบเทียบกับตัวอ่อนที่ระยะบลาสโตซิสที่ได้จาก IVF ขณะที่ระดับการแสดงออกของ
HDAC1 ในกลุ่มตัวอ่อนโคลนนิ่งทั้งสองกลุ่มโดยเฉพาะอย่างยิ่งในกลุ่มตัวอ่อน iSCNT ที่ใช้เซลล์
ไฟโบรบลาสของกระทิงเพศผู้เป็นเซลล์ต้นแบบมีการแสดงออกที่เพิ่มขึ้นในกลุ่มตัวอ่อน IVF ดังนั้น
ในการศึกษาครั้งนี้สรุปได้ว่าการเจริญของตัวอ่อนและการเปลี่ยนแปลงของ epigenetic ในกลุ่มตัว
อ่อนโคลนนิ่งที่ใช้เซลล์ต้นแบบที่มีเพศแตกต่างกันนั้นไม่มีความแตกต่างกัน แต่ในการแสดงออก
ของยีนในกลุ่มรูปแบบการฝังจำบนสายดีเอ็นเอ (imprinting gene) ในกลุ่มตัวอ่อน SCNT และ
iSCNT ที่ใช้เซลล์ต้นแบบที่มีเพศแตกต่างกันนั้น มีผลกระทบอย่างมีนัยสำคัญทางสถิติต่อการ
แสดงออกของยีน *Igf2* และ *Igf2r* ดังนั้นจีโนมของนิวเคลียสเซลล์ร่างกายที่มาจากเซลล์ต้นแบบที่มี
เพศแตกต่างกันใน SCNT และ iSCNT จะมีการแสดงออกของยีนรูปแบบการฝังจำบนสายดีเอ็นเอที่
ผิดปกติซึ่งมีผลกระทบต่อการเจริญของตัวอ่อน โคลนนิ่งทำให้เกิดความผิดปกติในตัวอ่อนระยะหลัง
การฝังตัว

สาขาวิชาเทคโนโลยีชีวภาพ   ลายมือชื่อนักศึกษา_____
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SUTATIP JIRUNDORN : THE EPIGENETIC PROFILES OF GAUR INTER-SPECIES SOMATIC CELL NUCLEAR TRANSFER EMBRYOS USING DIFFERENT DONOR CELL GENDER. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 86 PP.

INTRA-SPECIES SCNT (SCNT)/INTER-SPECIES SCNT (iSCNT)/SOMATIC CELL NUCLEI/DONOR CELL GENDER/EPIGENETIC MODIFICATION

Interspecies somatic cell nuclear transfer (iSCNT), is a technique for producing inter-species cloned embryos which are applied in endangered species using a somatic cell as a donor cell and transferred into the enucleated oocyte of a closely related species or different species. However, the SCNT technique is still extremely inefficient leading to abnormality of fetus development caused by aberrant expression of epigenetic modification in somatic cell nuclei. Thus, these studies investigated the different donor cell gender on embryonic development and transcript abundance of pluripotency and epigenetic modification genes in SCNT bovine and iSCNT gaur embryos. Embryos derived from *in vitro* fertilization (IVF) were used as the control group. Male and female bovine and gaur fibroblasts were transferred into matured enucleated bovine oocytes and the developmental competence of reconstructed embryos was investigated. Furthermore, the pluripotent (*Oct4*) and epigenetic genes (*Hat1, Hdac1, Dnmt1, Dnmt3a, Dnmt3b, Igf2* and *Igf2r*) of these embryos were analyzed. Histone modifications (AcH4K5 and HDAC1) were analyzed by the immunostaining method. In Experiment 1, the blastocysts formation rates of cloned embryos were significantly decreased in SCNT (male SCNT and female SCNT; 25% and 23.1%, respectively) and iSCNT (male iSCNT and female iSCNT; 27.5% and 25.8%, respectively) embryos when compared with IVF embryos (34.6%, $P < 0.05$).

The cell numbers of the inner cell mass (ICM) and trophectoderm (TE) of SCNT and iSCNT embryos were significantly decreased compared with IVF embryos ($P<0.05$), whereas the blastocyst rate and the ICM, TE and total cell numbers in SCNT and iSCNT embryos with different donor cell gender were not significantly different ($P>0.05$). In Experiment 2, the mRNA level of *Oct4* and *Hat1* activity was dramatically lower; whereas the expression of *de novo* DNA methylation (*Dnmt3a*, and *DNMT3b*) was significantly increased in SCNT and iSCNT blastocysts reconstructed with different donor cell gender when compared with IVF blastocysts ($P<0.05$). Moreover, the relative transcription levels in SCNT and iSCNT blastocysts had similar expression patterns of *Oct4, Hat1, Hdac1* and *Dnmt3a* genes. Interestingly, the transcription levels of *Igf2* and *Igf2r* genes in SCNT and iSCNT blastocysts derived from different donor cell gender were significantly different ($P<0.05$). In Experiment 3, the histone acetylation levels of AcH4K5 were downregulated in iSCNT male gaur blastocysts compared with IVF control group; whereas the relative HDAC1 activity in all cloned embryos remarkably increased, especially in iSCNT male gaur compared with the IVF blastocysts control group. From this study, it can be concluded that the embryonic development and epigenetic modification of different somatic donor cell genders did not have a significant influence, except the *Igf2* and *Igf2r* imprinting genes expression did have an effect on SCNT and iSCNT embryos derived from the different donor cell gender. Thus, the somatic cell nuclei genome derived from different of donor cell gender in SCNT and iSCNT embryos would have an aberrant expression of imprinting genes which causes abnormality cloned embryos at post-implantation development.

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Academic Year 2017   Advisor's Signature

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ART	Assisted reproductive technique
BSA	Bovine serum albumin
CB	Cytochalasin B
CD	Cytochalasin D
CHX	Cyclohexymide
CT	Cycle threshold
ºC	Degree celsius
COCs	Cumulus-oocyte complexes
Ø	Diameter
DCCC	Donor cell-cytoplast couplet
DNMT	DNA methyltransferase
FBS	Fetal bovine serum
HAT1	Histone acetyltransferase 1
HDAC1	Histone deacetyltransferase 1
ICM	Inner cell mass
iSCNT	Interspecies somatic cell nuclear transfer
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IGF2	Insulin-like growth factor 2
IGF2r	Insulin-like growth factor receptor 2

LIST OF ABBREVIATIONS (Continued)

LOS	Large offspring syndrome
mSOFaa	modified oviduct synthetic fluid with amino acids
SCNT	Somatic cell nuclear transfer
SEM	Standard error means
199H	Tissue culture medium 199 supplement with HEPES buffer
TE	Trophectoderm

CHAPTER I

INTRODUCTION

1.1 Significance of this study

In somatic cell nuclear transfer (SCNT), the donor nucleus of somatic cell is injected into enucleated oocyte to produce cloned embryo. SCNT technique has been applied to several mammalian species including rhesus monkey (Mitalipov et al. 2002), mouse (Wakayama et al., 1998), bovine (Kato et al., 1998), goat (Baguisi et al., 1999), pig (Onishi et al., 2000), rat (Hayes et al., 2001), cat (Shin et al., 2002), rabbit (Chesné et al., 2002), horse (Galli et al., 2003), mule (Woods et al., 2003), dog (Lee et al., 2005), water buffalo (Lu et al., 2005) and ferret (Li et al., 2006). Mammalian somatic cell cloning generated of genetically modified animals for agricultural and biomedical purposes. Gaur (Bos gaurus) is the largest species of wild bovine, which can be found in South Asia and Southeast Asia and the number of gaur has been decreasing due to wild meat hunting and hunting for horns trading. Interspecies SCNT (iSCNT) is the technique for producing inter-species cloned embryos which had been applied on endangered species (Soulé et al., 1991).

This technique transfers somatic cell into the enucleated oocyte which is a closely related species or another subspecies (Corley-Smith and Brandhorst, 1999). After transfer donor cell into enucleated recipient oocyte, the donor cell nuclei could be reprogrammed to full totipotency and initiate another round of embryonic development (Dominko et al., 1999). Therefore, the cellular reprogramming between

the somatic cell nuclei and oocyte cytoplasm is important for embryo development from iSCNT technique (Lagutina et al., 2013).

The successful of nuclear reprogramming in SCNT depends on the efficiency of oocyte cytoplasm component, which can be reprogrammed of the differentiated somatic cell nuclei, especially in the iSCNT embryos, which can be changed into a totipotent state. Several changes to epigenetic pattern and embryonic profile in cloned embryos to normal development beyond to tissue generation and organogenesis during post-implantation development (Cibelli et al., 2002; Yang et al., 2007). However, the incomplete reprogramming regularly occurs in cloned embryos might be due to an aberrant of gene expression and epigenetic modification pattern such as DNA methylation, histone modification, genomic imprinting and non-imprinted gene (Dean et al., 2001; Kang et al., 2001; Bourc'his et al., 2001; Daniels et al., 2000; Wrenzycki et al., 2001; Han et al., 2003), which resulting in the abnormality cloned embryo and offspring by faulty embryonic and fetal gene expression (Rideout et al., 2001; Xue et al., 2002) including low birth rate, placental dysfunctions and large offspring syndrome in cloned animals. Previous study was reported that the DNA methylation and gene expression patterns of donor cell have a significant effect on the early cloned embryos development (Santos et al., 2003). Several studies have reported the aberrance of imprinted reprogramming in many cloned embryos such as mouse, sheep and bovine embryos indicating the faulty expression of imprinted gene due to the incomplete reprogramming of epigenetic modification (Humpherys et al., 2001; Xue et al., 2002; Zhang et al., 2004).

The SCNT technology has an inefficiency to produce cloned offspring, which might be caused by the donor cell nucleus remains reprogramming.

The reprogramming of donor cell genome is an important step for the successful of SCNT technique. Various types of somatic cell have different phenotype and epigenetic profile, which will lead to the different reprogrammed and efficiency in SCNT embryonic development. Accordingly, several attempts have been made to improve efficiency in cloned animals by selecting appropriate donor cells nuclear. There are several types of somatic cells that used as donor cell in SCNT procedure such as cumulus (Kato et al., 2000), embryonic stem (ES) cells (Jeanisch et al., 2002), fetal and adult fibroblast (Arat et al., 2002), granulosa (Arat et al., 2001), myoblast (Gao et al., 2003), neurons (Zawada et al., 1998), and sertoli cells (Inoue et al., 2003). Moreover, several reports have been attempted to improve the efficiency of developmental competence in cloned embryos by using different types of donor cell or different gender of donor. There was no significant difference from different donor cell types of male and female origin on embryo development in cloned bovine (Kato et al., 1998), porcine (Yoo et al., 2017) and sheep (Hosseini et al., 2008) embryos. However, it was reported that the gene expression can be influenced by the reprogramming of donor cell genome on post-implantation development (Santos et al., 2002). Currently, limited information are available on this topic. Thus, in the present study, we investigated the effect of donor cell gender on iSCNT embryos by comparing the embryonic development, gene expression of epigenetic modification including pluripotency (*Oct4*), histone modification (*Hat1* and *Hdac1*), DNA methylation (*Dnmt1, Dnmt3a* and *Dnmt3b*) and imprinted genes (*Igf2* and *Igf2r*). As well as, the histone acetylation and histone deacetylation by using immunostaining of SCNT and iSCNT embryos with different gender of donor cell were compared for improving our knowledge of the relationship of embryonic development and

epigenetic modification of SCNT and iSCNT embryos derived from different of donor cell genders related to post-implantation developmental.

1.2 Research objectives

1. To examine the effects of donor cell gender on development and the cell number of the inner cell mass (ICM), trophectoderm (TE) and total cell number of SCNT bovine and iSCNT gaur embryos.

2. To investigate the effect of donor cell gender on gene expression and histone modification of SCNT bovine and iSCNT gaur embryos compare with IVF bovine embryos.

3. To evaluate the effects of donor cell gender on histone modification of SCNT bovine and iSCNT gaur embryos.

1.3 Scope and limitation of the study

1. The effects of different donor cell gender on embryo development of iSCNT, SCNT and IVF bovine embryos were examined. The inner cell mass (ICM), trophectoderm (TE) and total cell number in each blastocyst were determined.

2. The effect of different donor cell gender on gene expression of iSCNT, SCNT and IVF bovine embryos were examined by using the quantitative PCR (qPCR).

3. The effect of different donor cell gender on histone modification expression of iSCNT, SCNT and IVF bovine embryos were examined by using the immunostaining analysis.

CHAPTER II

LITERATURE REVIEWS

2.1 Gaur

Gaur (Bos gaurus), a member of the Bovidae family, is the largest species of wild bovine (male 700-1000 kg, female 550-700 kg) which is endangered species. The gaur can be found in Bangladesh, Bhutan, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, Thailand and Viet Nam and Sri Lanka (Duckworth et al., 2008). The number of gaur in Southeast Asia has been dramatically decreased because of hunting for meat and horns trading. In Thailand, gaur has been declared as the protected animal by Thai government and currently listed as vulnerable the IUCN Red List of Threatened Species (Duckworth et al., 2008). Gaur has a low fertility and illegal hunting (Johnston et al., 1994). Due to the limitation of oocytes and surrogate mothers in endangered species, iSCNT can facilitate the rescue of endangered species.

The reproductive technology could increase the number of gaur. In 1981, Stover and colleagues have successfully produced gaur offspring by transferred gaur embryos into bovine recipients. Moreover, IVF was applied in gaur production by using in vitro maturation and fertilization of gaur oocytes. After transferred 12 IVF-derived gaur blastocysts into 9 natural estrus bovine recipients, 3 recipients carried fetuses to term, with one recipient diagnosed as carrying twins. Two gaur offspring died within 26 h after birth and two gaur offspring still born (Johnston et al., 1994). Frozen-thawed gaur semen was used to AI estrus synchronized bovine and hybrid gaur-bovine calf was born (Junior et al., 1990).

Lanza and colleagues (2000) reported the first gaur calf born from iSCNT procedure by using gaur skin fibroblasts fused with enucleated bovine oocytes. Six hundred ninety-two reconstructed embryos developed to blastocysts at day 7 after in vitro culture (12%). Forty-four blastocysts were transferred into thirty-two bovine recipients. Eight recipients were pregnant (25%). Three fetuses from two recipients were removed early for tissue examination. Four recipients had spontaneous abortions and one recipient had a late-term abortion at day 202 of gestation. Only one recipient was developed to term. Noah, cloned gaur was born with 36 kg birth weight. The cloned gaur was healthy at birth but died 2 days after birth (Vogel, 2001). In 2007, Mastromonaco and colleagues reported that a gaur nucleus within a bovine cytoplasmic environment may not be properly capable of directing embryo development in the later pre-implantation stages leading to the poor blastocyst development. The developmental delayed, low cell numbers, aberrant apoptotic and related gene expression profiles were observed. In 2012, the male cloned gaur calf was born by using male gaur fibroblast as a donor cell with non TSA-treated. Nine of non TSA-treated embryos were transferred into three recipients. One recipient was pregnant (33.3%) and developed to term. The cloned gaur calf appeared healthy with normal cardiac rhythm but the tactile hair along the body was not well developed. Three hours after birth, the cloned gaur newborn showed sign of dyspnea and finally died at 12 h after birth (Srirattana et al., 2012).

2.2 Interspecies somatic cell nuclear transfer (iSCNT)

In current trends, some threatened or endangered vertebrate species will likely to be extinct (Corley-Smith and Brandhorst, 1999). The assistant reproductive technologies (ART) including AI, embryos transfer, IVF, gamete/embryo

micromanipulation, embryos sexing and semen sexing applied to restore and manage in endangered species (Srirattana et al., 2012); however, the developmental competence is quite low (Cibelli et al., 2014).

The iSCNT procedure has potential to produce endangered species animals by transferring a nucleus of donor cell from one species into enucleated oocyte cytoplasm from another species. The successful of iSCNT that could produce cloned embryos derived from iSCNT in bovidae family. After transferred embryos to bovine recipients led to pregnancies ingaur (Lanza et al., 2000), banteng (Sansinena et al., 2005) and yak (Li . et al., 2006). However, live birth of one gaur and two banteng calves were obtained but one gaur and two banteng died after birth (Vogel et al., 2001, Sansinena et al., 2005). The successful production of African wild cat iSCNT using skin of African wild cat as donor cells and domestic cat oocytes as recipient cytoplasts resulted in a total of seventeen cloned kittens born, seven were stillborn, eight died within hours of delivery or up to 6 weeks of age, and two were alive and healthy (Go´mez et al., 2004). Moreover, the production of gray wolves from iSCNT by using the wolf as donor cells and domestic dog oocyte as recipient cytoplasts has obtained two cloned gray wolves (Kim et al., 2007). An extinct mountain goat, which is called bucardo (Capra pyrenaica pyrenaica) were produced by using bucardo fibroblasts as donor cells reconstructed with enucleated domestic goat oocytes. Only one live birth cloned bucardo was obtained but it died few minutes after birth due to a physical defect in the lungs (Folch et al., 2009). On the other hand, the production of iSCNT embryos were not capable of cloned embryo development to blastocyst stage in llama (Sansinena et al., 2003), whale (Ikumi et al., 2004), cat (Thongphakdee et al., 2008), crab-eating monkey (Lorthongpanich et al., 2008), chimpanzee (Wang et al., 2009).

In iSCNT procedure must be use it is capable of reprogramming the somatic cell nucleus into totipotent stage and forming an embryo as the bovine, sheep, and rabbit cytoplasm (Beyhan et al., 2007). From previous reports, they found that the bovine and porcine cytoplasm of oocytes could support nucleoli formation in xeno-nuclei (Dominko et al., 1999). Moreover, cytoplasm of bovine oocytes are able to support nucleoli formation in the nuclei of closely related species such as domestic sheep (Dominko et al., 1999) indicated that the cytoplasm of bovine oocyte has efficiency of cellular reprogramming with donor cell nucleus. Thus, the cytoplasm of bovine oocyte can facilitate to use because the ovaries from local slaughterhouse are accessible for use in experiment. Additionally, the knowledge of in vivo and in vitro development of bovine embryos is comprehensible. The bovine cytoplasm has so far played an important role of iSCNT for production of blastocysts (Chen et al., 2003) that are called "universal recipients" for iSCNT.

2.3   Fibroblast donor cell

The somatic cell nuclear transfer (SCNT) technology has low efficiency for produced cloned animal development due to the ability of reprogramming in the donor nucleus remains to be a major issue. However, many attempts were made to find an appropriate type of donor cells to achieve success outcome in SCNT. Generally, the fibroblastss are most popular as the donor cell nucleus in somatic cell cloning because of the readily available and well maintained under *in vitro* culture.

Fibroblastss are the most common cell in animal connective tissue that has the ability to synthesize the extra cellular matrix and collagen that which has the active form of fibrocyte are large, flat, elongated spindle-like cells which possess many processes extending out from its body that forming of connective tissue.

Moreover, the fibroblastss are produce collagen, and all component of extracellular matrix, the ground substance which is an amorphous gel-like matrix fills the spaces between cells in tissue and variety of fibers. As well as, which are appear to play important role in wound healing. In embryo, fibroblasts, like any type of connective tissue cells, are derived from primitive mesenchyme. In addition, the fibroblasts were used to be the source of nucleus in studies related to mammalian aging which could reverse cellular ageing by SCNT. Adult fibroblast was also used by a study for cloning that compared between efficiency of donor cells in SCNT. Cells were collected from mouse tail-tip tissue at 2-4 months age, the percentage of developed offspring per embryo transferred (ET) in male had been 1.9% and 0.7% in female, this percentage is quite efficient compared to other cell types used (1.5% for cumulus cell and 4.7% for sertoli cell) (Inoue et al., 2006). Previous study, the fibroblast was used in cloning related to telomere length and telomerase activity to investigate the effect of donor cells in SCNT efficiency, although the nucleus to be transferred is senescent; effectively normal healthy animals were cloned with elongation in their telomere length and extension of life-span (Lanza et al., 2000). The efficiency of developmental rate of offspring cloned from adult fibroblastss were less than that of immature sertoli cells and cumulus cells (Inoue et al., 2006). Fetal fibroblast efficacy is also less than mesenchymal stem cells (Jin et al., 2007; Kumar et al., 2007) but it had proved to be more efficient than other cells such as mammary epithelial cells (Wilmut et al., 1997).

2.4   Gene expression in clone embryos of iSCNT

The important factor of cloned embryo from the SCNT depends on the reprogramming of somatic cell nucleus that affected on the efficiency of cloned embryo development. In addition, the somatic cells from different tissue, different cell type and different age will have reprogrammed and affected to embryo development differently. Thus, the reprogramming of donor cell type is significant for iSCNT, which result in the incomplete embryos development (Cho et al., 2002). Unlike the conventional offspring, cloned animals are less likely to survive. In cloned embryos, they have a correct activation of genes for early embryonic development and suppressed differentiation-associated genes that were transcribed in the original donor cell. Meanwhile, the iSCNT embryos should be reprogrammed to the expression of embryonic genes and the silencing of somatic cell (Canovas and Cibelli, 2014).

In iSCNT technique, the efficiency depends on the compatibility between donor cell nucleus and recipient cytoplasm originates from different species. The activation of embryo nuclei support the genetic information of one species that depend on the proteins and transcripts in the recipient cytoplasm derived from another species during the maternal embryonic transition (MET) (Minami et al., 2007). This can effect on the gene transcription and the absence or the species incompatibility that leads to block the transcription (Heix et al., 1997).   However, the donor cells nuclei were incomplete reprogramming that caused the silencing of somatic genes, it could affect to the survival rates (Niemann et al., 2002). Moreover, reprogramming events in zygotic activation are also different among animal species (Arat et al., 2003).

Embryo development in IVF and SCNT embryo is supported by embryonic genomic activation (EGA) that is supported by stored proteins and maternal transcripts present in the oocytes (Schultz, 1993). The iSCNT embryos stop development when

EGA occurs and exhibit defective EGA and improper silencing of somatic genes (Wang et al., 2011) In addition, the gene expression pattern of pluripotent genes depend on EGA. During the cleavage process, the EGA activate cellular differentiation and segregation of developmental lineage at the end of cleavage leading to formation of blastocysts (Nichols et al., 1998).

The inner cell mass (ICM) are cells that have the capability to develop into pluripotent progenitors of nontrophoblast extraembryonic tissues and all of fetal cell types including germ cells. Blastomeres can distinguish the expression of pluripotent in early embryo cells by the POU factor Oct4. *Oct4* is regulated expression of multiple genes (Saijoh et al., 1996) that can act to activate and to repress target gene transcription (Lenardo et al., 1989). Moreover, the fibroblast growth factor-4 (*FGF4*) has been demonstrated to respond as a co-expressed with *Oct4* in the ICM and epiblast (Ma et al., 1992). The current study, the reprogramming and gene expression in the transcription pattern of *Oct4* and *FGF4* in embryos derived from IVF and SCNT embryos using granulosa cells as donor cell nucleus were investigated. The *Oct4* gene showed similar patterns in comparison between IVF and SCNT embryos during pre-implantation stage. Nevertheless, the morula and blastocyst stage embryos derived from SCNT showed abnormal transcription in *FGF4* gene that demonstrate the pluripotent genes have different expression in different technique for embryos production (Daniels et al., 2000).

Moreover, the *Oct4* gene can express in ICM that have segregation of lineages divides into the primitive ectoderm (epiblast) and the primitive endoderm (PE) that forms the extra-embryonic endoderm layer, which can detected by expression of Nanog and Gata6 genes. The Nanog-positive cells are determined the by epiblast and the PE that depends on species determines Gata6-positive cells. (Kuijk et al., 2008).

At the compacted morula stage the segregation has occurred, the outer layer of cells formed the trophectoderm (TE) in blastocyst stage. But the gene expression of early morula results in cdx2-expressing and *Oct4*-expressing cells (Kuijk et al., 2008). In addition, the gene expression pattern of pluripotent genes from iSCNT embryos depends on EGA that can express during mainly down regulated. Chung and colleagues (2009) found the pluripotency-related genes as *Oct4, Nanog* and *SOX2*, which are express in iSCNT embryo using human cells and oocytes from cows and rabbits. However, the Oct4 and Nanog gene are express in rhesus-bovine iSCNT embryos (Wang et al., 2011). Pluripotent genes in iSCNT embryos have shown the lack of somatic cell silencing associated genes (Stadtfeld and Hochedlinger, 2010). Nonetheless, during iSCNT, the reactivation of *Oct4, Nanog* and some somatic genes remain expressed (Wang et al., 2011). In 1995, Christian et al investigated the gene expression of iSCNT embryos derived from the bovine cytoplasm and mouse fibroblast nuclei at the 8-cell stage of embryo development. The *Oct4* gene associated with the bovine recipient cytoplasm and non-reprogrammed donor cell nucleus of a mouse in iSCNT (Arat et al., 2003). This result is in agreement with Lagutina et al. (2013) that reported the bovine cytoplasm can block *de novo* transcription of a fibroblast-specific gene of the donor nucleus that demonstrated the absence of transcription of *COL6A1* gene. Moreover, the aberrant gene expression pattern was found in iSCNT offspring due to transcriptional gene silencing in the donor somatic cell (Imsoonthronruksa et al., 2010).

In conclusion, the majority of cloned animal derived from SCNT is cloned embryos die after implantation because of cloned embryos are lack pluripotent genes, which play an important role in control of development pluripotency, develop to the blastocyst stage and also die after implantation (Bortvin et al., 2003). Thus, aberrant

gene expression is frequently observed in cloned embryos which probably results in abnormal development and contributes to early loss of cloned fetuses (Daniels et al., 2000).

2.5   Epigenetic reprogramming in cloned embryos

The major problem of endangered species offspring from iSCNT is the incomplete of epigenetic reprogramming (Yan Jun et al., 2014). The capability of donor cell reprogramming is necessary to achieve successfully in SCNT. Furthermore, the stage of donor cell cycle is associated with the success of SCNT. The difference of donor cell types is the factor of the efficiency of reprogrammed. In cloned embryos, the expression profile of a differentiated cell is discontinued and generated a new embryo-specific expression profile, which drives embryonic and fetal development (Niemann et al., 2008). Form these results, the epigenetic reprogramming in cloned embryos that consist of pre-zygotic and post-zygotic expression patterns that caused the reprogramming which involves the removal of epigenetic marks from the nuclear DNA and provides another set of epigenetic mark into the DNA that are called the epigenetic modification including DNA methylation, histone modification, X-chromosome inactivation and imprinted genes (Hochedlinger and Jaenisch, 2003). Most of epigenetic modification is important for embryonic development and involved in establishing patterns of gene repression during embryo development.
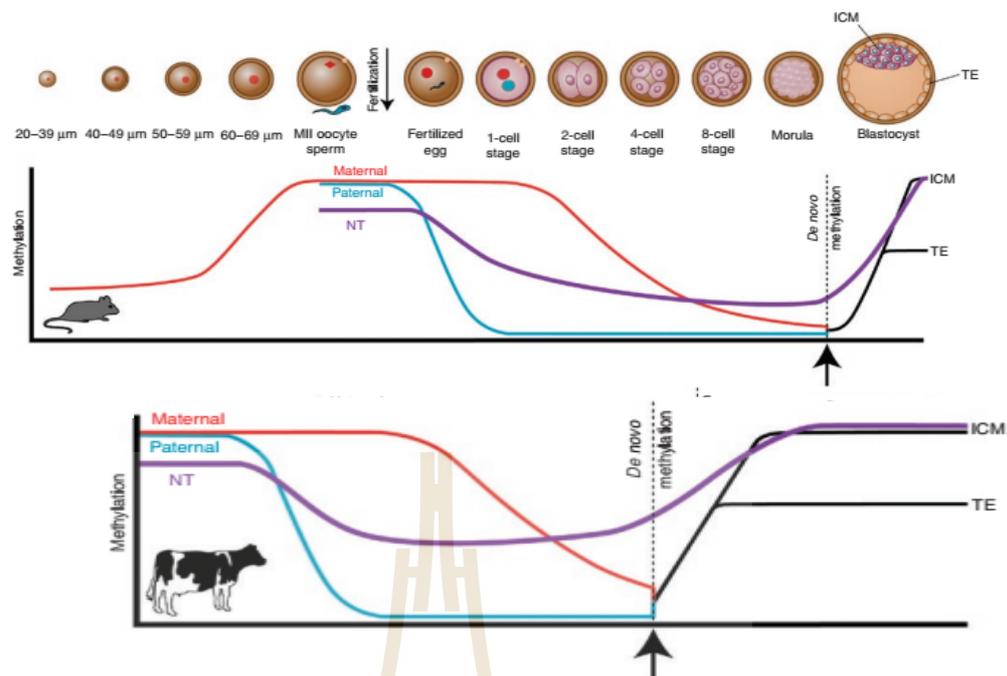
Figure 2.1 Methylation dynamics during normal early embryonic development and in clones embryos.

Epigenetic modification are the processes that influence on the function of genes without change DNA sequence including global changes in DNA methylation and histone modification as well as imprinted gene expression, which are related to transcriptional regulation (Suteevun et al., 2006). During the pre-implantation that had expressed the many of genes and are then shut down in somatic cell by epigenetic regulation of specific genes expression pattern (Ehrlich, 1982; Imamura et al., 2001). The epigenetics can be modified by the environment and transmitted to progeny including the two major mechanisms are DNA methylation and histone modification (Suteevun et al., 2006).

DNA methylation can interact with other histone modifications and modulate the other regulatory mechanisms (Li, 2002) that cause the blocking of transcription factors on the DNA, which leads to the condensation of the DNA into a chromatin conformation result in transcription repression (Reik et al., 2003). The process of

DNA methylation is the methyl groups bind to the carbon at position 5 of the pyrimidine ring of cytosine base on mammalian DNA by DNA methyltransferase (Dnmts) enzymes. These enzymes have been classified into 5 types; *Dnmt1, Dnmt2, Dnmt3a, Dnmt2b* and *Dnmt3l*. The main catalytic enzymes of the DNA methylation mechanism in *in vivo* are *Dnmt1, Dnmt3a* and *Dnmt3b* (Suteevun et al., 2006).

The *Dnmt1* is the major methyltransferase enzyme used in somatic cells, which is predominantly involved in passive DNA methylation by maintaining methylation through mitosis, DNA replication and in the DNA repair process of the cell (Leonhardt et al., 1992). Characteristic of *DNMT1* has a 5 to 30 fold preference for adding methyl groups to hemimethylated CpG sites, which are established by *Dnmt3a*, *Dnmt3b* (Yoder et al., 1997). In bovine, *Dnmt1* is excluded from the embryonic nucleus in the first few cleavages divisions and is allowed to enter the embryonic nucleus for short period during the 8-cell to 16-cell stage before disappearing again (Howell et al., 2001) that results in the disruption of the *Dnmt1* gene. It effects on the genome-wide DNA demethylation and cause embryonic lethality after gastrulation (Li et al., 1993). Further, the second type of DNA methyltransferase enzyme are *Dnmt3a* and *Dnmt3b* enzyme are involved in *de novo* methylation (active methylation), and are expressed at high levels during embryogenesis. The gene expression patterns in bovine, the *Dnmt3a* and *Dnmt3b* have only been observed in the blastocyst stage and are described to have high expression levels of both genes at this stage (Wrenzycki et al., 2005). In addition, the timing of *Dnmt3a* and *Dnmt3b* activity appears to differ in bovine embryos, occurring at the 8–16 cell stage as opposed to after the blastocyst stage in mice, indicating species-specific differences in these potential reprogramming events (Fairburn et al., 2002). Furthermore, the DNA methylation patterns can effect on roles in imprinting, X-chromosome inactivation, genome stability, silencing of

retrotransposons and inactivation of genes that leads to death of cloned embryos (Santos et al. 2002).

Histone modifications are another epigenetic mechanism that can effect on gene activity through the modification of histone proteins including acetylation, methylation, phosphorylation and ubiquitination of the highly conserved core histones, H2A, H2B, H3, and H4, influenced the control gene expression of chromosomes (Bártová, et al. 2008). Histone modifications are a post-translational modification, which plays an important role in regulating gene activity through chromatin conformation. According, acetylation of histones can change the chromatin organization that caused nucleosomes to loose, leading to gene expression. However, the methylation modification can either increase or decrease transcription of gene by transferring of methyl groups to amino acid of histone protein. Methylation mechanism is mostly shut off genes and leads to transcription repression. In 2003, Santos and colleges have been reported about epigenetic correlates with developmental potential in cloned bovine embryos that showed the majority of cloned embryos exhibit H3-K9 hypermethylation associated with DNA hypermethylation that the cause of failure of reprogramming.

Figure 2.2 Nucleosome with histone posttranslational modifications.

In addition, the genomic imprinting is an epigenetic phenomenon by parent of origin specific gene repression. Generally, one allele from the maternal or the paternal is expressed normally and is suppressed by addition of methyl groups to cytosine residues in CpG dinucleotide called "Imprinting Control Regions (ICRs)", catalyzed by the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3l*, and later propagated by the maintenance DNA methyltransferase *Dnmt1* (Abramowitz et al., 2012; Bartolomei et al., 2009; Delaval et al., 2004). The ICR region is methylation marks and maintain in the germline, whereas somatic DMRs (differentially methylated region) leading to imprinting are methylated in post-implantation embryos (Abramowitz et al., 2012; Royo et al., 2008) to achieve monoallelic expression lead to abnormality animals (Constancia et al., 2004).The proper allelic expression and the level of imprinted gene expression had effect on the fetal and placental growth that cause of developmental aberrations after SCNT. Imprinted gene has found in bovine including IGF2 (paternally expressed), IGF2r (maternally expressed), H19 (maternally expressed) and Nut genes that had variation of imprinted gene expression in different species. Some

imprinted genes are not imprinted in primates whereas which may be conserved the epigenetic regulation in other species (Killian et al., 2001). Therefore, the aberrant of imprinted gene expression in cloned embryos can cause the accumulation of fetal growth factor that lead to the occurrence of large offspring syndrome (LOS) (Suteevun et al., 2006).

2.6 Expression of imprinted genes in embryonic development

Genomic imprinting can be occurring on most mammalian chromosomes whose expression is dependent on the parental inheritance of the allele that establish during gametogenesis determine whether the imprinted gene expressed. Imprinting is epigenetic characterized primarily by DNA methylation marks on special CpG-rich regulatory elements called Imprinting Control Regions (ICRs), catalyzed by the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3l*, and later propagated by the maintenance DNA methyltransferase *Dnmt1* (Abramowitz et al., 2012; Bartolomei et al., 2009; Delaval et al., 2004) that the appropriate allele is silenced throughout embryonic development. During fertilization, the complementation of haploid genomes are contain an epigenetic mark conduce to produces a diploid genome of post-implantation development. After that, genome-wide erasure of existing epigenetic modifications on both DNA and histones can occur throughout the pre-implantation embryo. During tissue generation, the imprinting marks are later followed by further epigenetic changes during development that the result show a subset of genes being expressed monoallelically from either the maternal or paternal chromosome. However, in the primordial germ cells, the remaining parental imprinting marks are erased and their germline descendants reestablish imprinting marks in the cell cycle (Bartolomei et al., 2009; Delaval et al., 2004).
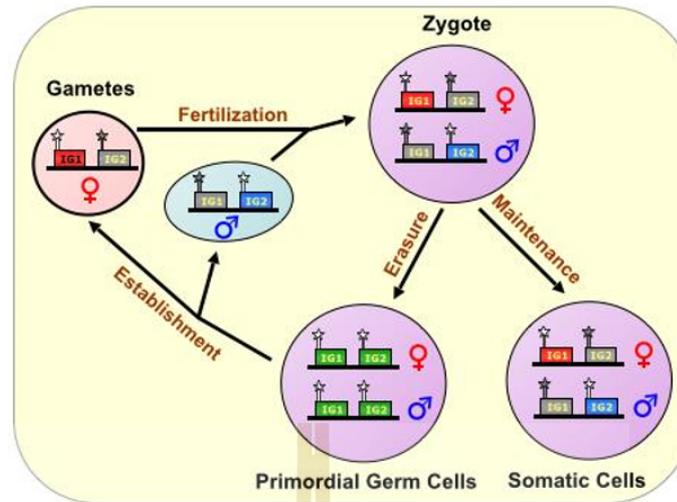
Figure 2.3 The imprinting cycle in normal fertilization embryo.

In SCNT cloned embryos to inappropriate conditions may changes in the expression of imprinted genes that abnormality proliferation and growth and placental development appears to be particularly vulnerable to these perturbations. IGF family members are low-molecular-weight peptide growth factors that function primarily as regulators of cell growth and differentiation, as well as acting as survival factors that protect against apoptosis (Brison, 2000; Byrne et al., 2002). Insulin-like growth factor 2 (*Igf2*) and Insulin-like growth factor 2 receptor (*Igf2r*) are imprinted genes that play important roles in preimplantation development (Jang et al., 2005). Previous study was reported the overexpression of *Igf2* in the mouse results in fetal and placental overgrowth and oversize of certain organs (Eggenschwiler et al. 1997), similar to those that are often reported to be affected in cloned bovine and ovine fetuses. Moreover, *Igf2r* is maternally expressed during peri-implantation development may depend on when the epigenetic modifications are established after early zygotic demethylation and specific expression of transcription factors with enhancers or regulatory elements of these imprinted genes. As well as, the expressions of *Igf2r* gene

gene have been changed reported for large offspring syndrome in sheep (Young et al. 2001). In the mouse, the absence of *Igf2r* expression results in excessive fetal growth lead to failure of the placenta to cease growing in late gestation (Lau et al. 1994).

Thus, the many aberrant phenotypes described in SCNT cloned fetuses or offspring abnormalities are associated with either deletion in imprinted genes or aberrant expression of these imprinted genes. The major cause of the large offspring syndrome (LOS) could be occurred the incomplete reprogramming of the donor cell nucleus, which cause chromosomal abnormalities that lead to the abnormal expression of imprinted gene (Jaenisch, 1997; Mizuki et al., 2001; Young et al., 2001) during preimplantation development resulting in perturbed embryonic and fetal gene expression patterns are thought to be involved in the syndrome (Wrenzycki et al., 2004). In addition, the imprinted genes are expressed predominantly in the brain that is important role in higher-order brain processes, such as learning and behavior (Davies et al., 2005; Chamberlain et al., 2012; Wilkinson et al., 2007). Perturbations of imprinted loci can result in pathological manifestations including Prader-Willi Syndrome, Angelman Syndrome, Beckwith-Wiedemann Syndrome, and Silver-Russell Syndrome (Santoro et al., 2011;Li and Sasaki, 2011), as well as some cancers (Uribe-Lewis et al., 2011) in human.
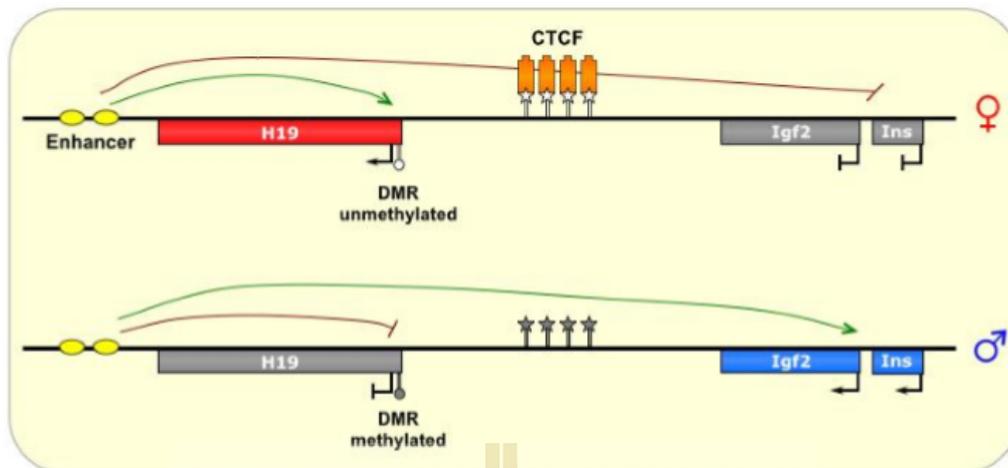
Figure 2.4 The ICR of this imprinted locus is intergenic, located between the *Igf2*

gene and the noncoding *H19* gene on the maternal and paternal allele.
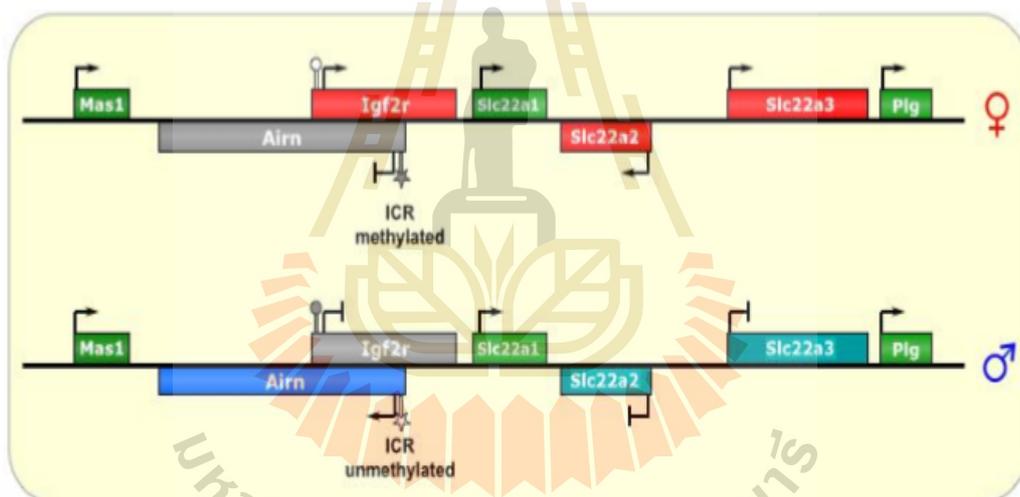
Figure 2.5 The ICR of this imprinted locus is at the promoter of the lncRNA Airn

and *Igf2r* on the maternal and paternal allele.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All chemicals and media used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless stated otherwise.

3.2 Methods

3.2.1 Preparation of fibroblast donor cells

Gaur and bovine ear skins tissue were biopsied from male and female gaurs and bovine. Skin of gaurs and bovine was isolated from cartilage and cut into small pieces before placed a 60-mm culture dish (Nunc, Denmark), then covered with a glass slide. The skins were cultured in αMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air. The fibroblasts monolayer were frozen at the fourth passage of cell culture in αMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO, Merck, Germany) and kept in liquid nitrogen. Frozen-thawed donor cells were cultured in a 35-mm culture dish (Nunc) for 2-3 days before used as a donor cells. A few minutes before injection, the proliferating fibroblasts were harvested by standard trypsinization and resuspended in Syngro® holding medium.

3.2.2 Oocyte preparation

Bovine ovaries from local slaughterhouse were collected and transported to the laboratory in 0.9% NaCl at room temperature. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (2-8 mm diameter) with an 18-gauge needle connected to a 10 ml syringe. COCs were selected and washed five times with modified Dulbecco's phosphate buffer saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP). Then, groups of 20 COCs were washed three times and cultured in 100 µl droplets of *in vitro* maturation (IVM) medium which consisting of TCM 199 powder supplemented with 10% FBS, 50 IU/ml hCG, 0.02 AU/ml FSH (Antrin®, Denka Pharmaceutical, Japan) and 1µg/ml 17β-estradiol (ADD P/S) overlaid with mineral oil and cultured at 38.5°C under humidified atmosphere of 5% CO_2 in air for 22 hours. After IVM, the cumulus cells were mechanically removed by repeat pipetting in 0.2% hyaluronidase (Sigma, USA) and subsequently washed five times in the Syngro® holding medium. Metaphase II oocytes that extruded the first polar body were selected for enucleation.
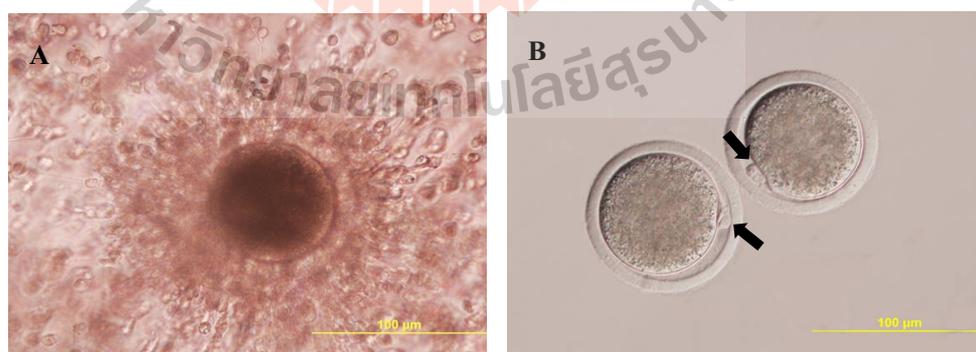
Figure 3.1 Bovine oocyte after *in vitro* maturation 22 h (A). After cumulus cells the removal, the first polar body (arrows) were extrudes from the matured oocytes (B) (magnification 200x).

3.2.3 Somatic cell nuclear transfer

Matured bovine COCs were placed in Syngro® holding medium containing 5 µg/ml cytochalasin B for 5 minutes. The zona pellucida above the first polar body was slit by cutting pipette and the polar body was squeezed out about 5-10% of the cytoplasm volume (Figure 3.2). The intraspecies bovine somatic cell nuclear transfer (SCNT) and interspecies gaur somatic cell nuclear transfer (iSCNT) process were operated by the micromanipulator (Narishige, Japan, model M0188NE) under inverted microscope (Olympus, Japan, model IX71) at 200x magnification. The enucleated oocytes were confirmed by staining the squeezed out cytoplasm with 5 µg/ml Hoechst 33342 (Figure 3.3). A single donor cell (diameter 14-16 µm) of male and female gaur or male and female bovine were inserted into the perivitelline space of each enucleated bovine oocytes (Figure 3.4). Fusion of bovine and gaur donor cells and bovine enucleated oocytes were performed in Zimmermann fusion medium (Zimmermann and Vienken, 1982) with the tips of fusion electrode (Figure 3.4) and electrostimulated by two direct current pulses (24 V, 15 µsec) generated by a fusion machine (SUT F-1, Suranaree University of Technology). After fusion, the reconstructed embryos were subsequently washed six times in Syngro® holding medium, activated by 7% ethanol (Carlo Erba, Italy) in Syngro® holding medium for 5 minutes at room temperature and subsequently with modified oviduct synthetic fluid (mSOF, Gardner et al., 1994) supplemented with 1.25 µg/ml cytochalasin D (CD) and 10 µg/ml cycloheximide (CHX) at 38.5°C under humidified atmosphere of 5% CO_2 in air for 5 hours (Muenthaisong et al., 2007).
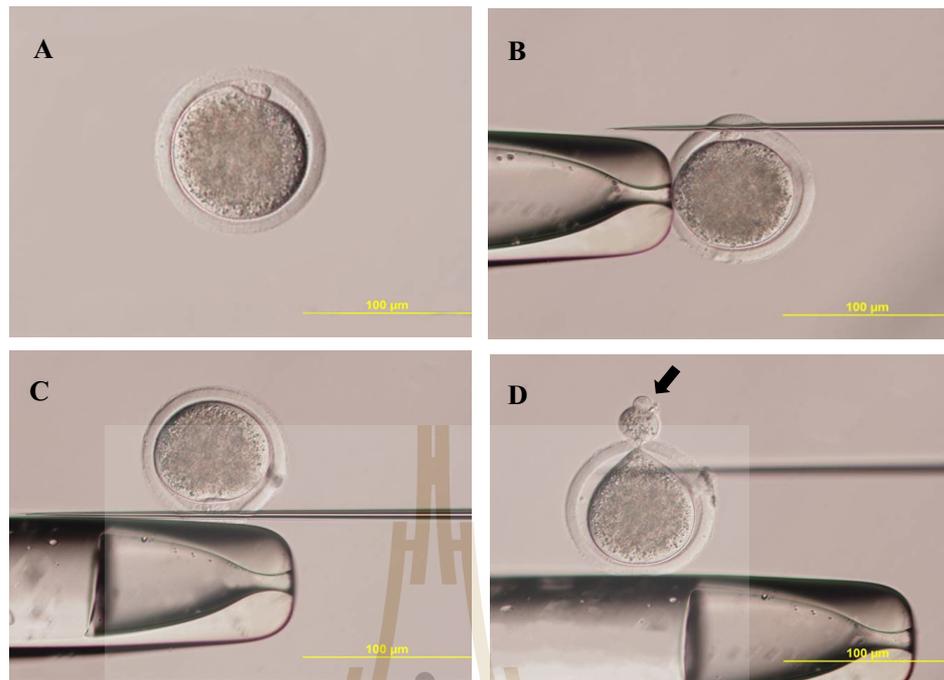
Figure 3.2 Enucleation procedure of bovine oocyte. Arrow indicated the first polar

body (magnification 200x).
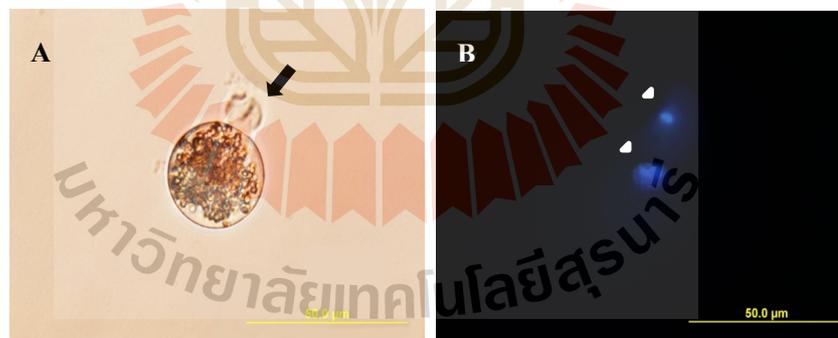
Figure 3.3 The squeezed out cytoplasm of MII oocyte after stained with Hoechst

33342. Bright field (A) and fluorescent image (B) of the first polar body

(arrow). The successful of enucleation show metaphase spindle removal

(circle) (magnification 400x).
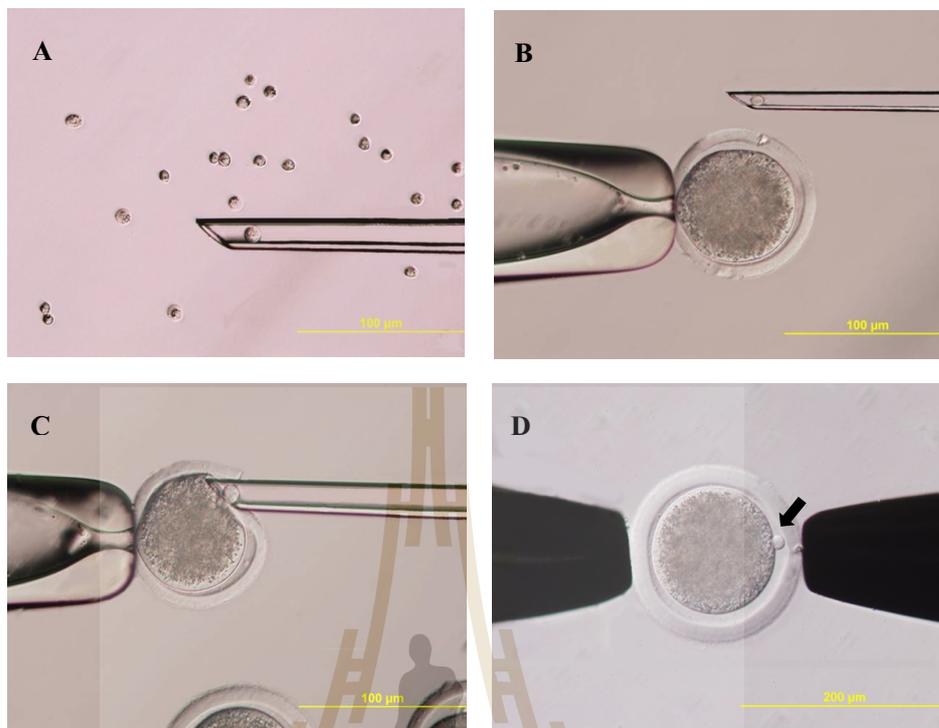
Figure 3.4 The injection and fusion procedure; the donor cell after trypsinized and separated into single cell (A). The donor cell was inserted into the perivitelline space of enucleated oocytes. Arrow indicated the donor cell (B,C). The cytoplasm/cell couplet during placed between both tips of fusion electrode (D) (magnification 200x).

3.2.4 *In vitro* embryo culture (IVC)

The reconstructed embryos were cultured in SOFaa (Glucose free) medium + 3 mg/ml BSA at 38.5°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 2 days. Eight-cell stage embryos were selected and co-cultured with bovine oviductal epithelial (BOEC) cells in SOFaa (0.25 mg/ml glucose) medium + 3 mg/ml BSA at 38.5°C under humidified atmosphere of 5% CO_2 in air for 2 days. Half volume of SOFaa (0.5 mg/ml glucose) medium + 3 mg/ml BSA was replaced with

fresh medium every day. The data of embryo development was recorded at approximately 144, 168 and 192 hours after culture, respectively.

3.2.5 *In vitro* fertilization (IVF)

Frozen semen was thawed at 39ºC for 30 sec. One hundred μl of thawed semen was placed to the bottom of a snapped tube containing 2 ml of Tyrode's Albumin Lactate Pyruvate (TALP,) medium supplemented with 1 mmol caffeine, 100 μg/mi heparin, 20 mmol/l penecilamine, 10 mmol/l hypotaurine and 20 mmol/l ephinephrine and kept at a 45º angle in a humidified atmosphere of 5% CO_2 in air at 38.5ºC for 30 minutes. Then the supernatant was collected and placed into 15 ml of conical tube and centrifuged at 500xg for 5 minutes. After centrifugation, supernatant was discarded and 3 ml of TALP medium were added for washing. The sperm concentration was calculated by using a hemocytometer and adjusted the final concentration of sperm into $2x10^6$/ml. On the other side, the cumulus cells was partially removed from bovine oocytes after IVM culture by gentle pipetting with 0.1% hyarulonidase. Groups of 10 oocytes was washed 3-4 times in TALP medium and placed in each 100 μl/drop of TALP medium and co-cultured with sperm at 38.5ºC under humidified atmosphere of 5% CO_2 in air for 13-14 hours. Then the presumptive zygotes were cultured in mSOFaa medium under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5ºC for 48 hours. Eight-cell stage embryos were selected and co-cultured with bovine oviductal epithelial (BOEC) cells in SOFaa (0.25 mg/ml glucose) medium + 3 mg/ml BSA at 38.5˚C under humidified atmosphere of 5% CO_2 in air for 2 days. Half volume of SOFaa (0.5 mg/ml glucose) medium + 3 mg/ml BSA was replaced with fresh medium every day. The data of embryo development was recorded at approximately 144, 168 and 192 hours after culture, respectively.

3.2.6 Differential staining of blastocysts

The SCNT and iSCNT blastocysts harvested from day 7 were counter-stained to evaluate of embryos quality by counted the cells of trophectoderm (TE) and inner cell mass (ICM) according to the method reported previously with some modifications (Thouas et al., 2001). Briefly, the blastocysts were washed 3 times in PBS buffer and treated with 10 μg/ml propidium iodide and triton X-100 for 60 seconds. Then, the IVF and cloned blastocysts were placed in 25 μg/ml Hoechst 33342 dissolved in 99.5% ETOH for 3-5 minutes and mounted on glass slides in glycerol (Merck) droplets, flattened by cover slips. The numbers of ICM cells (blue) and TE cells (red) were examined with fluorescence microscopy (Olympus).

3.2.7 Gene expression analysis

The SCNT, iSCNT and IVF embryos at 1-cell, 8-cell, morula and blastocyst stages were collected at approximately 12, 48, 120, 144, 168 and 192 h after cultured. The fibroblastss derived from bovine male, female and gaur were collected for analysis. These samples were placed in microcentrifuge tube containing 50 μl of RNase-free water and extracted mRNA by used Oligo (dT) 25 nucleotide attached with magnetic bead (Dynabeads mRNA purification kit, Dynal) following the manufacturer's instructions. The fibroblast donor cells of male and female bovine and gaur were also extracted mRNA. The mRNA was synthesized complementary DNA (cDNA) by used iScript reverse transcription super mix kit (Biorad, USA) with Eppendorf Mastercycler DNA Engine Thermal Cycler PCR. The quantification of all gene transcripts was done by quantitative PCR (qPCR). All of embryos were analyzed at least three times for every stage. The primer for *Oct4, Hat1, Hdac1, Dnmt1, Dnmt3a, Dnmt3b, Igf2* and *Igf2r* genes were analyzed gene expression while Histone

H2A.2 was used as the reference gene. The qPCR was executed with Chromo Four-Color Real-Time PCR Detection System using SYBR green incorporation. The gene expression was compared the relative level between the cloned and IVF embryos, the real-time data was analyzed by using the comparative CT method, ΔΔCT (Livak, and Schmittgen, 2001). This method calculates the quantity of starting material (cDNA) in fold change relative to a calibrator sample and this change is given by $2^{-\Delta\Delta CT}$ where ΔΔCT = (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. The 1/CT values were used to examine the pattern of gene expression throughout preimplantation development (Imsoonthornruksa et al., 2010).
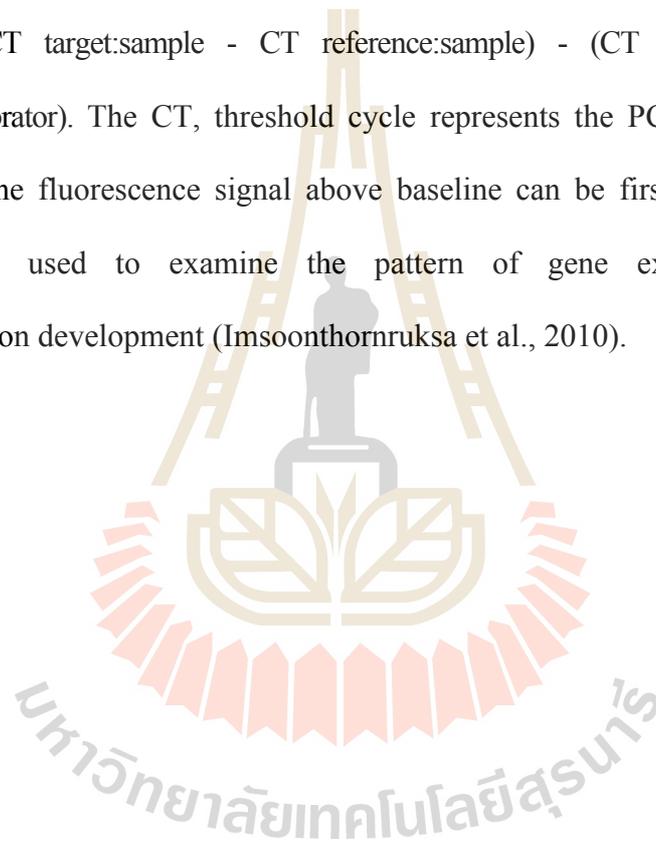
Table 3.1 Primer sequences used for qPCR.

Genes	Primer sequences 5'-3'	Accession number	References
Oct4	forward-CCACCCTGCAGCAAATTAGC reverse-CCACACTCGGACCACGTCTT	NM174580	Iager et al., 2008
DNMT1	forward-GAGGGCTACCTGGCTAAAGTC reverse-CATTCGCTTCCCGACTGAAA	AY173048	Suteevun et al., 2006
DNMT3a	forward-CGAGGTGTGTGAGGACTCCAT reverse-ACGTCCCCGACGTACATGA	AY271299	Suteevun et al., 2006
DNMT3b	forward-AGCATGAGGGCAACATCAAAT reverse-CACCAATCACCAAGTCAAATG	AY244713	Suteevun et al., 2006
HAT1	forward-CTTCAGACCTTTTTGATGTGGTTTATT reverse-GCGTAGCTCCATCCTTATTATACTTCTC	BT021536	Suteevun et al., 2006
HDAC1	forward-GCACTGGGCTGGAACATCTC reverse-GGGATTGACGACGAGTCCTATG	AY504948	Suteevun et al., 2006
IGF2r	forward-ACAGCGGGTACGTGTTTGAT reverse-CATCATTGCAGACGAAGCGG	NM174352	Goodall and Schmutz, 2007
IGF2	forward-CAGCCTGCAAACTGGACATTA reverse-AATCGCTGGATGCCTTGGAA	NM174087.3	Goodall and Schmutz, 2007
HistoneH2A.2	forward-GAGGAGCTGAACAAGCTGTTG reverse-TTGTGGTGGCTCTCAGTCTTC	BF076713	Ross et al., 2010

3.2.8 Immunostaining of histone modification analysis

The IVF, SCNT and iSCNT embryos were collected at 2-cell, 4-cell, 8-cell, morula and blastocysts stage and fibroblastss from male and female bovine and gaur were collected and investigated for histone modification analysis. Briefly, the samples of this study were washed in phosphate-buffer saline (PBS) + 0.1% PVP and fixed in 4% paraformaldehyde for 30 minutes. The permeabilizations of the samples were transferred in PBS + 0.5% triton X-100 for 1 hour. After that, the DNA was denatured by incubation in 2N HCl for 1 hour at 37ºC and then blocked in PBS + 10% BSA (blocking solution) to prevent non-specific binding for 1 hour. After incubation, the samples were stained with blocking solution + primary antibody (Ac-H4K5 and HDAC1, company, country) at 4ºC overnight. The samples were incubated with PBS + 0.1% PVP + secondary antibody for 1 hour at room temperature. Then, the samples were washed in PBS + 0.1% PVP and stained with DAPI for stained DNA. In case of fibroblasts donor cell derived from different gender, immunofluorescence staining was carried out as described previously step except that washing and incubation in 2N HCl solutions of embryos staining step. After incubated in blocking solution, each donor cell type was analyzed for each epigenetic marker. The histone modification of embryos and fibroblasts were mounted on slides and observed under ultraviolet light provide by 100W high-pressure mercury burner (Olympus, model BH2-RFL-T3) for fluorescence microscopy and quantified fluorescence intensity of all nuclei in the embryo by using image J software.

3.2.9 Experimental design

3.2.9.1 Experimental 1: carried out to examine the effects of donor cell gender on development of cloned gaur and cloned bovine using bovine oocytes as recipient cytoplasts. The fusion, cleavage and *in vitro* developmental rates were evaluated. Moreover, the effects of donor cell gender on ICM and TE cell numbers of iSCNT gaur, SCNT bovine and IVF blastocysts were evaluated by using differential staining.

3.2.9.2 Experimental 2: carried out to evaluate the effects of donor cell gender on gene expression of iSCNT gaur, SCNT bovine, IVF embryos and fibroblastss of male and female bovine and gaur. The cloned and IVF embryos of 3 embryos at each stage of embryo development at 1-cell, 8-cell, morula and blastocyst stages were collected and extracted mRNA. The cDNA derived from each treatments was synthesized and quantification analyzed by using quantitative PCR (qPCR).

3.2.9.3 Experimental 3: carried out to study the effects of donor cell gender on histone modification analysis of SCNT bovine, iSCNT gaur embryos and fibroblastss of male and female bovine and gaur. The IVF, SCNT bovine, iSCNT gaur embryos and fibroblastss were collected for immunostaining method by using Ac-H4K5 and HDAC1 as primary antibody for detect the protein on all nuclei in the embryos. The histone modification was analyzed fluorescence intensity of each group by using image J software.

3.2.10 Statistical analysis

The fusion rate, embryo developmental rate, gene expression levels and the relative intensity of histone acetylation and deacetylation of gaur iSCNT, bovine SCNT and IVF embryos were evaluated by One-way Analysis of Variance (ANOVA) using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed. The relative quantification of target genes expression level is presented as fold-difference. The transcription expression of these genes between cloned embryos and their donor cells types of each groups were estimated by student T-Test analysis. Data are presented as mean ± SEM. P value of less than 0.05 was considered to be significant. The gene expression experiment was repeated at least three times. The numbers of metaphase II oocytes in this experiment were used approximately 500 oocytes per treatments in five treatments. The IVF embryos production experiments were repeated at least five times and least eight times in SCNT and iSCNT embryos.

CHAPTER IV

RESULTS

4.1 Effect of donor cell gender on development and ICM and TE cell numbers of SCNT bovine and iSCNT gaur embryo

The developmental ability and ICM and TE cell numbers of SCNT bovine and iSCNT gaur embryos were investigated. These results showed that the fusion rate of male and female bovine and gaur fibroblasts with enucleated bovine oocytes were no significantly difference (P>0.05). The fusion rate of male and female bovine fibroblasts with bovine recipient cytoplasts were 84.2% and 84.1%, respectively. The fusion rates of iSCNT gaur embryos were showed 83.8% and 80.8%, respectively.

The cleavage and embryo development to 8-cell, morula and lastocyst stages in SCNT bovine and iSCNT gaur embryos were investigated. The cleavage development rate of IVF embryos (89.94%) was significantly higher than iSCNT female gaur (83.1%, P<0.05) whereas no significantly different with other cloned groups (SCNT male bovine, SCNT female bovine and iSCNT male gaur; 85.2%, 86.4%, and 84.4%, respectively).The development to 8-cell stage of IVF, SCNT bovine and iSCNT gaur embryos were not significantly different (P>0.05).The development to blastocyst stage of IVF embryos (34.3%) was significantly higher than SCNT embryos derived from male and female fibroblasts (25.0% and 23.1% respectively, P<0.05) and iSCNT gaur embryos derived from male and female fibroblasts (27.5% and 25.8% respectively, P<0.05).

The ICM and TE cell numbers and total cells derived of IVF embryos were significantly higher than SCNT bovine and iSCNT gaur embryos. However, the cloned embryos fields were not significantly different between SCNT and iSCNT embryos of the cell number of ICM, TE and total cell at blastocyst stage. A summary of these results are showed in Table 4.1
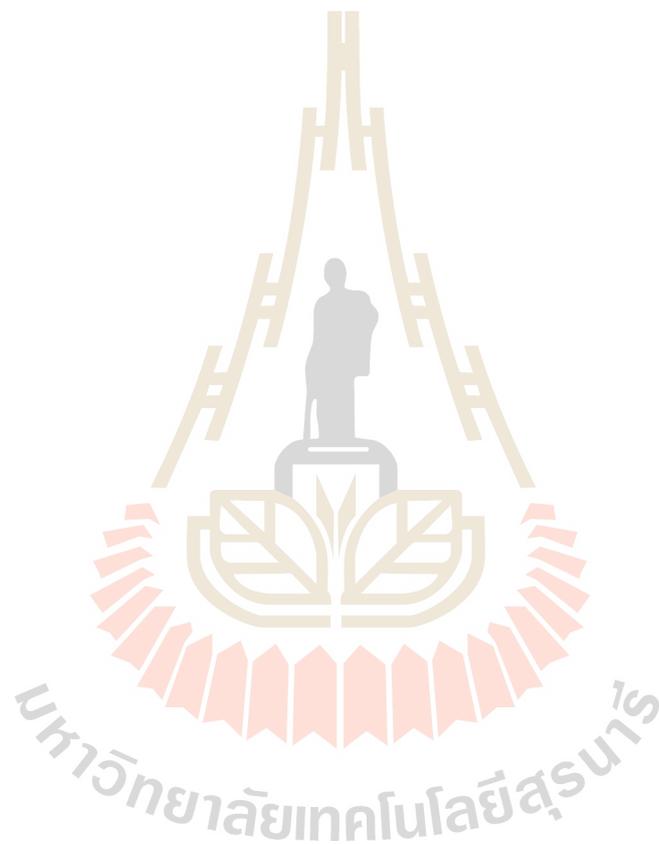
Table 4.1 The development of ability and ICM and TE cell numbers of SCNT bovine and iSCNT gaur embryos derived from donor cell

gender.

time	Treat.	Fusion (%)	No. cultured	Cleaved (%)	No. (%) embryo developed to			Cell numbers		
					8-C	Morula	Blastocyst	ICM	TE	Total cell
IVF			159	143/159 (89.94)[a]	88/159 (55.3)[a]	61/159 (38.4)[a]	55/159 (34.6)[a]	36.4± 3.3 [a]	94.4 ± 13.3 [a]	130.9±15.5 [a]
SCNT	male bovine	176/209 (84.2)[a]	176	150/176 (85.2)[a]	89/176 (50.6)[a]	49/176 (27.8)[bc]	44/176 (25.0)[b]	32.3± 2.5[b]	83.3± 4.5 [b]	115.8± 6.8 [b]
	female bovine	169/201 (84.1)[a]	169	146/169 (86.4)[ab]	92/169 (54.4)[a]	44/169 (26.0)[c]	39/169 (23.1)[b]	32.1± 1.6 [b]	78.1 ± 4.8 [b]	110.2± 5.1 [b]
iSCNT	male gaur	160/191 (83.8)[a]	160	135/160 (84.4)[ab]	88/160 (55.0)[a]	53/160 (33.1)[ab]	44/160 (27.5)[b]	34.6± 2.9 [b]	79.3 ± 1.9 [b]	113.9± 2.3 [b]
	female gaur	159/197 (80.9)[a]	159	132/159 (83.1)[b]	79/159 (49.7)[a]	47/159 (29.6)[abc]	41/159 (25.8)[b]	33.5± 0.5 [b]	80.0± 2.8 [b]	113.6± 2.5 [b]

[a,b,c] Values have different superscripts are significantly different at P<0.05 using one-way ANOVA.
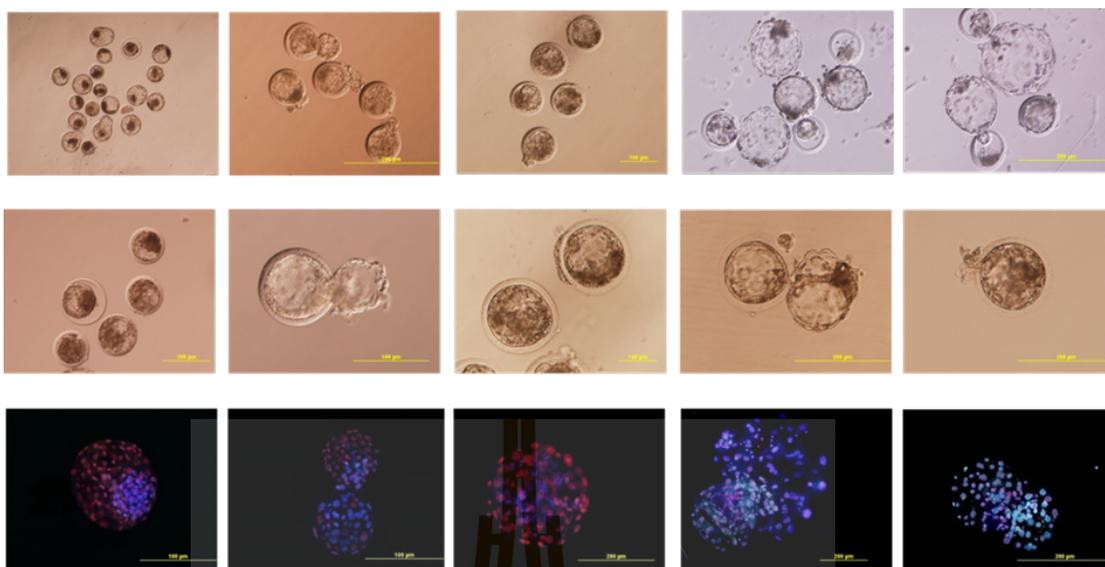
Figure 4.1 Representative results of embryonic development and quality by used differential staining method of various embryo types (A) *in vitro* fertilization embryos (B) iSCNT embryos derived from male bovine fibroblasts (C) SCNT embryo derived from female bovine fibroblasts (D) iSCNT embryos derived from male gaur fibroblasts (E) iSCNT embryos derived from female gaur fibroblasts.

4.2 The effects of donor cell gender on gene expression of SCNT bovine, iSCNT gaur embryos and fibroblasts

To study the effects of donor cell gender on gene expression of SCNT bovine embryos, iSCNT gaur embryos and fibroblasts. From Figure 4.2, the expression patterns of *Oct4* levels in all groups of embryos were incrementally expressed from 1-cell to blastocyst stage, which increased the *Oct4* mRNA levels at blastocyst stage. At 1-cell stage of SCNT female bovine was significantly higher transcript levels (1.5 fold; $P<0.05$) than IVF (0.7 fold), SCNT male bovine (0.9 fold) and iSCNT gaur

(male = 0.05 fold, female = 0.09, respectively). And the iSCNT gaur embryos reconstructed with male and female gaur fibroblasts were lowest expression levels of this gene. However, the IVF and SCNT male bovine embryos (0.8 fold and 1.4 fold, respectively; $P<0.05$) were significantly higher transcript levels than the other types of embryos at 8-cell stage. At morula stage, the iSCNT female gaur embryo (9.0 fold; $P<0.05$) showed significantly higher mRNA levels than the other types of embryos. Conversely, the mRNA levels of Oct4 gene showed significantly higher transcript levels in IVF embryo (37.6 fold; $P<0.05$) than SCNT bovine (male = 12.0 fold, female = 7.7 fold, respectively) and iSCNT gaur (male = 25.4 fold, female = 23.8 fold, respectively) embryos at blastocyst stage. Moreover, the expression of Oct4 mRNA levels were no significantly different transcript levels in SCNT bovine and iSCNT gaur embryos reconstructed with different donor cell gender.

The relative quantitative levels of histone acetylation *(Hat1* gene) were low transcript levels at cleavage stage. The 8-cell stage until morula and blastocyst stage showed high expression level of this gene (Figure 4.3). The mRNA levels of *Hat1* gene in IVF embryo showed significantly higher transcript levels than SCNT bovine embryos reconstructed with male and female bovine fibroblasts at 1-cell and 8-cell stage. The relative transcription levels of IVF embryo was no significantly different with iSCNT gaur embryos at 1-cell stage and showed similar pattern of *Hat1* gene with iSCNT female gaur embryos at 8-cell stage. At 8-cell stage, the iSCNT female gaur embryo (0.73 fold; $P<0.05$) was significantly higher transcript levels than iSCNT male gaur embryo (0.5 fold). However, the mRNA levels of *Hat1* showed no significantly different in IVF (6.7 fold), SCNT male bovine (5.6 fold) and iSCNT gaur embryos (male = 6.8 fold, female = 6.9 fold, respectively) at morula stage. But the SCNT

female bovine embryo (1.2 fold; P<0.05) was significantly lower transcript levels than

the other types of embryos. At blastocyst stage, IVF embryo (17.8 fold; P<0.05) was

significantly higher transcript levels than SCNT bovine (male =8.2 fold, female = 5.8

fold, respectively) and iSCNT gaur (male = 7.8 fold, female = 9.8 fold, respectively)

embryos. Accordingly, the SCNT bovine and iSCNT gaur embryos reconstructed with

different donor cell gender showed no significantly different transcript levels at this

stage (Figure 4.3).
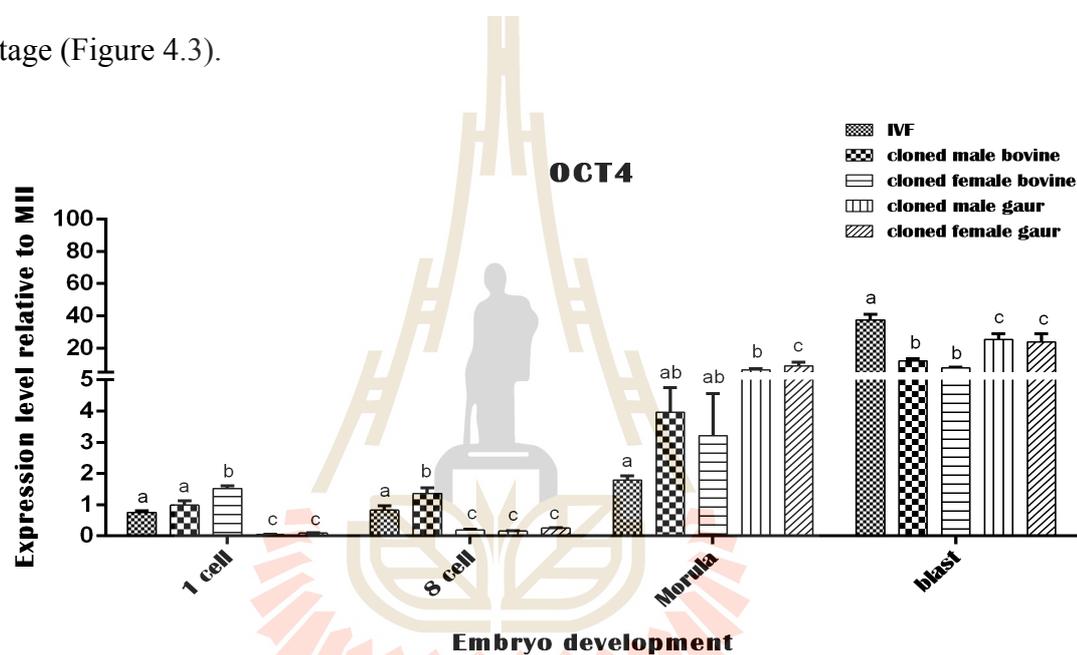
Figure 4.2 Relative expression levels of several candidate genes related with *Oct4* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c] Values have different superscripts are significantly different at P<0.05 using one-way ANOVA.
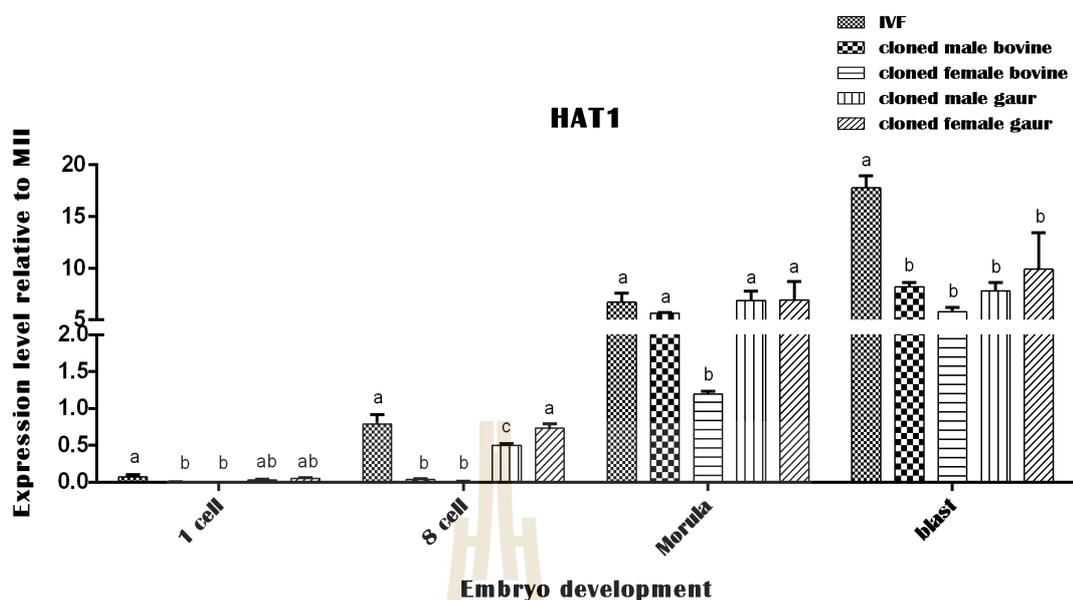
Figure 4.3 Relative expression levels of several candidate genes related with *Hat1* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c] Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.

Moreover, the relative expression of histone deacetylation (*Hdac1* gene) was variance of mRNA levels at all development stage of embryonic development (Figure 4.4). In IVF embryo was lower level of mRNA expression at 4-cell to 8-cell stage and was increased at morula and blastocyst stage. While, the iSCNT male and female embryos showed an increase of the transcription levels of this gene during 8-cell to the blastocyst stage. The mRNA levels of *Hdac1* in IVF embryo showed significantly higher transcript levels at 1-cell (0.2 fold) and 8-cell stage (0.07 fold; $P<0.05$) than other type of embryos. However, the SCNT male and female bovine embryos were significantly lower at 1-cell (male = 0.005 fold, female = 0.004 fold, respectively) and 8-cell stage (male = 0.004 fold, female = 0.002 fold, respectively; $P<0.05$) than other type of embryos. Moreover, the mRNA level of *Hdac1* in SCNT bovine and iSCNT

gaur embryos reconstructed with male and female gaur fibroblasts were significantly of transcript levels at 1-cell and 8-cell stage. At morula stage, the iSCNT male gaur embryo showed higher transcript levels than the other types of embryos. While, IVF embryo (0.11 fold) was no significantly different with SCNT bovine (male = 0.15 fold, female = 0.05 fold, respectively) and iSCNT gaur (male = 0.18 fold, female = 0.07 fold, respectively) embryos. However, the SCNT bovine and iSCNT gaur embryos were significantly of transcript levels when compared with different donor cell gender. At the blastocyst stage, the iSCNT male gaur embryo (0.35 fold) was higher transcript levels than SCNT bovine (male = 0.23 fold, female = 0.22 fold, respectively) and iSCNT female gaur (0.27 fold) embryos but showed significantly higher than IVF embryo (0.2 fold; $P<0.05$).
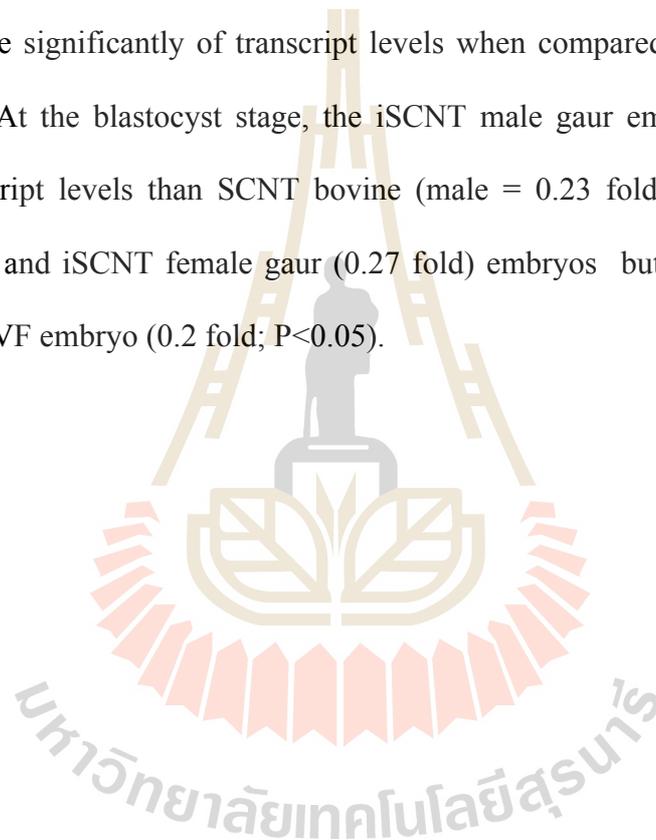
Figure 4.4 Relative expression levels of several candidate genes related with *Hdac1* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c,d]Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.
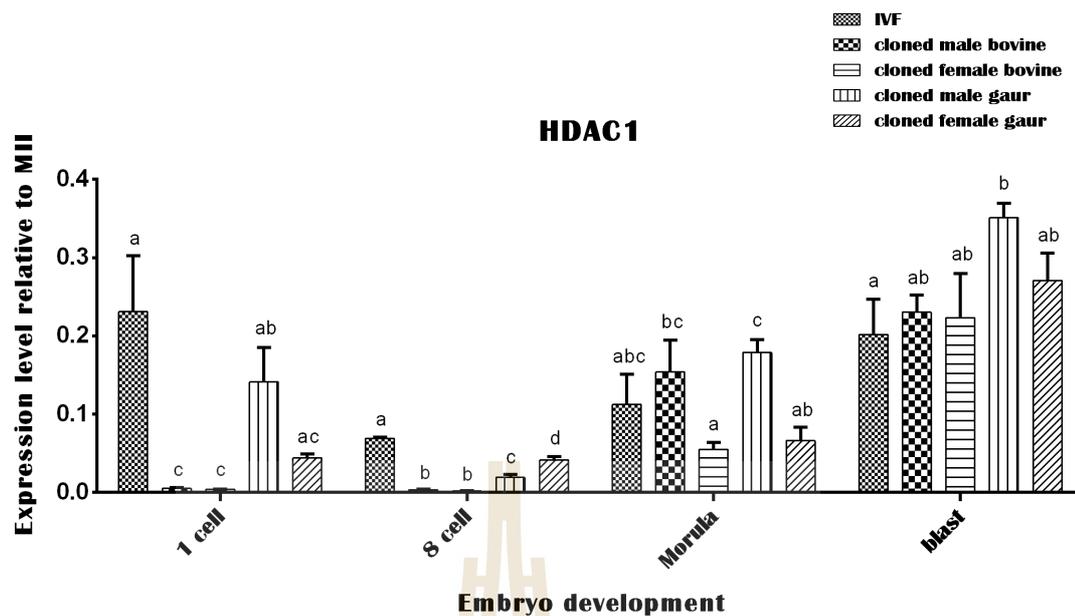
The expression level of passive DNA methylation (*Dnmt1*) showed highest at 1-cell to 4-cell stage of SCNT bovine and iSCNT gaur embryos; those were decreased at 8-cell stage until blastocyst stage of cloned embryos (Figure 4.5). Furthermore, the mRNA levels of *Dnmt1* in IVF embryo was extremely lower level at all stage of embryo. This results demonstrated that the correctly epigenetic remodeling of global DNA methylation. The SCNT bovine embryos reconstructed with male and female bovine fibroblasts (male = 3.5 fold, female = 4.7 fold, respectively; $P<0.05$) were significantly higher mRNA level than other types of embryos at 1-cell stage. At 8-cell stage, the SCNT male bovine (0.49 fold) and iSCNT male gaur (0.52 fold; $P<0.05$) embryos were significantly higher transcript levels than other types of embryos. IVF embryo showed significantly lower mRNA level at 8-cell to blastocyst stage.

However, the expression of *Dnmt1* gene showed significantly expression in SCNT and iSCNT embryos reconstructed with male and female fibroblasts at 8-cell stage ($P<0.05$) but were not significantly mRNA levels of this gene at blastocyst stage.

Figure 4.5 Relative expression levels of several candidate genes related with *Dnmt1* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c,d] Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.

In order to examine the activities of *De novo* DNA methylation (*Dnmt3a* gene) of each embryo types, the transcription levels were become increased from cleavage to blastocyst state (Figure 4.6). The relative expression of this gene in IVF embryo (2.9 fold; $P<0.05$) was significantly lower transcript levels than the SCNT bovine embryos (male = 15.7 fold and female = 14.9 fold; $P<0.05$) but was no significantly different with the iSCNT gaur embryos (male = 1.5 fold and female = 6.3 fold; $P>0.05$) at 1-cell stage. At 8-cell stage, the IVF embryo showed no significantly different with SCNT bovine and iSCNT gaur embryos. Whereas the SCNT male bovine embryo

(30.8 fold; P<0.05) showed significantly higher transcript levels than other types of embryos at this stage. At morula stage, the iSCNT female gaur embryo (224.6 fold; P<0.05) was significantly transcript levels than other types of embryos. However, the SCNT bovine and iSCNT gaur embryos showed higher mRNA level than IVF embryos at morula and blastocyst stage. At blastocyst stage, the iSCNT male gaur embryo showed higher transcript levels than the SCNT bovine and iSCNT female gaur embryos (P>0.05).
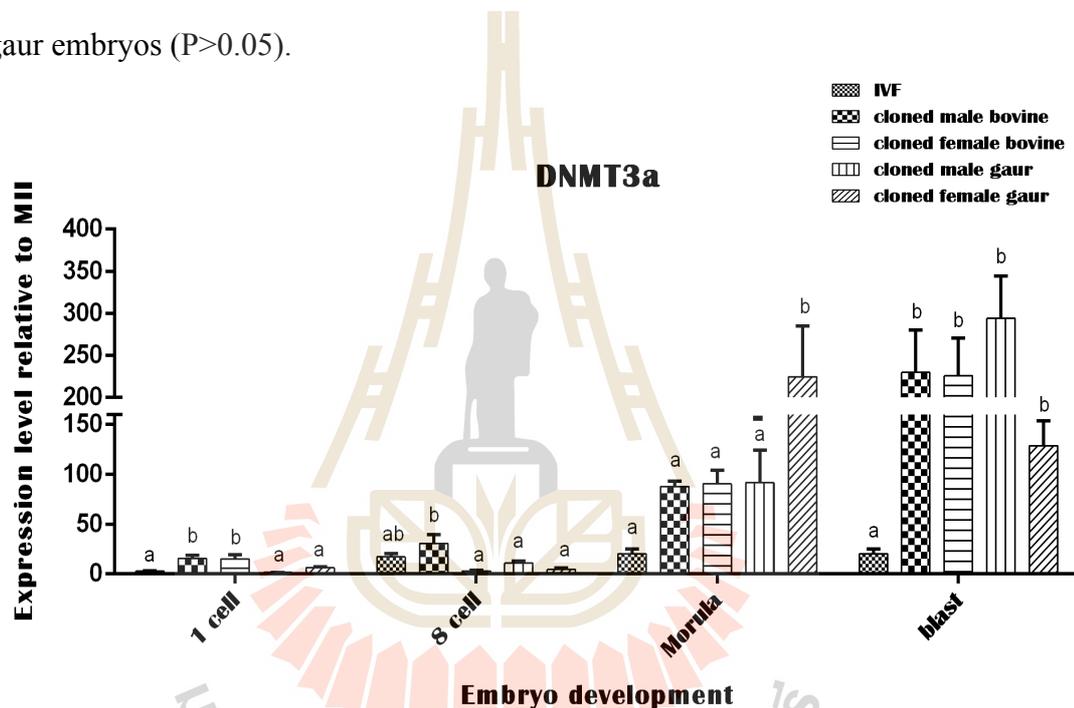
Figure 4.6 Relative expression levels of several candidate genes related with *Dnmt3a* gene derived from IVF, SCNT bovine and iSCNT gaur embryos atvarious stage of embryonic development. [a,b] Values have different superscripts are significantly different at P<0.05 using one-way ANOVA.
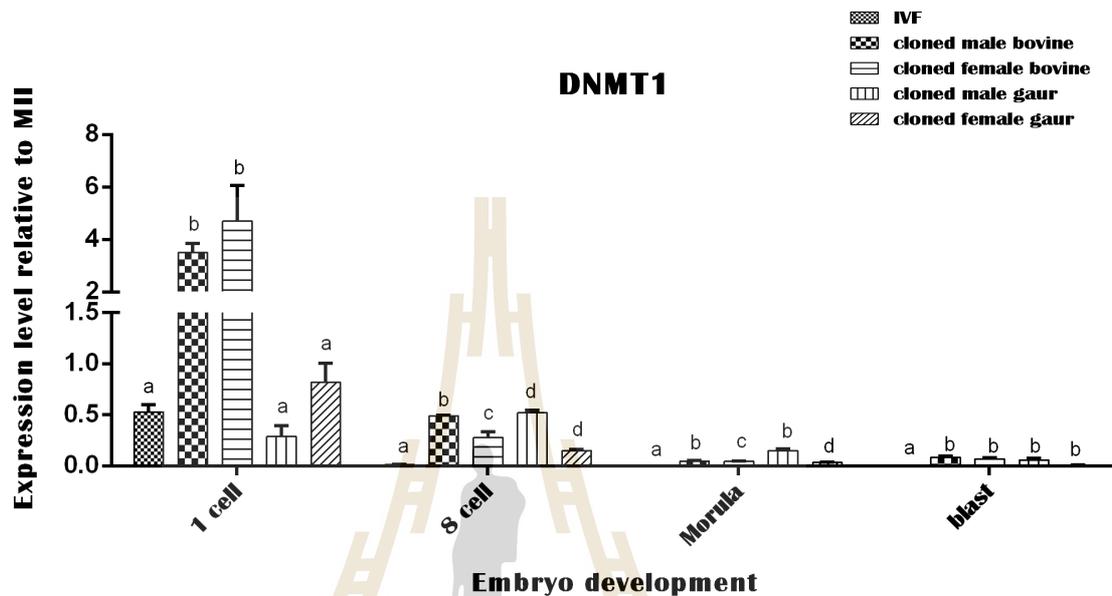
Figure 4.7 Relative expression levels of several candidate genes related with *Dnmt3b* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c,d] Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.

Furthermore, the relative transcription levels of *Dnmt3b* were variable of mRNA levels at all stage of embryonic development in all embryos groups (Figure 4.7). At 1-cell and 8-cell stage, the mRNA level of *Dnmt3b* gene in iSCNT female gaur embryo (3.5 fold and 0.18 fold, respectively; $P<0.05$) was significantly higher than the other types of embryos. Moreover, the IVF embryo (0.9 fold; $P>0.05$) was no significantly different with SCNT bovine embryos (male = 0.41 fold, female = 0.09 fold) and showed significantly higher transcript levels than iSCNT male gaur embryo (0.48 fold; $P<0.05$). At morula and blastocyst stage, the IVF embryo was no significantly different with SCNT bovine and iSCNT gaur embryos. However, the iSCNT male gaur embryo (0.52 fold and 1.36 fold, respectively; $P<0.05$) was significantly higher transcript levels than other types of embryos at these stage. Surprisingly, the relative

of transcription levels of *Dnmt3b* gene was significantly at all stage of embryonic development in iSCNT gaur embryos with different of donor cell gender (P<0.05).

Accordingly, the mRNA level expression of *Igf2* (paternal imprinted) gene was lower variable at early stage of embryo development in all embryo types (Figure 4.8). The expression of this gene in IVF embryo (115.3 fold; P<0.05) was significantly higher transcript levels than iSCNT female gaur embryo (18.5 fold) whereas similar expression pattern with the other type of embryos at 1-cell stage. However, the SCNT male bovine embryo (147.7 fold; P<0.05) showed significantly higher mRNA level than the other types of embryos at 8-cell stage. The SCNT male bovine (272.2 fold and 367.8 fold, respectively; P<0.05) and iSCNT male gaur (195.9 fold and 1751.7 fold, respectively; P<0.05) embryos were significantly higher transcript levels than IVF (122.4 fold and 145.1 fold, respectively), SCNT female bovine (67.0 fold and 41.8 fold, respectively) and iSCNT female gaur (131.4 fold and 203.6 fold, respectively) embryos at morula and blastocyst stage. Moreover, the relative transcription of *Igf2* gene in SCNT bovine and iSCNT gaur embryos reconstructed with different donor cell gender showed significantly at morula and blastocyst stage.
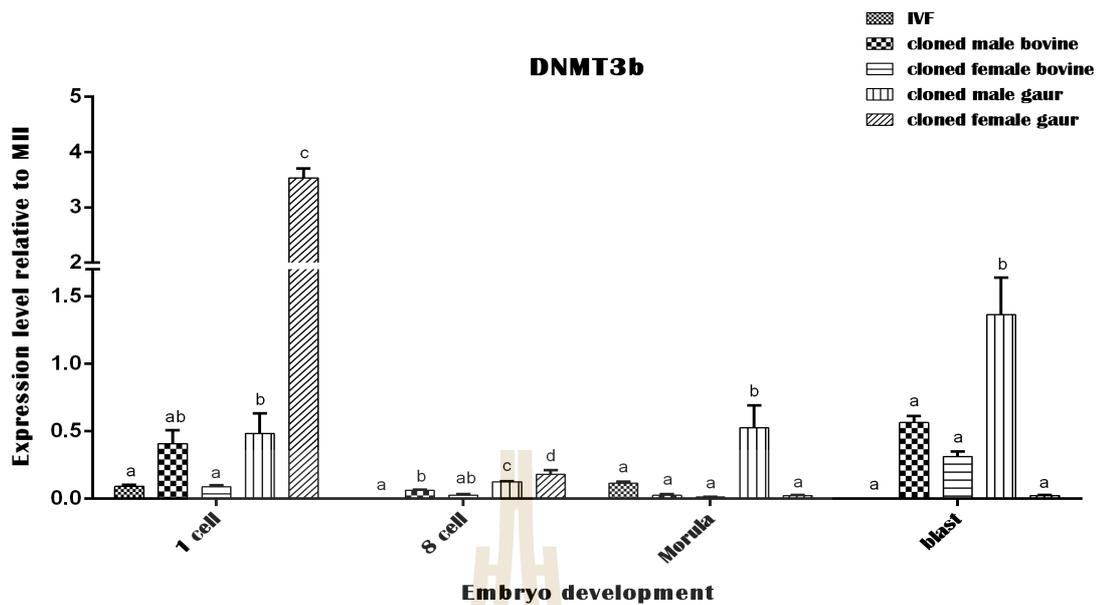
Figure 4.8 Relative expression levels of several candidate genes related with *Igf2* gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c] Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.
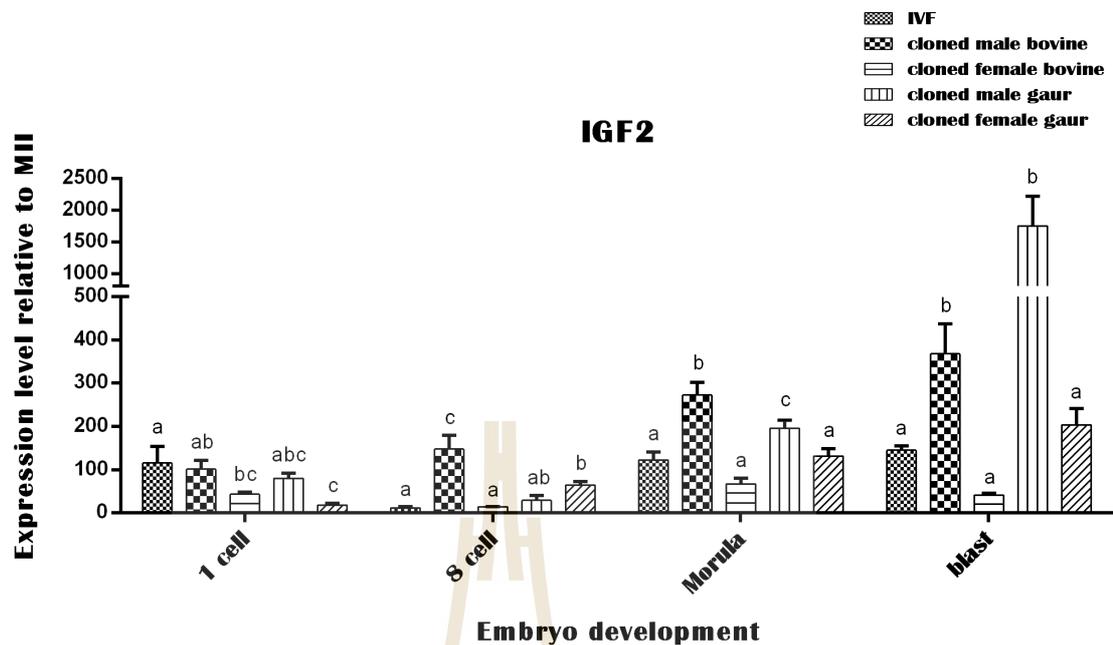
The relative expression level of *Igf2r* gene of female iSCNT embryo (1.2 fold and 0.26 fold, respectively; $P<0.05$) significantly higher than other types of embryos whereas the IVF embryo was no significantly different with SCNT bovine and iSCNT male gaur embryos at 1-cell, 8-cell and morula stage (Figure 4.9). At the blastocyst stage, the transcript levels of this gene showed no significantly different with SCNT female bovine (0.63 fold), iSCNT female gaur (0.87 fold) and IVF (0.51 fold) embryos. While, the SCNT male bovine (0.21 fold; $P<0.05$) and iSCNT male gaur (0.05 fold; $P<0.05$) embryos showed significantly lower transcript levels than the other types of embryos at this stage. Moreover, the mRNA levels of *Igf2r* gene were significantly in SCNT bovine embryos reconstructed with different donor cell gender

at blastocyst stage and the iSCNT gaur embryos showed significantly at 1-cell, 8-cell, morula and blastocyst stage. So, we were indicated that the aberrantly expression of imprinted genes were significant high influenced in SCNT and iSCNT embryos derived from different of donor cell gender.
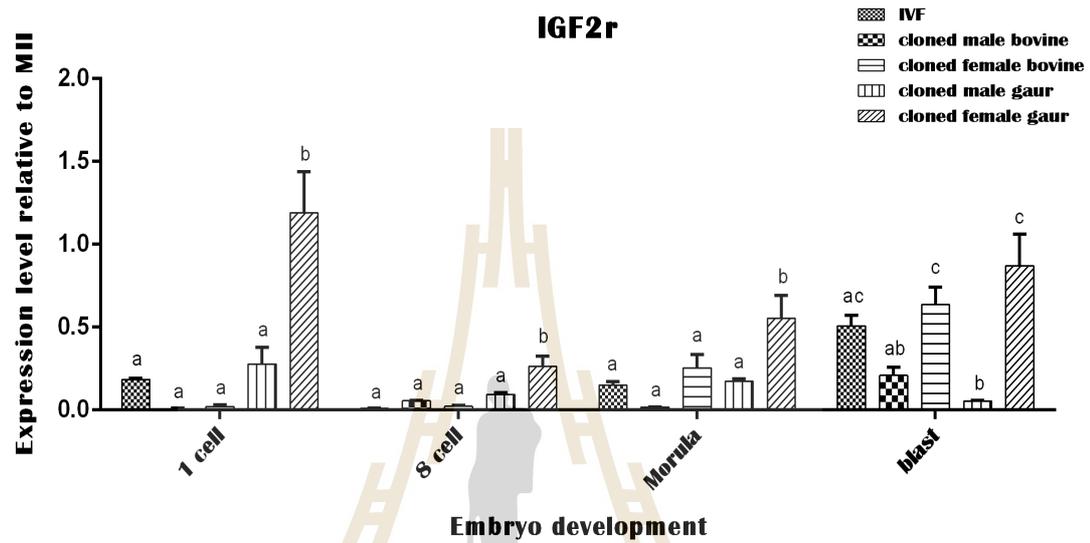
Figure 4.9 Relative expression levels of several candidate genes related with *Igf2*r gene derived from IVF, SCNT bovine and iSCNT gaur embryos at various stage of embryonic development. [a,b,c] Values have different superscripts are significantly different at $P<0.05$ using one-way ANOVA.

In addition, we examined the mRNA expression levels of pluripotent and epigenetic modification genes of fibroblast donor cells including male and female bovine and gaur. The expression of *Oct4* and *Hat1* mRNA level of fibroblasts derived from male and female gaur were higher mRNA level than male and female bovine fibroblasts ($P<0.05$). Moreover, the expression of *Hdac1* gene was higher mRNA level in male and female bovine than male and female gaur fibroblasts ($P>0.05$).

The relative transcription of *Dnmt1* gene in male and female bovine fibroblasts were significantly higher expression level than male and female gaur fibroblasts (P<0.05). However, the female bovine fibroblasts showed significantly higher mRNA level of *Dnmt3a* and *Dnmt3b* genes than the other types of fibroblasts (P<0.05). The expression mRNA level of *Igf2* gene in male bovine and male gaur fibroblasts were significantly higher of mRNA levels than female bovine and gaur fibroblasts (P<0.05). According, the expression transcription levels of *Igf2r* gene in female bovine and gaur fibroblasts were also significantly higher transcript levels than the male bovine and gaur fibroblasts (P<0.05).
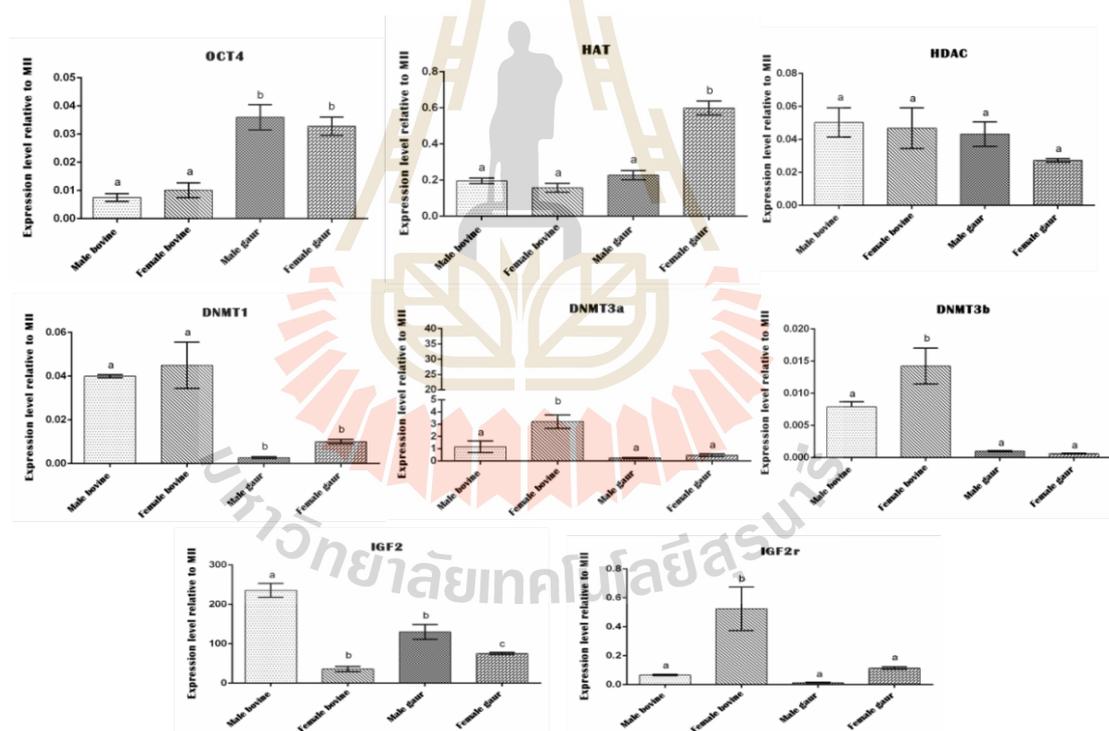
Figure 4.10 Relative expression levels of *Oct4, Hat1, Hdac1, Dnmt1, Dnmt3a, Dnmt3b, Igf2* and *Igf2r* genes of fibroblasts derived from male and female bovine and gaur. [a,b,c] Values have different superscripts are significantly different at P<0.05 using one-way ANOVA.

4.3 The effects of donor cell gender on histone modification of SCNT bovine and iSCNT gaur embryos

In order to examine the effect of donor cell gender on histone modification by immunostaining analysis, we were investigated the mean relative levels of histone acetylation (AcH4K5) and histone deacetylation (HDAC1) in IVF embryo, cloned embryos derived from SCNT bovine and iSCNT gaur embryos and also investigated fibroblasts derived from male and female bovine and gaur. The relative fluorescence intensity levels of histone acetylation (AcH4K5) in nuclei of IVF embryo and cloned embryos were similar pattern that showed variance of AcH4K5 levels at 2-cell to 8-cell stage following the acceleration of AcH4K5 levels at blastocyst stage. IVF embryo was higher levels of AcH4K5 intensity than cloned embryos derived from SCNT bovine and iSCNT gaur embryos (P>0.05). Moreover, the relative fluorescence intensity levels of AcH4K5 of different of donor cell gender was showed not significantly different in male and female SCNT bovine and iSCNT gaur embryos at blastocyst stage of embryonic development.

Normal embryo   Cloned embryo
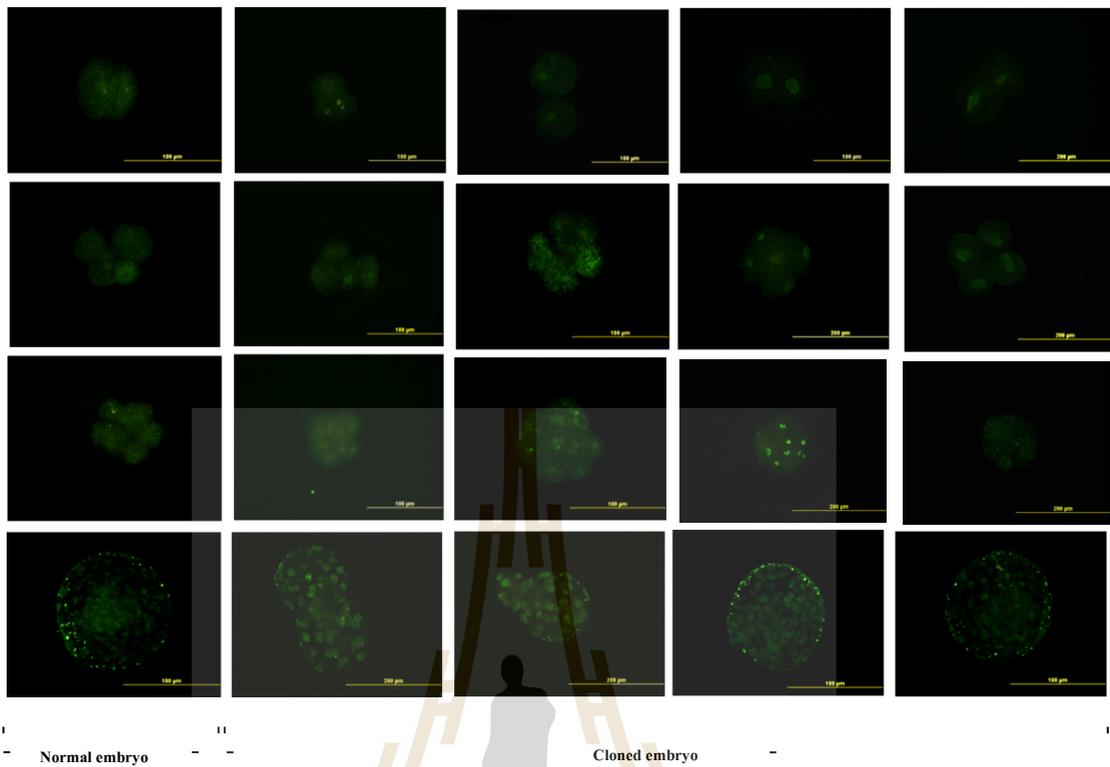
Figure 4.11 Immunostaining of histone acetylation (AcH4K5) of various embryo
types; (column A) in IVF embryo (column B) SCNT male bovine
embryo (column C) SCNT female bovine embryo (column D) iSCNT
male gaur embryo (column E) iSCNT female gaur embryo with anti-
AcK4H5 antibody.

Normal embryo   Cloned embryo
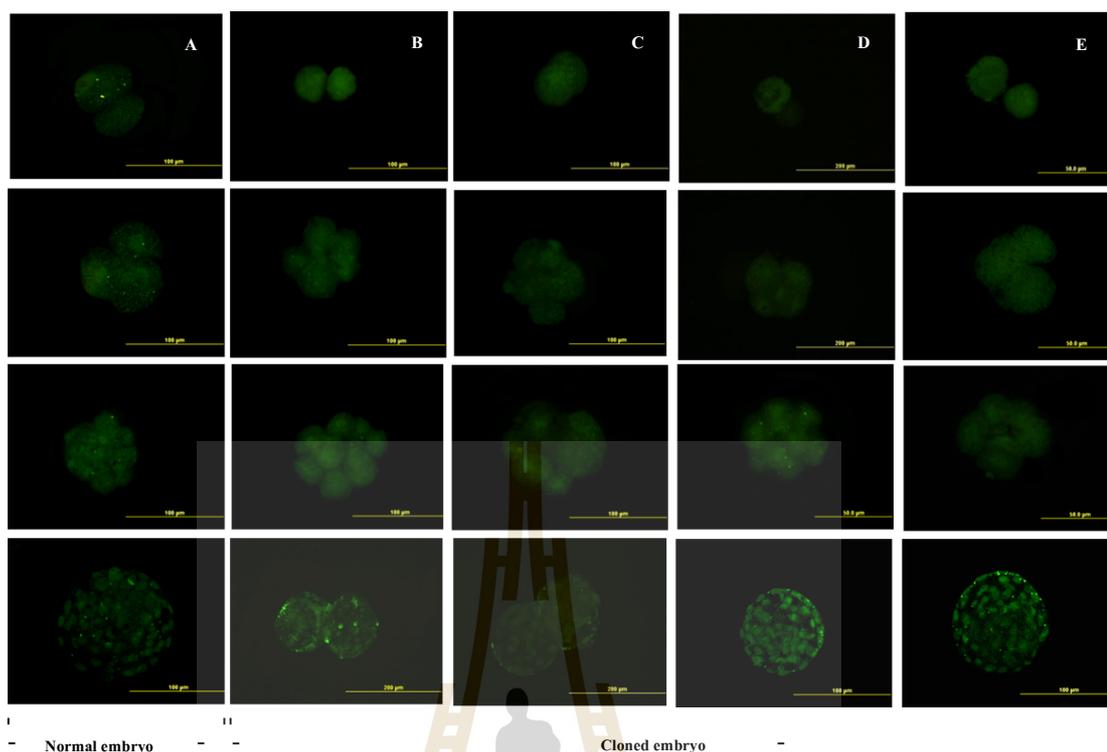
Figure 4.12 HDAC1 immunostaining of cloned at various stages of embryonic
development. The relative intensity levels of HDAC1 derive from
(column A) IVF embryo (column B) SCNT male bovine embryo
(column C) SCNT female bovine embryo (column D) iSCNT male gaur
embryo (column E) iSCNT female gaur embryo.

Accordingly, relative fluorescence intensity levels of HDAC1 demonstrated that
the iSCNT male bovine embryo was significantly highest of relative levels than
another embryo type (P<0.05), the IVF embryo was showed lowest relative levels of
histone deacetylation when compared with cloned embryos. At the blastocyst stage,
the iSCNT male gaur embryo showed significantly higher of HDAC1 levels than
iSCNT female gaur embryos (P<0.05). However, the SCNT bovine embryos
reconstructed with male and female bovine fibroblasts were not significantly different
of HDAC1 intensity levels at the blastocyst stage (P>0.05).
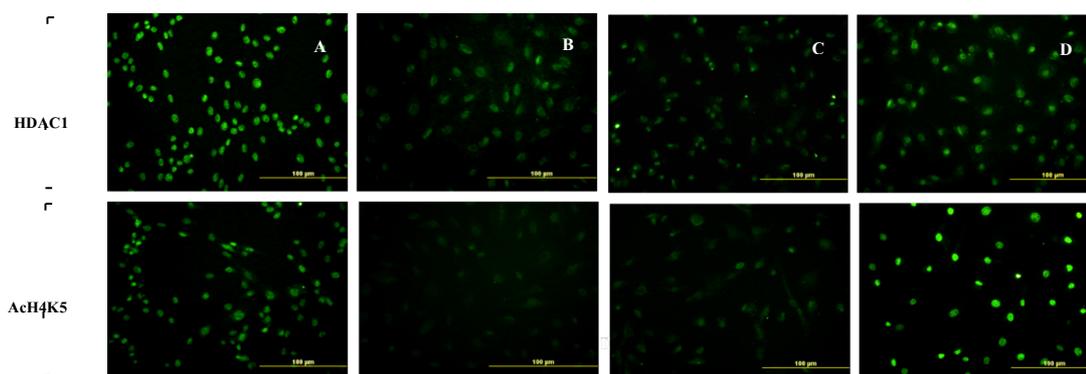
Figure 4.13 The relative intensity of histone acetylation and deacetylation with anti

HDAC1 and anti- AcK4H5 antibody, respectively. The fibroblast donor

cells were evaluated derived from (column A) male bovine fibroblast

(column B) female bovine fibroblast (column C) male gaur fibroblast

(column D) female gaur fibroblast.

Moreover, we investigated the intensity levels of histone acetylation and
deacetylation on donor cells derived from male and female bovine and gaur
fibroblasts, this result showed not significantly different of relative levels (P>0.05).
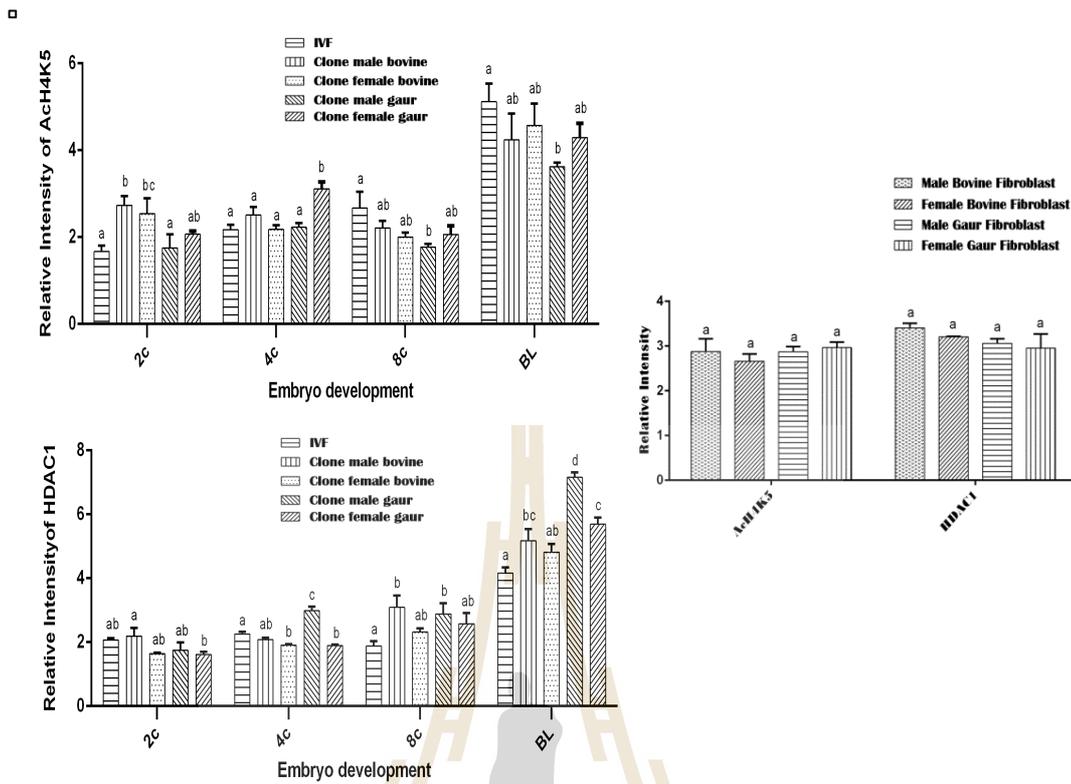
Figure 4.14 The relative intensity of histone acetylation and deacetylation with anti HDAC1 and anti- AcK4H5 antibody of all embryos types and fibroblasts. (A) represented the relative intensity of histone acetylation of IVF and cloned embryos derived SCNT bovine and iSCNT gaur embryos, (B) represented the relative intensity of histone deacetylation of IVF and cloned embryos derived SCNT bovine and iSCNT gaur embryos, (C) represented the relative intensity of histone acetylation and deacetylation of fibroblasts types. [a,b,c] Values have different superscripts are significantly different at P<0.05.

CHAPTER V

DISCUSSION AND CONCLUSION

The several studies were investigated the somatic cell nuclear transfer production of genetically animal offspring (Inoue et al., 2003; Tamada and Kikyo, 2004), which indicated the low efficiency of cloned embryos developmentally due to the incomplete reprogramming of somatic donor cell nuclei that might be faulty reprogrammed of differentiated cell into potency status of embryonic developmentally become to abnormality of cloned embryos (Beyhan et al., 2007). Thus, the improvement of efficiency in SCNT technique was investigated, previous studies were investigated the effect of different donor cell nuclei origin and/or type including adult, newborn, or fetal somatic cells that could be indicated the cloned blastocysts development derived from using the different types of donor cell nuclei were different percentages of development in SCNT cattle (Cho et al., 2002; Powell et al., 2004) while the different of donor cell has been little reported for increment of cloning efficiency , especially interspecies SCNT. Thus, we could be investigated the different of donor cell gender of cloned embryos on embryonic development and gene expression from the present study.

The results of fusion rate and blastocyst developmental of SCNT and iSCNT embryos derived from male and female bovine and gaur donor cell nuclei reconstructed with bovine oocyte cytoplasm were similar pattern of each somatic donor cell gender in their groups, which is correlated with previous study

(Kato et al., 2000; Vogel, 2001; Sangngam et al., 2005; Srirattana et al., 2012). This result indicated that the different of donor cell gender did not effect on the fusion rate and the blastocyst in *in vitro* culture developmental of both cloned embryo groups. While, the embryonic development of IVF embryo at blastocyst stage demonstrated high percentage rate than SCNT bovine and iSCNT gaur embryos that correlated with earlier studies (Srirattana et al., 2012), they have been reported that the aberrant of developmentally important gene expression of somatic cell nuclei is the key factor that cause to abnormality of cloned embryonic development (Daniels et al., 2000;). Moreover, this experiment show the iSCNT gaur embryo can develop to the blastocyst stage that there are supported with present study (Lanza et al., 2000; Vogel, 2001; Sangngam et al., 2005; Srirattana et al., 2012), which result indicate that the iSCNT in gaur can used the oocyte recipient from domestic cow and fused with somatic cell nuclei from gaur (*Bos gaurus*) by iSCNT technique (Lanza et al., 2000). The cell number of IVF blastocyst showed the high number of ICM and TE cells than both group of cloned blastocyst, which were accordant with previous study (Srirattana et al., 2012). As well as, our results showed the low number of ICM, TE and total cell in cloned embryos that these results correlated with previous reported in cloned mouse (Rybouchkin et al., 2002) and bovine (Fahrudin et al., 2002) blastocyst stage embryos, which was indicated the cloned embryos has low viability of blastocyst stage than IVF embryos (Van Soom et al., 1997). However, the cell number of inner cell mass (ICM) and trophectoderm (TE) cells between the SCNT and iSCNT blastocysts derived from different of donor cell gender were similar pattern that could be indicated the different of donor cell gender did not effect on the blastocyst formation or quality of cloned blastocysts development. So, these results concluded that the bovine recipient

cytoplasm could be remodeled when transferred the male and female bovine and gaur fibroblasts.

The transcription expression of cloned embryos is a valuable tool for the investigation of nuclear reprograming of the differentiated cell should be reprogramed into enucleated oocytes. Moreover, the SCNT technique can be produce cloned animals from different cell types of male and female donor cells in cloned sheep (Hoesseini et al., 2008) and cloned bovine (Kato et al., 2000) embryos. From these results indicated that the somatic cell cloning by using somatic cell nuclei derived from different donor cells (male and female) did not affect the embryonic development of cloned embryos but it has not been reported the influenced on the gene expression of epigenetic modification lead to abnormality cloned embryos. Thus, we were investigated the gene expression in cloned embryos derived from different of donor cell gender (male and female fibroblast). In experimental 2, we investigated the pluripotency (*Oct4*) and epigenetic modification (*Hat1, Hdac1, Dnmt1, Dnmt3a, Dnmt3b, Igf2 and Igf2r*) genes in IVF, SCNT and SCNT blastocysts by quantitative PCR (qPCR) technique.

The expression of the pluripotent gene as *Oct4* gene (The POU domain, class 5, transcription factor 1) is a transcription factor that is critical particularly important for establishment and maintenance embryonic pluripotency development (Niwa et al., 2000). These results were demonstrated the expression of *Oct4* gene in IVF embryo is dramatically increased and show high levels than cloned embryos, which is correlated with previous study (Boiani et al., 2002) at the blastocyst stage as our results. This may indicate the IVF embryo is demonstrated pluripotency characteristic to embryonic development (Wu and Schöler, 2014; Yeom et al., 1996; Imsoonthornruksa

et al., 2010) while our results showed the expression of *Oct4* gene has effect on cloned embryo derived from SCNT bovine and iSCNT gaur blastocyst development by low level of *Oct4* gene expression. These are cause of *Oct4* gene inability lead to the development defect of cloned embryos viability involved the low blastocysts development, which supported the present result with previous study (Bortvin et al., 2003; Mitalipov et al., 2003; Lorthongpanich et al., 2008) due to the silencing of a somatic gene is binding to promoter regions (Stadtfeld and Hochedlinger, 2010) lead to transcription repression in cloned embryos.

The histone modification is post-translational modifications of histone tail that provide an epigenetic marking system to regulate gene expression and chromatin remodeling (Turner, 2000), which is also important of epigenetic expression of cloned embryos (Tamada and Kikyo, 2004). The expression of histone acetylation and deacetylation were expressed on various stage of embryonic development in bovine oocyte and clone bovine embryo (McGraw et al., 2003). The transcription levels of *Hat1* and *Hdac1* genes in this study during preimplantation embryonic development, which is similar expression with previous reported (Suteevun et al., 2006), the increase of histone acetylation can enhance the reprogrammed in the epigenetic status for promoting in IVF blastocysts development (Enright et al., 2005). While, the somatic donor cells are incompetence of epigenetic reprogrammed by the erasure of epigenetic mark that causes from the histone acetylation is decrease the transcription mRNA levels in blastocysts stage (Imsoonthornruksa et al., 2010). These results demonstrated the somatic genome reprogramming had influenced on the variable of histone modification levels in cloned embryos. However, this results of the transcription level of *Oct4*, *Hat1* and *Hdac1* genes in SCNT bovine and iSCNT gaur

embryos derived from different of donor cell gender at blastocyst stage, which are show that the transcription expression of these genes were similar pattern even though the clone embryos reconstructed the different of donor cell origin as sex genotype. These results indicate that the different of sex-specific chromosome manner of donor cell did not influence on the aberrant expression of pluripotency (*Oct4* gene) and histone modification (*Hat1* and *Hdac1* genes) in SCNT bovine and iSCNT gaur blastocysts but the transcription of pluripotent gene has effect on the different groups of cloned embryos that the iSCNT gaur blastocysts are exhibit the correct expression of this gene in each somatic cell nuclei than the SCNT bovine blastocysts because the iSCNT gaur embryo was demonstrated the high mRNA levels of *Oct4* gene than the SCNT bovine embryo at the blastocyst stage. Whereas, the transcription of *Oct4*, *Hat1* and *Hdac1* genes in the present study could be effect to different expression pattern in SCNT bovine and iSCNT gaur embryos at early stage when cloned embryos reconstructed with male and female fibroblasts of each groups.

The major of epigenetic modification is DNA methylation of the genome that can regulate during embryonic developmental (Reik et al., 2001; Imsoonthornruksa et al., 2010). DNA methylation is a process by the addition of a methyl group to the CpG dinucleotide by DNA methyltransferases (DNMTs). The DNA methyltransferases family mainly is dividing into three members: *Dnmt1, Dnmt3a and Dnmt3b*. *Dnmt1* is important role in the maintenance of DNA methylation pattern during DNA replication (Bostick et al., 2007; Sharif et al., 2007). *De novo* DNA methyltransferases are *Dnmt3a* and *Dnmt3b*, which are responsible for the initial establishment of new DNA methylation pattern (Okano et al., 1998; Okano et al., 1999). This present results show that the transcription of global DNA methylation (*Dnmt1* gene) of IVF and

cloned embryos are high expressed at the early stage following the reduction of *Dnmt1* gene relative levels until the blastocyst stage, which are correlate with early study (Wrenzycki et al., 2001). This result is indicated that the global DNA methylation is reduced progressively with cleavage division until blastocyst stage (Monk et al., 1987) by perturbation with by passive demethylations (Santos et al., 2003) that can be caused the global DNA methylation is reduced progressively with cleavage division (Monk et al., 1987). However, the cloned embryos derived from SCNT bovine and iSCNT gaur are exhibit the high *Dnmt1* transcriptional activity that is influence the global DNA methylation patterns of somatic cell nuclei (*Dnmt1*s), which can maintain the DNA methylation patterns from somatic cell genome throughout the embryonic development because the somatic cell genome is obstruent to the erasure of DNA methylation in clone bovine embryos (Bourc'his et al. 2001; Dean et al., 2001). While, the *de novo* DNA methylation is activate by *Dnmt3a* and *Dnmt3b* genes were occurred high expression levels in morula and blastocyst stage of cloned embryos by the reduction of DNA demethylation activities. *Dnmt3a* and *Dnmt3b* activities had begun at the 8-16 cell stage in cloned bovine embryos (Wrenzycki et al., 2005; Dean et al., 2001) by the reduction of DNA demethylation activities. The *de novo* DNA methylation of cloned blastocyst embryos have initiated in lineage-specific cells of blastocyst stage as an inner cell mass (ICM) is hypermethylated while trophectoderm (TE) is hypomethylated (Santos et al., 2002) that are a reason for the dramatically higher methylation levels in blastocyst stage development, as well as, the aberrant expression of somatic cell reprogramming and faulty of the DNMT activity are cause the abnormality of DNA methylation pattern for properly embryonic transcription activation (Imsoonthornruksa et al., 2010) ,which are accordant with the results of the

present study. Moreover, Kang and colleague (2001) were investigated the expression levels of DNA methylation in cloned bovine morula and blastocysts stage by bisulfite analysis that showed higher methylation levels and unique sequences than normal embryos that could be recapitulate on the DNA methylation was expressed from the remodeling of epigenetic status in somatic nuclei (Kang et al., 2001). Previous study had been reported the DNA methylation level of cloned embryos depending on the embryonic stages examined, detection methods used, and donor cell type targeted DNA sequences (Kang et al., 2001). Moreover, the sex-specific methylation pattern of donor cell nuclei is effect on the epigenetic modification in preimplantation embryonic development. Previous study, the IVF bovine embryos were investigated the DNA methylation on male and female bovine embryos that the results showed the similar expression level of *Dnmt1* gene while the female embryo was lower expression of *Dnmt3a* and *Dnmt3b* than the male embryo (Bermejo-A'lvarez et al., 2008), which this reported was correlated with results of the present study of the expression pattern of *Dnmt3a* and *Dnmt3b* genes. The transcription levels of *Dnmt3a* and *Dnmt3b* genes were decreased in female embryos because it had been reported the XX ES cell line of DNA methylation pattern, the XX chromosome is overexpression of these genes that can lead to the reduction of *Dnmt3a* and *Dnmt3b* expression and is hypomethylation in XX chromosome germline (Zvetkova et al., 2005). However, our results show that the SCNT bovine blastocysts were showed not significant lower the mRNA levels of *Dnmt3a* and *Dnmt3b* genes in SCNT female bovine embryo while the iSCNT female gaur embryo had different of gene expression with iSCNT male gaur embryo of these genes that may be occurred the defect of the incompatibility between the somatic cell nuclei and nuclear component of oocyte recipient (Kim et al., 2004), which indicated

was distinctly different the transcription levels of the *de novo* DNA methylation pattern in iSCNT gaur embryos. Thus, the sex-specific of donor cell genome and/or the incompatibility between oocyte cytoplasm and transplanted donor cell nuclei in SCNT bovine and iSCNT gaur embryos are influence on the DNA methylation expression at the blastocyst stage development.

Genomic imprinting is epigenetic phenomenon and is important for development in normal mammalian from maternal and paternal contribution are expressed and regulated by DNA methylation and histone modification (Hu et al., 2000; Svensson et al., 1998). The expression of imprinted reprogramming is observed in cloned embryo development. The present study was investigated the transcription expression of insulin-like growth factor 2 (*Igf2*) as paternally imprinted and insulin-like growth factor 2 receptor (*Igf2r*) as maternally imprinted. The results of this study showed high levels of the transcription *Igf2* gene in iSCNT male gaur embryo following with SCNT male bovine embryo than the IVF embryo at the blastocyst stage, which indicated that the imprinted reprogrammed of somatic cell derived from male-specific genome was incomplete reprogrammed and overexpressed of the IGF2 allele lead to the monoallelic transcription and aberrant expression of embryonic developmental (Mizuki et al., 2001; Han et al., 2003), which was accordant with early reported (Han et al., 2003). Previous study was investigated the methylation level at the *Igf2* intragenic of differentially methylated region (DMR) in male and female blastocysts derived from *in vivo* and SCNT embryos that the male blastocyst derived from both groups showed high levels of this gene. So, the male embryo is aberrantly expression of *Igf2* imprinted gene while the female embryo was correct to the methylation level

at the *Igf2* intragenic reprogramming pattern in cloned embryos (Gebert et al., 2009).

The present study of the transcription levels of *Igf2r* maternally imprinted gene was showed similar pattern between IVF embryos and SCNT embryos at blastocyst stage, which was accordant with previous study (Han et al., 2003). Previous studies reported that the *Igf2r* transcript level in SCNT, IVF and *vivo* embryos were exhibited not different of expression level (Sawai et al., 2005), which accordant with our results of *Igf2r* gene expression at the blastocyst stage of cloned embryos. Moreover, the SCNT bovine and iSCNT gaur embryos from female fibroblasts were demonstrated high transcription level of *Igf2r* (maternally imprinted gene) at the blastocyst stage of our results that could be deduced the SCNT bovine embryos occurred defect of maternally imprinted gene expression by methylated reprogrammed from DNA methylation status of oocyte cytoplasm (Reik and Walter, 2001). The DNA methyltransferase enzymes promote the aberrant expression of imprinted genes involved in *Dnmt1o* is oocyte-specific isoform, which is maintained maternal imprints and another type is *Dnmt3L* may support the reconstruct specific methylation imprints in the female germline and co-localizes with *Dnmt3a* and *Dnmt3b* (Bourc'his et al., 2001) that are a reason to supported the higher transcription levels of maternally imprinted genes at oocyte and blastocyst stage embryonic development. Thus, the epigenetic reprogramming of *Igf2r* imprinted gene had inappropriate to reprogramming in female germ cell line due to this gene expression of *Igf2r* was aberrant expression in female cloned embryo. Previous study had been reported the *in vitro* environment and the sex chromosome had distinguished effect on the imprinted gene expression (Saini et al., 2015).

Furthermore, the results of the transcription levels of each donor cells types as male and female bovine and gaur were exhibit the similar pattern of gene expression in pluripotent status, histone modification and imprinted expression that indicated the somatic cell chromatin of donor cell of each groups are ability for maintained epigenetic reprogrammed during pre-implantation development, which accordant with early study (Li et al., 2006). However, the present study showed the DNA methylation was different pattern of transcription levels between each donor cell groups and their cloned embryos groups that indicated the epigenetic pattern of somatic cell is incomplete reprogramming between the somatic cell and oocyte recipient might be faulty to reprogrammed (Li et al., 2006).

The aberrant expressions of histone H4 acetylation reprogramming is observed in cloned bovine embryos lead to the abnormality expression of histone modification relate genes, which can contribute to failure of cloned embryo development (Wee et al., 2006). In previous experiment, they found the level of acetylated H4K5 was dramatically decreased at 2-cell and morula stages of preimplantation development (Ma and Schultz, 2008). Moreover, another study was examined gene expression of histone acetylation that shown the low level of histone acetylation expression between the 1-cell and 8-cell stages (Wiekowski et al., 1997), which can indicate that the transcription expression of histone deacetylation is activate during cleavage stage of cloned embryos. In the histone H4 acetylation modification by evaluated the AcH4K5 intensity, we found that the relative intensity of AcH4K5 in IVF blastocyst showed highly levels than both of cloned blastocyst derived from SCNT bovine and iSCNT gaur embryos, which is accordance with the previous study (Wee et al., 2006). These results may be indicate that the transcription expression of histone deacetylation is

activated during cleavage stage of cloned embryos or the faulty of EGA in cloned embryos for embryonic development due to the low activity of histone acetylation (Wee et al., 2006). However, the results of present study showed higher levels of the AcH4K5 intensity in female SCNT bovine and iSCNT gaur embryos at blastocyst stage, which correlated with previous study (Walid E. Maalouf et al., 2008) that investigated the histone acetylation of H4K5 in bovine parthenotes embryos as absence of the male pronucleus, the results showed enrichment of the histone acetylation in the female pronucleus lead to hyperacetylation in female embryos. Thus, the different of donor cell gender can be influence on different histone modification activity in cloned embryos. In addition, HDACs are generally to act as transcriptional repressors, by removing acetyl groups at inactive genes (Brunmeiret al., 2009) lead to chromatin condensation that is cause the transcriptional repressors activity (Brunmeiret al., 2009; Suteevun et al., 2006). The expression status of HDAC1 and AcH4K5 acetylation are inverses of gene activity by the enlargement of acetylation of H4K5 lead to the progressive decrease with HDAC1 status (Ma and Schultz, 2008), which accordant with our results showed the HDAC1 intensity of cloned embryos were higher levels than IVF embryos at blastocysts stage. The reduction of HDAC1 expression is effect on abnormality embryonic development that must be occurred during 8-cell, morula and blastocyst stage (Ma and Schultz, 2008). So, the reduction of HDAC1 expression has reactivated the efficiency of histone acetylation lead to active transcription in the normal embryo. However, the intensity of HDAC1 levels has not been reported in cloned embryos by using the different of donor cell gender that cannot deduce. These results demonstrated iSCNT male gaur embryos showed significantly high level of HDAC1 intensity than iSCNT female gaur

embryos at blastocyst stage (P<0.05) that can indicated the histone deacetylation could be incomplete reprogramming in iSCNT gaur embryos reconstructed with different of donor cell gender.

In conclusion, this study showed the effect of different donor cell gender on the embryonic development and epigenetic modification in SCNT bovine and iSCNT gaur embryos. We found that the fusion rate and blastocyst development did not influence of cloned embryos reconstructed with different of donor cell gender. Subsequently, the transcription expression of pluripotent and epigenetic modification in bovine SCNT and gaur iSCNT embryos with different of donor cell gender at blastocyst stage showed aberrant gene expression of imprinted genes as *Igf2* and *Igf2r* due to the somatic cell nuclei derived from the different of donor cell gender could be different epigenetic reprogramming of each sex donor cell. These results provide the understanding the epigenetic reprogramming mechanism in intra- and interspecies SCNT with the different of donor cell gender that causes aberrantly of transcription expression, especially in imprinted gene, and may be one of the reasons the faulty of epigenetic reprogramming of donor cell gender can be influenced on the abnormality development in clone animals.

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