EFFECTS OF TRICHOSTATIN A ON FULL-TERM DEVELOPMENT OF CLONED BOVINE AND GAUR EMBRYOS

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ผลของ Trichostatin A ต่อการเจริญเติบโตจนครบกำหนดคลอดของตัวอ่อนโคโคลนนิ่งและตัวอ่อนกระทิงโคลนนิ่ง

นางสาวกนกวรรณ ศรีรัตนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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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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กนกวรรณ ศรีรัตนา : ผลของ Trichostatin A ต่อการเจริญเติบโตจนครบกำหนดคลอดของตัวอ่อนโคโคลนนิ่งและตัวอ่อนกระทิงโคลนนิ่ง (EFFECTS OF TRICHOSTATIN A ON FULL-TERM DEVELOPMENT OF CLONED BOVINE AND GAUR EMBRYOS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 106 หน้า

องค์การระหว่างประเทศเพื่อการอนุรักษ์ธรรมชาติได้ขึ้นทะเบียนกระทิงให้เป็นสัตว์ป่าที่มีความเสี่ยงต่อการสูญพันธุ์สูง เทคโนโลยีการผสมพันธุ์ไม่สามารถนำมาใช้ในการเพิ่มจำนวนสัตว์ใกล้สูญพันธุ์ได้ เนื่องจากขาดแคลนไข่และตัวรับ การย้ายฝากนิวเคลียสข้ามสายพันธุ์จึงเป็นอีกทางเลือกหนึ่งสำหรับการทำโคลนนิ่งสัตว์ใกล้สูญพันธุ์โดยใช้ไข่และตัวรับจากสัตว์ที่มีสายพันธุ์ใกล้เคียงกัน อย่างไรก็ตาม ความสำเร็จในการผลิตลูกสัตว์ยังต่ำอยู่มาก เนื่องจากกระบวนการ reprogramming ที่ไม่สมบูรณ์ของเซลล์ต้นแบบ จากการวิจัยที่มีมาก่อนพบว่า Trichostatin A (TSA) ซึ่งเป็นสารเคมีที่ทำหน้าที่ยับยั้งการดึงหมู่อะเซทิลบนโปรตีนฮีสโตนออก สามารถเพิ่มประสิทธิภาพการทำโคลนนิ่งในสัตว์หลายชนิด ดังนั้นวัตถุประสงค์ของการทดลองนี้คือ ศึกษาผลของ TSA ที่มีต่อการเจริญเติบโตจนครบกำหนดคลอดของตัวอ่อนโคที่ได้จากการย้ายฝากนิวเคลียสในสายพันธุ์เดียวกันและตัวอ่อนกระทิงที่ได้จากการย้ายฝากนิวเคลียสข้ามสายพันธุ์

เซลล์ต้นแบบที่ใช้ในการทดลองนี้มาจากเซลล์ไฟโบรบลาสของโคและกระทิงทั้งเพศผู้และเพศเมีย การผลิตตัวอ่อนโคและกระทิงทำโดยหลอมรวมเซลล์ต้นแบบเข้ากับไข่โคที่กำจัดสารพันธุกรรมออกแล้ว จากนั้นตัวอ่อนที่ได้จะถูกแบ่งออกเป็นสองกลุ่มคือ กลุ่มที่เติม TSA และกลุ่มที่ไม่เติม TSA สำหรับกลุ่มที่เติม TSA ตัวอ่อนจะถูกกระตุ้นให้เกิดการแบ่งตัวและเลี้ยงในน้ำยาที่มี 50 nM TSA เป็นเวลานาน 10 ชั่วโมง หลังจากนั้นทำการย้ายตัวอ่อนไปเลี้ยงในน้ำยาที่ไม่เติม TSA นาน 7 วัน สำหรับกลุ่มที่ไม่เติม TSA จะทำการกระตุ้นแล้วเลี้ยงในน้ำยาที่ไม่มีการเติม TSA จากผลการทดลองพบว่าอัตราการเชื่อมติดของเซลล์ต้นแบบกับไข่ อัตราการแบ่งตัว อัตราการเจริญสู่ระยะ 8 เซลล์ และมอรูล่าของตัวอ่อนโคและกระทิงไม่มีความแตกต่างกันในกลุ่มที่เติม TSA และกลุ่มที่ไม่เติม TSA แต่พบว่า TSA สามารถเพิ่มอัตราการเจริญในระยะบลาสโตซีสของตัวอ่อนโค แต่ไม่สามารถเพิ่มอัตราการเจริญของตัวอ่อนกระทิงได้ อีกทั้งคุณภาพของตัวอ่อนที่ได้ไม่มีความแตกต่างกันในแต่ละกลุ่มทดลอง นอกจากนี้ การใช้เซลล์ต้นแบบเพศผู้หรือเพศเมียไม่มีผลต่ออัตราการเจริญของตัวอ่อนระยะก่อนการฝังตัว

ในการทดลองนี้ยังได้ทำการศึกษาอัตราการเจริญระยะหลังฝังตัวของตัวอ่อนโคแช่แข็งด้วยวิธี vitrification แบบหยด ก่อนการย้ายฝากตัวอ่อนแช่แข็งจะถูกละลายและล้างในสารละลายที่เจือจางเป็นลำดับขั้น จากนั้นตัวอ่อนโค ตัวอ่อนโคแช่แข็ง ตัวอ่อนกระทิง จากกลุ่มที่เติมและไม่เติม TSA จะถูกย้ายฝากให้โคตัวรับโดยวิธีไม่ผ่าตัด จากการทดลองพบว่า TSA ช่วยเพิ่มอัตราการ

ตั้งท้องในวันที่ 45 หลังการย้ายฝากในกลุ่มของตัวอ่อนโค แต่ไม่สามารถเพิ่มอัตราการตั้งท้องในตัวอ่อนกระทิงและตัวอ่อนโคแช่แข็งได้ อีกทั้ง TSA ไม่สามารถเพิ่มอัตราการเจริญของลูกอ่อนจากระยะหลังฝังตัวในวันที่ 45 ไปจนถึงครบกำหนดคลอดของลูกอ่อนในทุกกลุ่มทดลอง การใช้เซลล์ต้นแบบเพศผู้และเพศเมีย ไม่มีผลต่ออัตราการเจริญของตัวอ่อนกระทิงระยะหลังฝังตัว นอกจากนี้อัตราการเจริญระยะหลังฝังตัวของตัวอ่อนโคแช่แข็งไม่แตกต่างจากตัวอ่อนโคที่ไม่ได้แช่แข็ง แม้ว่าพบการแท้งของโคตัวรับที่ระยะ 3 เดือนแรก และ/หรือ ระยะ 3 เดือนสุดท้ายของการตั้งท้อง แต่มีโคตัวรับที่สามารถตั้งท้องจนครบกำหนดคลอดได้ สุดท้ายได้ลูกโคเพศผู้จากกลุ่มที่เติม TSA จำนวน 3 ตัว แต่ลูกโคตายระหว่างคลอด 1 ตัว และได้ลูกโคแฝดเพศผู้จากตัวอ่อนโคแช่แข็งกลุ่มที่เติม TSA นอกจากนี้ยังได้ลูกกระทิงเพศผู้จากกลุ่มที่ไม่เติม TSA จำนวน 1 ตัว แต่ลูกกระทิงมีความผิดปกติภายในปอด มีชีวิตได้เพียง 12 ชั่วโมง ผลการวิเคราะห์สารพันธุกรรมยืนยันได้ว่าลูกโค ลูกอ่อนกระทิงจากการแท้ง ลูกอ่อนกระทิงที่เป็นมัมมี่ และลูกกระทิงที่เกิดขึ้น มีสารพันธุกรรมมาจากเซลล์ต้นแบบ ลูกโคทั้ง 4 ตัว มีการเจริญเติบโตเป็นปกติและยังมีชีวิตอยู่จนถึงปัจจุบัน การทดลองนี้สรุปได้ว่า TSA สามารถเพิ่มอัตราการเจริญในระยะก่อนฝังตัว และอัตราการตั้งท้องของตัวอ่อนโคย้ายฝากนิวเคลียสในสายพันธุ์เดียวกันได้ แต่ไม่สามารถเพิ่มอัตราการเจริญของตัวอ่อนกระทิงย้ายฝากนิวเคลียสข้ามสายพันธุ์จากระยะตัวอ่อนไปจนถึงครบกำหนดคลอดได้

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา กนกวรรณ ศรีรัตนา
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

KANOKWAN SRIRATTANA : EFFECTS OF TRICHOSTATIN A ON FULL-TERM DEVELOPMENT OF CLONED BOVINE AND GAUR EMBRYOS. THESIS ADVISOR : ASST. PROF. RANGSUN PARNPAI, Ph.D., 106 PP.

INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER/ TRICHOSTATIN A/GAUR/BOVINE/FULL-TERM DEVELOPMENT

The International Union for Conservation of Nature has classified a gaur (*Bos gaurus*) as an endangered species and listed as vulnerable. Artificial reproduction as somatic cell nuclear transfer (SCNT) in endangered species has been limited due to the lack or shortage of oocytes and recipients. Interspecies somatic cell nuclear transfer (iSCNT) is an alternative technique for cloning endangered species using an oocyte and a recipient from related domestic species. However, the success rate of live offspring produced by this technique is still low because there was an incomplete reprogramming of donor cells. From previous studies, trichostatin A (TSA), a histone deacetylase inhibitor, can improve cloning efficiency in several species. Therefore, the objective of this study was to investigate the effects of TSA on full-term development of bovine SCNT and gaur iSCNT embryos.

Male and female fibroblasts from bovine and gaur were used as the donor cells. The bovine SCNT and gaur iSCNT embryos were produced by the fusion of an individual donor cell with enucleated bovine oocytes. After that, the embryos were divided into two groups: TSA-treated and non TSA-treated. For TSA-treated group, the embryos were chemically activated and cultured in culture medium supplemented

with 50 nM TSA. After 10 h of TSA treatment, embryos were continuously cultured in culture medium without TSA supplementation for 7 days. For non TSA-treated group, the embryos were activated and cultured in culture medium without TSA for 7 days. The results indicated that TSA treatment had no positive effects on the rates of fusion, cleavage, development to 8-cell and morula stages of bovine SCNT and gaur iSCNT embryos. However, TSA could enhance the blastocyst rate of bovine SCNT embryos but not in the gaur iSCNT embryos. Similar qualities of blastocysts were found in all treatment groups. Moreover, there were no differences on pre-implantation development of embryos derived from male or female fibroblasts.

The effect of TSA on post-implantation development of vitrified/thawed bovine SCNT embryos was also examined. The vitrified/thawed embryos were produced by the vitrification of bovine SCNT embryos using microdrop method and thawed by a stepwise dilution method. The bovine SCNT, vitrified/thawed bovine and gaur iSCNT embryos of TSA-treated and non TSA-treated groups were non-surgically transferred into synchronized bovine recipients. The results showed that TSA treatment could increase the pregnancy rate on day 45 after embryo transfer of bovine SCNT embryos. However, no beneficial effects were found in gaur iSCNT or vitrified/thawed bovine SCNT embryos. TSA could not enhance the fetal development after day 45 until term of all embryo types. No differences were found in the post-implantation development of gaur iSCNT embryos derived from male or female fibroblasts. Moreover, vitrified/thawed embryos have similar post-implantation developmental potential as fresh embryos. Although abortions at the first and/or third trimester of gestation were found, some recipients could maintain pregnancy status until term. Finally, three cloned calves from TSA-treated bovine

embryos were delivered. However, one calf of those died during veterinary-assisted delivery. Furthermore, twin cloned calves from vitrified/thawed embryos of TSA-treated group were born. One cloned gaur from male gaur iSCNT embryos in the non TSA-treated group was born. The cloned gaur died 12 h after birth with pulmonary disorder. DNA microsatellite analysis confirmed that all cloned bovine calves and gaur aborted fetus, mummified fetus and newborn were genetically identical to the donor cells. Up to now, the four cloned bovine calves have normal growth and are still alive.

In conclusion, TSA could enhance the pre-implantation development and pregnancy rate in bovine SCNT embryos. But no beneficial effects were found in full-term development of gaur iSCNT embryos.

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LIST OF ABBREVIATIONS

AI	=	artificial insemination
ART	=	assisted reproductive technique
COCs	=	cumulus-oocyte complexes
DCCC	=	donor cell-cytoplast couplet
ET	=	embryo transfer
HAT	=	histone acetyltransferase
HDAC	=	histone deacetylase
ICM	=	inner cell mass
iSCNT	=	interspecies somatic cell nuclear transfer
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
LOS	=	large offspring syndrome
mSOFaa	=	modified oviduct synthetic fluid with amino acids
NaBu	=	Sodium butyrate
SCNT	=	somatic cell nuclear transfer
S.E.M	=	standard error of mean
TE	=	trophectroderm
TSA	=	trichostatin A

CHAPTER I

INTRODUCTION

1.1 Background

Gaur (*Bos gaurus*), a member of the Bovidae family, is one of the protected animals in Thailand and listed as vulnerable animal by the International Union for Conservation of Nature (Duckworth et al., 2008). The number of gaur in Southeast Asia has been dramatically decreased because of wild meat hunting and hunting for horns trading.

Somatic cell nuclear transfer (SCNT) involves the transferring of the donor nucleus into an enucleated oocyte. This technique provides a unique tool for preservation of valuable individuals, livestock propagation, genetically modified animal production and research of biomedicine. SCNT has been applied to several mammalian species including sheep (Wilmut et al., 1997), 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). However, SCNT technique is almost impossible for the endangered species production because of the lack of oocytes and recipients. Interspecies somatic cell nuclear transfer (iSCNT) which the donor cells and oocytes are derived from different species, would be an alternative tool for the preservation of endangered species

(reviewed by Beyhan et al., 2007). Previous study found that bovine (*Bos taurus*) oocyte could support *in vitro* development of embryos derived from sheep, pig, monkey and rat (Dominko et al., 1999). Moreover, bovine oocytes have been used as recipient oocytes in many non-domestic bovids such as gaur (Lanza et al., 2000), buffalo (Kitiyanant et al., 2001), bongo (Lee et al., 2003), banteng (Sansinena et al, 2005) and takin (Li et al., 2006). The advantages of bovine oocyte are the availability of ovaries from slaughterhouse and the well understanding of *in vivo* and *in vitro* embryo culture (reviewed by Mastromonaco and King, 2007). To date, successful gaur iSCNT blastocyst production using bovine enucleated oocytes have been reported (Lanza et al., 2000; Sang-ngam et al., 2005; Mastromonaco et al., 2007). Lanza and colleagues (2000) successfully produced several pregnant recipients after transferring gaur iSCNT embryos to bovine recipients. However, only one cloned gaur was obtained but it died within 2 days after birth with diarrhea (Vogel, 2001). Although the offspring productions from iSCNT have been reported in several endangered species such as mouflon (Loi et al., 2001), gaur (Lanza et al., 2000; Vogel, 2001), Afican wild cat (Gómez et al., 2003), gray wolf (Kim et al., 2007) and bucardo (Folch et al., 2009), however, the birth rate is still low.

Several factors influence the success rate of iSCNT such as the iSCNT procedure, donor nucleus-recipient cytoplasm and fetal-maternal compatibilities (reviewed by Beyhan et al., 2007). The low pregnancy rate after transferring gaur embryos into bovine recipients cause by several pregnancy associated problems including abnormal placental development, fetal malnourishment and hypoxia (Hammer et al., 2001). The low overall success rates of SCNT and iSCNT productions consist of the incidents of poor embryo development, low pregnancy rate,

high frequency of early and late gestation losses, peri- and post-natal losses (Hill et al., 2000; Heyman et al., 2002). The live offspring production depends on the successful reprogramming of donor nuclear resulting in proper embryonic initiation and fetal gene expression (reviewed by Mastromonaco and King, 2007). Therefore, the developmental failure of cloned embryos and fetuses involve with incomplete reprogramming during nuclear transfer process, mainly due to errors at the epigenetic level (Jeanisch et al., 2002; Shi et al., 2003). Nearly 50% of cloned bovine and sheep blastocysts show epigenetic errors in both DNA methylation and histone acetylation (Santos et al., 2003; Shiota and Yanagimachi, 2002; Han et al., 2003).

In theory, assisted reprogramming of the donor nucleus via SCNT might improve embryo development (Wilmut et al., 2002). Many studies have attempted to explore the alterations in methylation and acetylation of donor nucleus before and after the SCNT process. Previous study indicated that the treatment of donor cells with histone deacetylase inhibitor, TSA, could increase the developmental rate to blastocyst of cloned bovine embryos (Enright et al., 2003). TSA could enhance the combination of acetylated histones (Yoshida et al., 1990) and DNA demethylation (Hattori et al., 2004) which also act as an effective inducer of phosphorylation (Zhong et al., 2003). Hyperacetylation of histone increases the access of some transcription factors to nucleosomes resulting in overexpression of imprinted genes (Lee et al., 1993). TSA treatment of cloned embryos has improved the *in vitro* embryo development of many species such as mouse (Kishigami et al., 2006), bovine (Ding et al., 2008), rabbit (Shi et al., 2008) and pig (Zhang et al., 2007). However, no positive effect on pre-implantation development was seen in cloned rat (Mizumoto et al., 2008) after TSA treatment. TSA could enhance the live birth rate in cloned mouses

and the cloned pups had no obvious abnormality (Kishigami et al., 2006). In contrast, TSA had no positive effect on pregnancy and birth rates of cloned rabbit embryos (Meng et al., 2009). For iSCNT, TSA could not improve *in vitro* development of human-rabbit embryos (Shi et al., 2008). On the other hand, TSA treatment of dog-pig iSCNT embryos could increase blastocyst rate when tail tip cell was used as donor cell but no positive effect was found when dew claw cell was used (Sugimura et al., 2009). These results were in consistent with the previous report that indicated that the beneficial effects of TSA treatment on cloned mouse embryos depends on the donor cell type (Kishigami et al., 2006). The effects of TSA treatment on cloning efficiency have been debatable in several species. The effects of TSA treatment on full-term development have not yet been examined in any species other than that of mouse and rabbit. This research is the first report on full-term development of bovine SCNT and gaur iSCNT embryos after TSA treatment.

1.2 Research objectives

1.2.1 To examine the effects of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos.

1.2.2 To examine the effects of TSA treatment on post-implantation development of bovine SCNT, gaur iSCNT and vitrified/thawed bovine SCNT embryos after transferring to bovine recipients.

1.2.3 To examine the effects of sex of donor cell on full-term development of bovine SCNT and gaur iSCNT embryos.

1.3 Research hypotheses

1.3.1 TSA treatment could enhance pre-implantation development of bovine SCNT and gaur iSCNT.

1.3.2 TSA treatment might have beneficial effect on post-implantation development of bovine SCNT, gaur iSCNT and vitrified/thawed bovine SCNT embryos and healthy cloned offspring can be obtained.

1.3.3 Sex of donor cell has no effect on full-term development of bovine SCNT and gaur iSCNT embryos.

1.4 Scope of the study

1.4.1 The effects of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos were examined. The rates of fusion, cleavage and *in vitro* development to blastocyst stage of TSA-treated embryos were observed compared with non TSA-treated embryos. The pre-implantation development of bovine SCNT and gaur iSCNT derived from male and female fibroblasts were observed. The total numbers of trophectoderm (TE) and inner cell mass (ICM) and also the TE: ICM ratios in each blastocyst were determined as an indicator of embryo quality.

1.4.2 The effects of TSA treatment on post-implantation development of bovine SCNT and gaur iSCNT embryos were examined. The pregnancy rate on day 45 after embryo transfer and pregnancy status on day 60, 90, 120, 150, 180, 210, 240 and 270 of gestation were observed. The post-implantation development of gaur iSCNT embryos derived from male and female fibroblasts were compared. The effect of vitrified/thawed bovine SCNT embryos of TSA-treated and non TSA-treated group

were also examined.

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CHAPTER II

LITERATURE REVIEW

2.1 Somatic cell nuclear transfer (SCNT) and interspecies somatic cell nuclear transfer (iSCNT)

Assisted reproductive techniques (ART) such as artificial insemination, embryos transfer, *in vitro* fertilization (IVF), gamete/embryo micromanipulation, embryo sexing, semen sexing and SCNT have been developed to obtain offspring from genetically individuals or infertile animal (reviewed by Andrabi and Maxwell, 2007). SCNT is the transplantation of a nucleus from donor cell individual into an enucleated oocyte of another individual. After Dolly, the first cloned sheep produced by SCNT was born in July, 1996 (Wilmut et al., 1997), SCNT has been successfully applied to many mammalian species for the production of offspring of many species for example, 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; Polejaeva 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 Z. et al., 2006).

SCNT is inefficient for the production of endangered animals because of the limitation of oocytes and recipient. Therefore, iSCNT is an alternative technique to solve this problem by transferring a donor cell from one species into an enucleated oocyte of another species (reviewed by Beyhan et al., 2007). This technique is useful

for studying nucleus-cytoplasm interaction and conservation of the endangered species. The cytoplasm of oocytes from bovine, sheep, and rabbit have been shown to be capable of reprogramming somatic cell from other species and also support the growth of such iSCNT embryos to blastocyst stage (reviewed by Beyhan et al., 2007). Bovine oocyte has been used to support embryo development of various species (Table 2.1), because of the availability of ovaries from local slaughterhouse and a great understanding of *in vivo* and *in vitro* development of bovine embryos (Reviewed by Mastromonaco and King, 2007). The majority of iSCNT experiments published to date report the production of at least blastocyst stage embryos, however, iSCNT embryos of llama (Sansinena et al., 2003), whale (Ikumi et al., 2004), cat (Thongphakdee et al., 2008), Crab-eating monkey (Lorthongpanich et al., 2008) and chimpanzee (Wang et al., 2009) were not capable of development past through blastocyst stage. But iSCNT of sheep (Dominko et al., 1999), gaur (Lanza et al., 2000), banteng (Sansinena et al., 2005) and yak (Li Y. et al., 2006) were established and pregnancies of surrogate mother were obtained. However, only gaur and banteng resulted in live birth. But the one gaur and two bantengs died within a few days after birth (Vogel, 2001; Holden, 2003). As with the other endangered species, mouflon has been successfully produced by mouflon-sheep iSCNT (Loi et al., 2001). Surprisingly, successful production of African wild cat iSCNT using domestic cat oocytes as recipient cytoplasts resulted in 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 (Gómez et al., 2004). Two cloned gray wolves were obtained by iSCNT of wolf cell and domestic dog oocyte (Kim et al., 2007). Moreover, an extinct mountain goat, bucardo (*Capra pyrenaica pyrenaica*), were produced by fusing of

bucardo fibroblasts with enucleated domestic goats. One cloned bucardo was obtained but died few minutes after birth due to physical defects in the lungs (Folch et al., 2009).

Although successful production of cloned animals from SCNT and iSCNT has been achieved, the efficiency of development to live offspring has remained very low due to developmental abnormalities occurring throughout embryo and fetal development and following birth. The success depends on the ability of the differentiated donor nucleus to be completely reprogrammed to initiate proper embryonic and fetal gene expression (reviewed by Mastromonaco and King, 2007). The completed reprogramming is affected by several factors including suitable of donor cells and recipient cytoplasm, compatibility between the karyoplast and cytoplast, technical efficiency (enucleation, fusion, oocyte activation) and optimal culture condition (Campbell, 1999; Smith et al., 2000). Moreover, individual and species-specific differences in chromosome number, genome-related events and maternal-embryonic transition all influenced the outcome (reviewed by Mastromonaco and King, 2007). Previous report indicated that some genes essential for early embryonic development were abnormally expressed in cloned bovine embryos, suggesting that aberrant transcription patterns detected in cloned embryos may lead to abnormalities at various embryonic stages (Bourc'his et al., 2001; Daniels et al., 2001; Bureau et al., 2003). Following embryos transfer, gestational losses have been associated with placental abnormalities (Hill et al., 1999 Chavatte-Palmer et al., 2002) and aberrant gene expression patterns have been reported in the placenta of cloned mice (Humpherys et al., 2002). Following birth, the offspring have been reported with a range of abnormalities including increased size (large offspring

syndrome, LOS), specific adult phenotypes and shorter life-span (Wells et al., 2004). Previous studies demonstrated that gene expression in the embryo, fetus and placenta are abnormal and suggested that inefficient or incomplete nuclear reprogramming is the cause of the abnormalities observed (Humpherys et al., 2002). For iSCNT, live offspring have been obtained by combining of donor nucleus and recipient oocyte from closely related species (reviewed by Beyhan et al., 2007). However, very low numbers of live iSCNT have been produced to date. The failure of iSCNT production may be due to the incompatibility between oocyte proteins and the donor nucleus, the mitochondrial DNA incompatibility and the lack of fetal-maternal interactions between the embryo and the surrogate mother uterus (reviewed by Beyhan et al., 2007).

Table 2.1 List of interspecies somatic cell nuclear transfer using bovine oocyte as recipient cytoplasts.

Donor cell	Blastocyst	Pregnancy	Offspring	References
Pig (*Sus sucrofa*)	Yes	No	-	Dominko et al., 1999
Rat (*Rattus rattus*)	Yes	-	-	Dominko et al., 1999
Rhesus monkey (*Macaca mulatta*)	Yes	-	-	Dominko et al ., 1999
Sheep (*Ovis aries*)	Yes	Yes	No	Dominko et al., 1999
Gaur (*Bos gaurus*)	Yes	Yes	Yes	Lanza et al., 2000; Vogel, 2001
Korean tiger (*Panthera tigiris altaica*)	Yes	-	-	Hwang et al., 2001
Water buffalo (*Bubalus bubalis*)	Yes	-	-	Kitiyanant et al., 2001
Horse (*Equus caballus*)	Yes	-	-	Li et al., 2002
Saola (*Pseudoryx nghetinhensis*)	Yes	-	-	Bui et al., 2002
Black bear (*Ursus thibetanus*)	Yes	-	-	Ty et al., 2003
Giant eland (*Taurotragus derbianus*)	Yes	No	-	Damiani et al., 2003
Human (*Homo sapiens*)	Yes	-	-	Chang et al., 2003
Llama (*Lama glama*)	No	-	-	Sansinena et al., 2003
Mouse (*Mus musculus*)	Yes	-	-	Arat et al., 2003
Bongo (*Tragelaphus eurycuerus isaaci*)	Yes	-	-	Lee et al., 2004
Chicken (*Gallus gallus*)	Yes	-	-	Kim et al., 2004
Whale (*Balaenoputera bohaerensis*)	No	-	-	Ikumi et al., 2004
Banteng (*Bos javanicus*)	Yes	Yes	Yes	Sansinena et al., 2005
Dog (*Canis familiaris*)	Yes	-	-	Murakami et al., 2005
Takin (*Budorcas taxicolor*)	Yes	-	-	Li, Y., et al., 2006
Yak (*Bos grunniens*)	Yes	Yes	No	Li, Y., et al., 2006
Cat (*Felis catus*)	No	-	-	Thongphakdee et al., 2008
Crab-eating monkey (*Maccaca fascicularis*)	No	-	-	Lorthongpanich et al., 2008
Chimpanzee (*Pan troglodytes*)	No	-	-	Wang et al., 2009

-, not attempted

2.2 Improving somatic cell nuclear transfer techniques

As discussed above, the success rates of SCNT are still low. Many studies have attempted to improve the original techniques. Chemicals such TSA, 5-aza-2'-deoxycytidine or cellular extracts were used to improve embryo development to blastocyst and implantation rates (reviewed by Keefer, 2008)

TSA (Figure 2.1) was first isolated from *Streptomyces hygroscopicus* as an antifungal antibiotics active against *Trichophyton* (Tsuji et al., 1976; Tsuji and Kobayashi, 1978) but a wider potential was soon revealed. TSA could arrest the mammalian cell cycle and induces differentiation of tumor cells (Yoshida et al., 1987). Moreover, TSA treatment of cells was found to have hyperacetylation of their histones due to the decrease of deacetylation activity (Yoshida et al., 1990). TSA is known as a histone deacetylase (HDAC) inhibitor (Yoshida et al., 1995).

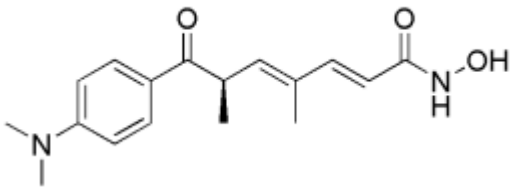

Figure 2.1 Chemical structure of TSA.

In eukaryotes, genomic DNA is package with histone proteins into chromatin, compacting DNA some 10,000-fold (reviewed by Grant and Berger, 2001). The basic repeating unit of chromatin is the nucleosome, typically composed of an octamer of the four core histone H2A, H2B, H3 and H4 and 146 basepairs of DNA wrapped around the histones (Luger et al., 1997). Each core histone is composed of a structured domain and an unstructured amino-terminal tail of 25-40 residues. The

acetylation and deacetylation of the ε-amino groups of conserved lysine residues present in histone tails has long been linked to transcriptional activity (Allfrey et al., 1964). When histones are acetylated by a protein called histone acetyltransferase (HAT), the histones will be neutralized of the positive charges on the tail regions, reducing their ability to bind to DNA and thus loosening the structure of chromatin which allows gene expression. After a short time, the histones will be commonly deacetylated by HDAC which causes the gene expression to stop. The balance of these activities contributes to transcriptional control (reviewed by Grant, 2001). TSA binds to HDAC to inhibit the HDAC action and thereby inhibits histone deacetylation, leading to hyperacetylation of chromatin (Figure 2.2). Highly acetylated regions are more accessible to the transcription factors and are actively transcribed (Lee et al., 1993).

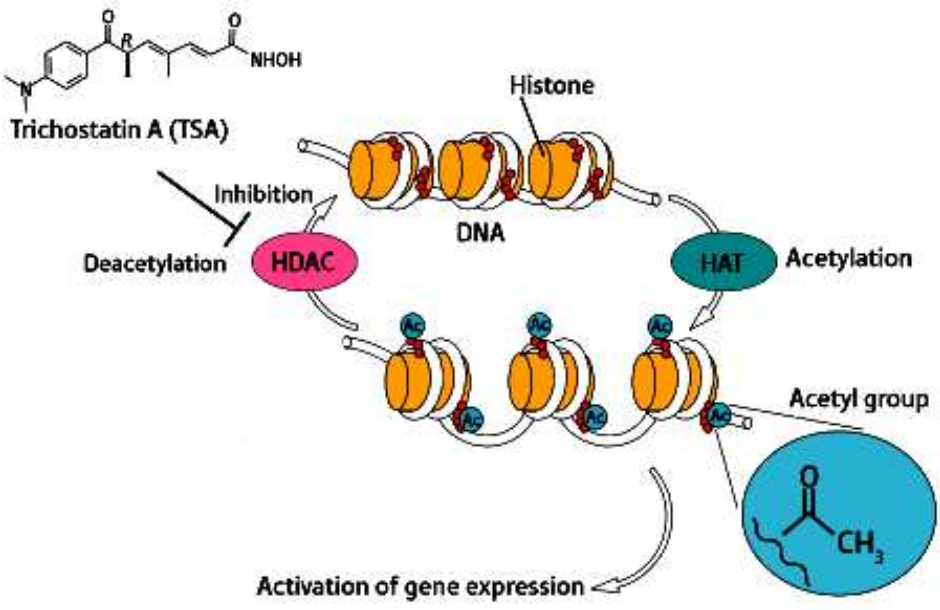

Figure 2.2 Inhibition of histone deacetylation by TSA (Yoshida, 2008).

Previous studies have reported that increasing the histone acetylation level in donor cells improves developmental potential of SCNT embryos (Enright et al., 2008). Kishigami and colleagues (2006) found that treatment of 5-50 nM TSA for 10 h following oocyte activation resulted in more efficient of *in vitro* development of mouse SCNT embryos to the blastocyst stage from two to five fold depending on the donor cell types. They used tail tip cells, spleen cells, neural stem cells and cumulus cells as donor cells. Moreover, adult male and female outbred mice were successfully cloned only when TSA was applied (Kishigami et al., 2007). The beneficial effect of TSA treatment in mouse SCNT was also found in other reports. Rybouchkin and colleagues (2006) found that extension of the TSA treatment (100 µM) beyond the activation point up to 9 h increased the blastocyst rate and quality of mouse SCNT embryos. Tsuji and colleagues (2009) reported that mouse SCNT oocytes treated with TSA for 8 to 12 h had higher rate of development to blastocyst and full-term fetuses were obtained. In contrast from previous reports, Maalouf and colleagues (2009) reported that the cleavage and blastocyst rates of TSA-treated mouse embryos were similar compared with non TSA-treated embryos. However, blastocysts from the TSA-treated embryos had a greater number of inner cell mass cells than those from the non TSA-treated embryos. The number of live pups obtained with TSA-treated embryos was higher than the non TSA-treated embryos (Maalouf et al., 2009). Li and colleagues (2008) demonstrated that treatment of mouse SCNT embryos with TSA influences the expression of chromatin structure- and DNA methylation-related genes at the blastocyst stage and selectively increases the expression level of *Sox2* and *cMyc*, which are responsible for embryonic development, at blastocyst stage.

In bovine, treating bovine donor cells with a low dose of TSA (0.08 µM)

increased development of cloned embryos to the blastocyst stage (Enright et al., 2003). Iwamoto and colleagues (2007) reported that treatment of bovine SCNT embryos with 50 nM TSA for 48 h post-activation increased the blastocyst rate. Relative levels of DNA methylation of TSA-treated cloned and IVF embryos did not differ but were lower than those of non TSA-treated cloned embryos. TSA treatment decreased the DNA methylation levels of cloned bovine embryos to the similar levels of IVF embryos, resulting in improved blastocyst development of the cloned embryos (Iwamoto et al., 2008). Ding and colleagues (2008) reported that treatment of donor cells, cloned embryos and continuous treatment of both donor cells and cloned embryos with 0.05 μM TSA in bovine SCNT increased the blastocyst rate. TSA treatment induced a higher level of histone acetylation and lower level of DNA methylation at the 2-cell stage in bovine SCNT embryos, which facilitates epigenetic reprogramming of the transferred somatic cell nucleus. Oh and colleagues (2009) reported that the short-term treatment of bovine embryos after activation with high concentration of TSA (100 nM for 4 h) improved the blastocyst formation. However, there is no beneficial effect on total cell number of blastocysts. Oliver and colleagues (2009) shown that treated with 5 nM TSA for 18 h after activation of bovine SCNT embryos, The blastocyst rate showed higher than control group but there was no difference in the pregnancy rate on day 150 of gestation. In contrast, Akagi and colleagues (2007) reported that treating with 50 nM TSA for 15 h after activation did not affect the *in vitro* developmental competence, but increased total cell number in bovine SCNT embryos. Iager and colleagues (2008) found that the treatment of bovine SCNT embryos with 50 nM TSA for 13 h could produce eight-cell embryos with levels of acetylation of histone H4 at lysine 5 similar to IVF embryos and greater

than in non TSA-treated SCNT embryos. However, the cleavage rate, blastocyst formation and total cell numbers of blastocyst in TSA-treated embryos were similar to IVF embryos and non TSA-treated SCNT embryos. Moreover, TSA treatment during bovine IVF does not affect blastocyst development but altered the cell number of ICM (Ikeda et al., 2009).

In other species, beneficial effects of TSA were found in pig SCNT. Zhang and colleagues (2007) showed that treatment of porcine SCNT embryos with 50 nM TSA for up to 24 h post-activation could improve blastocyst yield compared to the non TSA-treated embryos, where as similar cleavage rate and total cell number per blastocyst were observed. Li and colleagues (2008) found that the treatment of porcine embryos produced by handmade cloning technique with 37.5 nM TSA for 22-24 h post-activation increased the blastocyst rate compared to non TSA-treated embryos. However, the cell number per blastocyst did not differ between groups. The one hundred thirty of TSA-treated blastocysts were transferred into two recipients resulting one pregnancy and birth of one live and five dead piglets. Yamanaka and colleagues (2009) reported that TSA treatment of 5 nM for 15-20 h was optimal conditions for miniature pig SCNT embryos, increased the blastocyst rate and mean cell number as compared with the non TSA-treated group.

In rabbit, Shi and colleagues (2008a) found that the histone acetylation patterns of TSA-treated rabbit SCNT embryos appeared to be more similar to that of IVF embryos than non TSA-treated SCNT embryos. Meng and colleagues (2009) reported that no differences in the cleavage and blastocyst rates, cell number of blastocyst, pregnancy rate and term development rate were found between TSA-treated and non TSA-treated rabbit embryos. Similarly, no beneficial effect of TSA

treatment on the developmental potential was found in rat SCNT embryos (Mizumoto et al., 2008).

For iSCNT embryos, Shi and colleagues (2008b) reported that TSA treatment could not improve blastocyst rate of human-rabbit iSCNT embryos. On the other hand, TSA treatment could increase the blastocyst rates of rabbit SCNT more than two times when compare with non TSA-treated embryos. Sugimura and colleagues (2009) found that TSA treatment did not improve the blastocyst rates of dog-pig iSCNT embryos from dewclaw cells but did so in the embryos derived from tail-tip cells. They suggested that the beneficial effects of TSA treatment on dog iSCNT embryos depends on the donor cell type. This finding was consistent with a report by Kishigami and colleagues (2006). Furthermore, TSA treatment did not improve the embryo development of sei whale-bovine iSCNT embryos (Bhuiyan et al., 2009). The effects of TSA treatment on SCNT and iSCNT embryos were summarized in Table 2.2.

Table 2.2 The improvement of SCNT and iSCNT embryos after TSA treatment**.**

Experiment	TSA treatment	Improvement			References
		% Blastocyst[A]	Cell Number[B]	Pregnancy rate	
mouse SCNT	5-50 nM 10 h	Yes	-	Yes	Kishigami et al., 2006
	100 μM 9 h	Yes	No	Yes	Rybouchkin et al., 2006
	5 nM 10 h	No	Yes	Yes	Maalouf et al., 2009
	100 nM 8-12 h	Yes	-	Yes	Tsuji et al., 2009
bovine SCNT	50 nM 15 h	No	Yes	-	Akagi et al., 2007
	50 nM 48 h	Yes	-	-	Iwamoto et al., 2007
	0.05 μM 12 h	Yes	-	-	Ding et al., 2008
	50 nM 13 h	No	No	-	Iager et al., 2008
	100 nM 4 h	Yes	No	-	Oh et al., 2009
	5 nM 18 h	Yes	-	-	Oliver et al., 2009

A, percentage of embryo development to blastocyst stage; B, total cell numbers of blastocyst; -, not attempted.

Table 2.2 (Continued).

Experiment	TSA treatment	Improvement			References
		% Blastocyst[A]	Cell Number[B]	Pregnancy rate	
pig SCNT	50 nM 24 h	Yes	No	-	Zhang et al., 2007
	37.5 nM 22-24 h	Yes	No	-	Li et al., 2008
	5 nM 15-20 h	Yes	Yes	-	Yamanaka et al., 2009
rabbit SCNT	5 nM 10 h	No	No	No	Meng et al., 2008
	100 nM 6 h	Yes	-	-	Shi et al., 2008b
rat SCNT	5 nM 2 h	No	-	-	Mizumoto et al., 2008
human-rabbit iSCNT	100 nM 3, 6 h	No	-	-	Shi et al., 2008
dog-pig iSCNT	5 nM 10 h	Yes*	No	-	Sugimura et al., 2009

A, percentage of embryo development to blastocyst stage; B, total cell numbers of blastocyst; -, not attempted.

*, Blastocyst rate was increased when tail tip cells were used as donor cell.

2.3 Gaur

Gaur is the largest species of wild bovine. It is found in Bangladesh, Bhutan, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, Thailand, Viet Nam and Sri Lanka (Duckworth et al., 2008). The number of gaur is dramatically decreasing cause by the illegal hunting for horns and loss of suitable habitat. In thailand, gaur has been declared as the protected animal by Thai government and currently listed as vulnerable by the IUCN Red List of Threatened Species (Duckworth et al., 2008).

ARTs have been used to increase the number of gaur. Stover and colleagues (1981) has successfully produced gaur offspring by transferred gaur embryos into Holstein bovine recipients. The knowledge of artificial insemination (AI) technique in bovine has been applied for gaur production. The captive gaurs were synchronized by prostaglandin and AI was performed using frozen/thawed gaur spermatozoa and live gaur offspring were produced (Junior et al., 1990). Johnston and colleagues (1994) reported that immature gaur oocytes are capable of *in vitro* maturation and IVF with frozen/thawed sperm. Gaur embryos could develop to blastocyst stage after *in vitro* cultured for 7 days. The blastocysts were individually transferred into naturally estrus Holstein bovine recipient. A live-birth gaur was delivered by caesarian section on day 308 after embryos transfer. More recently, twelve gaur embryos derived from IVF were transferred into nine synchronized Holstein recipients and five pregnancies were obtained. Only three recipients carried fetus to term, with one recipient diagnosed as carrying twins. Two gaur offsprings died within 26 h after birth and two gaur offsprings were stillborn. Furthermore, abnormal placentas were found in interspecies gaur fetuses after transferred embryos to bovine recipients (Hammer et al., 2001). In 2000, Lanza and colleagues have performed iSCNT embryos using gaur skin

fibroblasts fused with enucleated bovine oocytes. Six hundred ninety two reconstructed embryos developed to blastocytes 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 late-term abortion at day 202 of gestation (Lanza et al., 2000). 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). Sang-ngam and colleagues (2005) had studied developmental potential of iSCNT gaur embryos derived from male and female cloned gaur fibroblasts reconstructed with enucleated bovine ooocytes. They found that the rates of fusion, cleavage and development to blastocyst stage at day 6 were not significant difference between male and female fibroblasts-derived embryos. However, embryos derived from male fibroblasts had higher percentage of blastocyst at day 7 than those female fibroblasts. Mastromonaco and colleagues (2007) 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. Poor blastocyst development (18.1%) accompanied by developmental delayed, decreased cell numbers, aberrant apoptotic and related gene expression profiles were observed.

As described herein, TSA treatment could enhance the development competence of SCNT embryos in mouse, pig, bovine and rabbit. However, the effects of TSA treatment on cloning efficiency are controversial in several species and some groups have reported that TSA treatment had various detrimental effects on development of SCNT embryos. Moreover, no beneficial effect of TSA was found in

iSCNT embryos. The conflicting results may be due to the difference of species, exposure time and concentration of TSA. There is no report about effect of TSA on gaur iSCNT embryos. Therefore, the beneficial effects of TSA on gaur iSCNT embryos are needed to be examined.

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CHAPTER III

EFFECT OF TRICHOSTATIN A ON PRE-IMPLANTATION DEVELOPMENT OF CLONED BOVINE AND GAUR EMBRYOS

3.1 Abstract

The success rate of gaur production by iSCNT is still low. Incomplete nuclear reprogramming leads to abnormal cloned embryo development. TSA, histone deacetylase inhibitor has been used for improving cloning efficiency in many species. This study was carried out to investigate the effect of TSA on pre-implantation development of bovine SCNT and gaur iSCNT embryos. The bovine enucleated oocytes were used as recipient cytoplasm for male and female bovine and gaur fibroblasts. Donor cell-cytoplast was fused by electrical pulses. After fusion, oocytes were divided into two groups, TSA-treated and non TSA-treated. For the TSA-treated group, oocytes were activated and cultured in culture medium supplemented with 50 nM TSA. After 10 h of TSA treatment, the reconstructed embryos were transferred to embryo culture medium without TSA. For non TSA-treated group, oocytes were activated and cultured with the same culture system except no TSA supplementation. The results showed that, there was no significant difference in fusion rate, cleavage rate, development to 8-cell and morular stage among treatment groups ($P>0.05$). Blastocyst formation of bovine SCNT embryos from TSA-treated group is significant

higher than other groups ($P<0.05$). The cell number of TE, ICM and the proportion of TE:ICM from bovine SCNT and gaur iSCNT blastocysts were not significant difference in either TSA-treated or non TSA-treated, male or female fibroblasts. Therefore, TSA treatment can improve development of bovine SCNT embryos but cannot improve the development of gaur iSCNT embryos.

3.2 Introduction

Gaur is the largest species of wild bovine (male 700-1000 kg, female 550-700 kg) found in South East Asia. Gaur becomes an endangered species because of it is low infertile and illegal hunting (Johnston et al., 1994). Due to the limitation of oocytes and surrogate mothers in endangered species, iSCNT can facilitates rescue of endangered species. iSCNT is a method involved transferring a donor cell from one species into a recipient oocyte of another species. Bovine, sheep and rabbit oocytes have been used for iSCNT (Dominko et al., 1999; White et al., 1999; Chen et al., 2002). The advantage of bovine oocyte is easy to obtain and well manipulated. Previous reports confirmed that the bovine cytoplasm has the ability to support embryo development of several mammalian species such as rat, sheep, pig, monkey (Dominko et al., 1999), gaur (Lanza et al., 2000), buffalo (Kitiyanant et al., 2001), human (Chang et al., 2003), mountain bango antelope (Lee et al., 2003), whale (Ikumi et al., 2004), banteng (Sansinena et al., 2005), yak, dog (Murakami et al., 2005), and takin (Li et al., 2006). However, the successful offspring of iSCNT have been observed in only closely related species such as mouflon (*Ovis orientalis musimon*)-sheep (*Ovis aries*) (Loi et al., 2001), gaur-bovine (Lanza et al., 2000, Vogel 2001), african wild cat (*Felis silvestris libica*)-domestic cat (*Felis silvestris catus*) (Gómez et

al., 2003), gray wolf (*Canis lupus*)-domestic dog (*Canis familiaris*) (Kim et al., 2007) and mountain goat, bucardo-domestic goat (*Capra aegagrus hircus*) (Folch et al., 2009) . Lanza and colleagues (2000) transferred gaur iSCNT blastocysts to bovine surrogate recipients. Eight recipients were pregnant (25%). Only one recipient developed to term. The cloned gaur was healthy at birth but died 2 days after birth due to fatal bacterial infection (Vogel, 2001). Sang-ngam and colleagues (2005) found that gaur iSCNT embryos derived from male fibroblasts showed higher percentage of blastocysts at day 7 when compared to iSCNT embryos derived from female fibroblasts. Mastromonaco and colleagues (2007) reported that the developmental delay, decreased cell numbers, aberrant apoptotic and aberrant related gene expression were found in gaur iSCNT blastocysts. Although gaur iSCNT embryo production using bovine oocyte has been accomplished and pregnancies were established, the blastocyst rate and the offspring were still low. Previous reports showed that TSA, a histone deacetylase inhibitor, has been used to improve cloning efficiency in many species such as mouse (Kishigami et al., 2006; Rybouchkin et al., 2006), bovine (Enright et al., 2003; Shi et al., 2003), porcine (Zhang et al., 2007). Moreover, no occurrence of LOS and an abnormal phenotype was observed in cloned mice after TSA treatment (Kishigami et al., 2006). Therefore, investigation of the effect of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos was the aim of this study.

3.3 Materials and methods

3.3.1 Donor cell preparation

Bovine fibroblasts: Ear skin tissues were biopsied from male and female

bovines (Figure 3.1A) and transported to the laboratory on ice in 0.9% NaCl (Carlo Erba, France, 479687). Skin tissues were removed from cartilage and cut into small pieces (about 1 mm^2, Figure 3.1B) before being placed in a 60-mm culture dish (Nunc, Denmark, 150288) and covered with a glass slide (Figure 3.1C). Five milliliters of αMEM (Minimum Essential Medium Eagle, Alpha modification, Sigma, USA, M-0644) plus 10% fetal bovine serum (FBS, Gibco, USA, 10270-098) was added to the dish and then cultured under a humidified atmosphere of 5% CO_2 in air at 37°C for 8-10 days. The medium was replaced every three days. At sub-confluence, the fibroblasts outgrowth (Figure 3.1D) were harvested using 0.25% trypsin (Sigma, T-4799) / EDTA (Sigma, E-4884) in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) and seeded in 5 ml αMEM plus 10% FBS of 75-cm^2 culture flask (Nunc, 156499). The fibroblasts were frozen at the fourth passage of cell culture in αMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO, Merck, Germany, 116743) and kept in liquid nitrogen.

Gaur fibroblasts: Skin tissues were biopsied from male and female gaurs (Figure 3.2A) by biopsied gun (Figure 3.2B) and transported to the laboratory on ice in 0.9% NaCl. Skin tissues (Figure 3.2C) were removed from adipose tissue and cut into small pieces (about 1 mm^2). Skin tissues were cultured and frozen as bovine fibroblasts.

Frozen-thawed donor cells were cultured in 35-mm culture dish (Nunc, 153066) for 2-3 days before used as donor cells. Only the fourth passage of cell culture of all cell types was used as donor cell for SCNT. A few minutes before injection the proliferating donor cells were harvested by standard trypsinization and resuspened in Emcare® holding medium (ICP bio, New Zealand, ECHM-500).

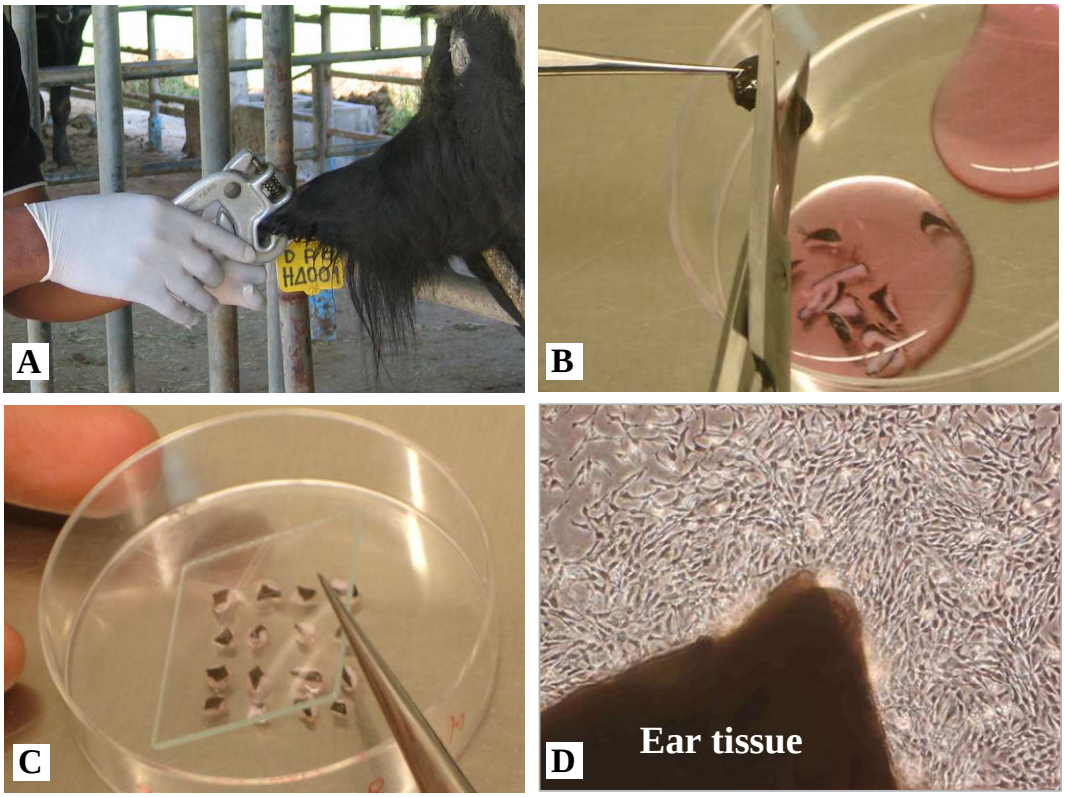

Figure 3.1 Bovine fibroblasts preparation. Ear skin was biopsied from bovine (A). Skin tissue was cut into small pieces (B) before being placed in culture dish and covered with a glass slide (C). Fibroblasts were outgrown from ear tissue after 8-10 days of cultured (D, magnification 40x).

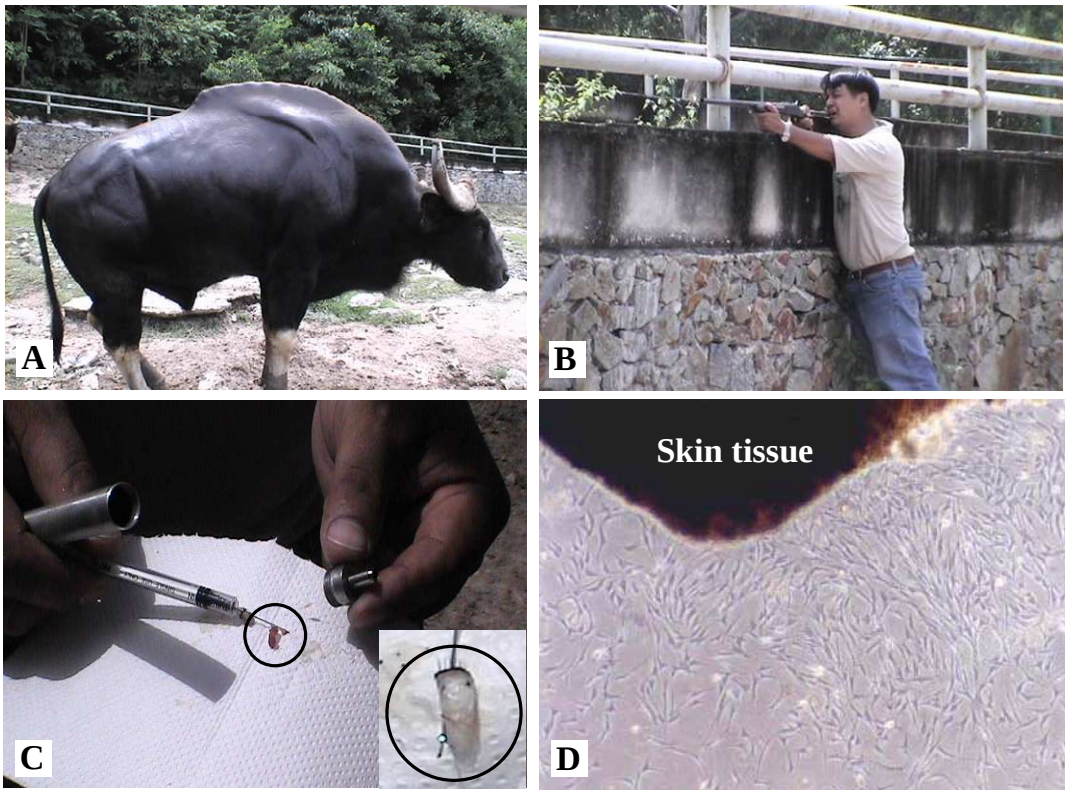

Figure 3.2 Gaur fibroblasts preparation. Skin tissue was biopsied from gaur (A) by biopsied gun (B). Skin tissue was removed from biopsied gun (C). Fibroblasts were outgrown from skin tissue after 8-10 days of cultured (D, magnification 40x).

3.3.2 Oocyte preparation

Bovine ovaries from local abattoir were collected and transported to the laboratory in 0.9% NaCl at room temperature. Cumulus-oocyte complexes (COCs) were collected by aspiration from follicles 2–8 mm diameter using an 18-gauge needle attached to a 10 ml syringe (Figure 3.3A). Collected COCs (Figure 3.3B) were washed five times with modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP, Sigma, P-0930) then washed three times in *in vitro* maturation (IVM) medium. The IVM medium consisted of

TCM199 (Sigma, M-5017) supplemented 10% FBS, 50 IU/ml hCG (Intervet, Netherlands, CDN781851), 0.02 AU/ml FSH (Antrin®, Denka Pharmaceutical, Japan) and 1 µg/ml 17β-estradiol (Sigma, E-8875). Each of 20 COCs were cultured in 100 µl droplets of IVM medium overlaid with mineral oil (Sigma, M-8410) under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 21 h (Parnpai et al., 1999). After IVM (Figure 3.3C), the cumulus cells were mechanically removed by repeat pipetting using a fine-tip pipette in 0.2% hyaluronidase (Sigma, S-3506) and were subsequently washed five times in Emcare® holding medium. Metaphase-II oocytes (Figure 3.3D) with first polar body were selected for enucleation.

3.3.3 Somatic cell nuclear transfer

In vitro matured bovine oocytes were placed in Emcare® holding medium containing 5 µg/ml cytochalasin B (CB, Sigma, C-6762) for 5 min. The zona pellucida above the first polar body was cut with a glass needle (Figure 3.4A-C). The small volume (about 5-10%) of the cytoplasm beneath the first polar body was squeezed out (Figure 3.4D). All processes were done by micromanipulator (Narishige, Japan, model M0188NE) under inverted microscope (Olympus, Japan, model IX71) at 200x magnification. Completed enucleation was confirmed by staining the squeezed out cytoplasm with 5 µg/ml Hoechst 33342 (Sigma, C-2261) (Figure 3.5). Individual donor cell (diameter 14-16 µm) of male and female bovine or gaur was inserted into the perivitelline space of enucleated bovine oocytes (Figure 3.6). Donor cell-cytoplast couplet (DCCC) was fused in Zimmermann fusion medium (Zimmermann and Vienken, 1982) between the tips of the fusion electrodes and electrostimulated by two direct current pulses (24 V, 15 µsec) generated by a fusion machine (SUT F-1, Suranaree University of Technology) (Figure 3.7). The DCCC

were subsequently washed six times in Emcare® holding medium. The success fusion was examined at 45 min after electrostimulated. The reconstructed embryos were activated by 7% ethanol (Carlo Erba, 414607) in Emcare® holding medium for 5 min at room temperature and cultured in modified oviduct synthetic fluid with amino acids medium (mSOFaa, Gardner et al., 1994) supplemented with 1.25 µg/ml cytochalasin D (CD, Sigma, C-8273) and 10 µg/ml cycloheximide (CHX, Sigma, C-6798) at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 5 h (Muenthaisong et al., 2007).

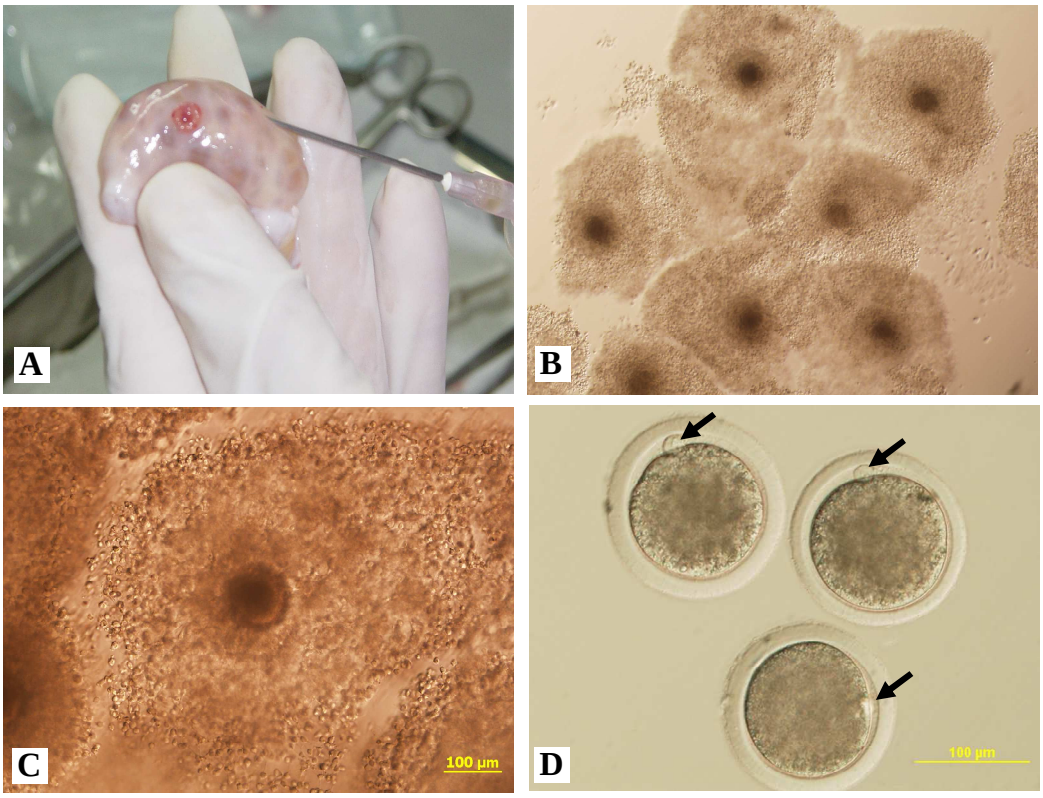

Figure 3.3 Oocyte preparation. Bovine ovaries (A) were collected from local abattoir. Cumulus-oocyte complexes (COC) were aspirated from ovaries (B). COC after 21 h of *in vitro* maturation (C). Metaphase-II oocytes with first polar body (arrow) (D, magnification 200x).

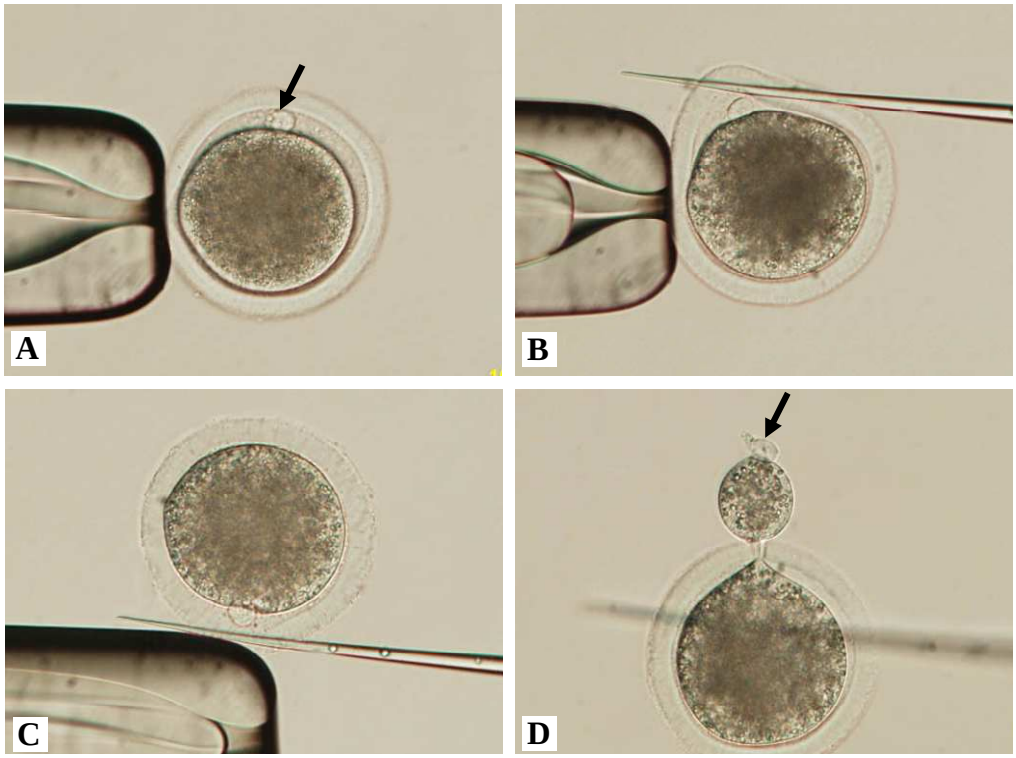

Figure 3.4 Enucleation procedures. Arrow indicated the first polar body (A). The glass needle was used to cut the zona pellucida above the first polar body (B-C). The first polar body and small volume of cytoplasm were squeezed out by placing the glass needle above oocyte (D, magnification 200x).

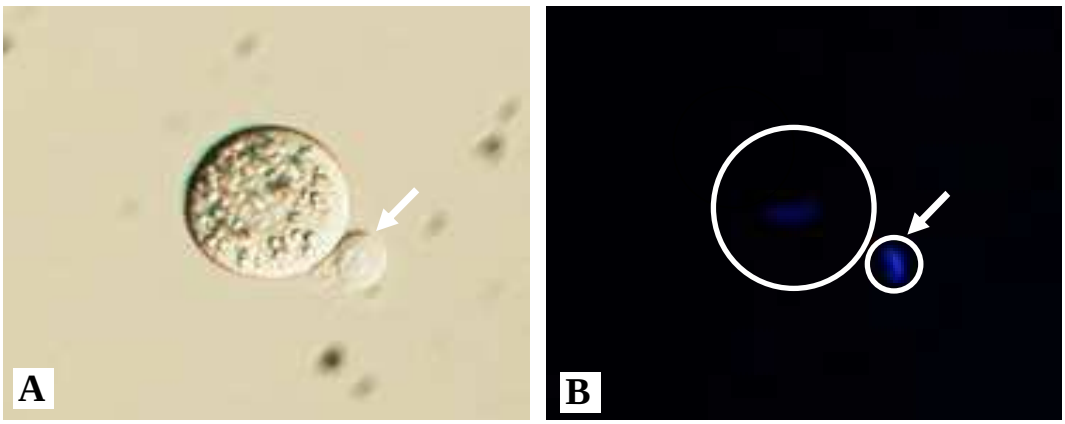

Figure 3.5 Hoechst 33342 staining. The squeezed out cytoplasm was stained by Hoechst 33342. Bright filed (A) and fluoresces image (B) of the squeezed out cytoplasm. Arrow indicated the first polar body. The completed enucleation was confirmed by fluorescence signal in the squeezed out cytoplasm (magnification 200x).

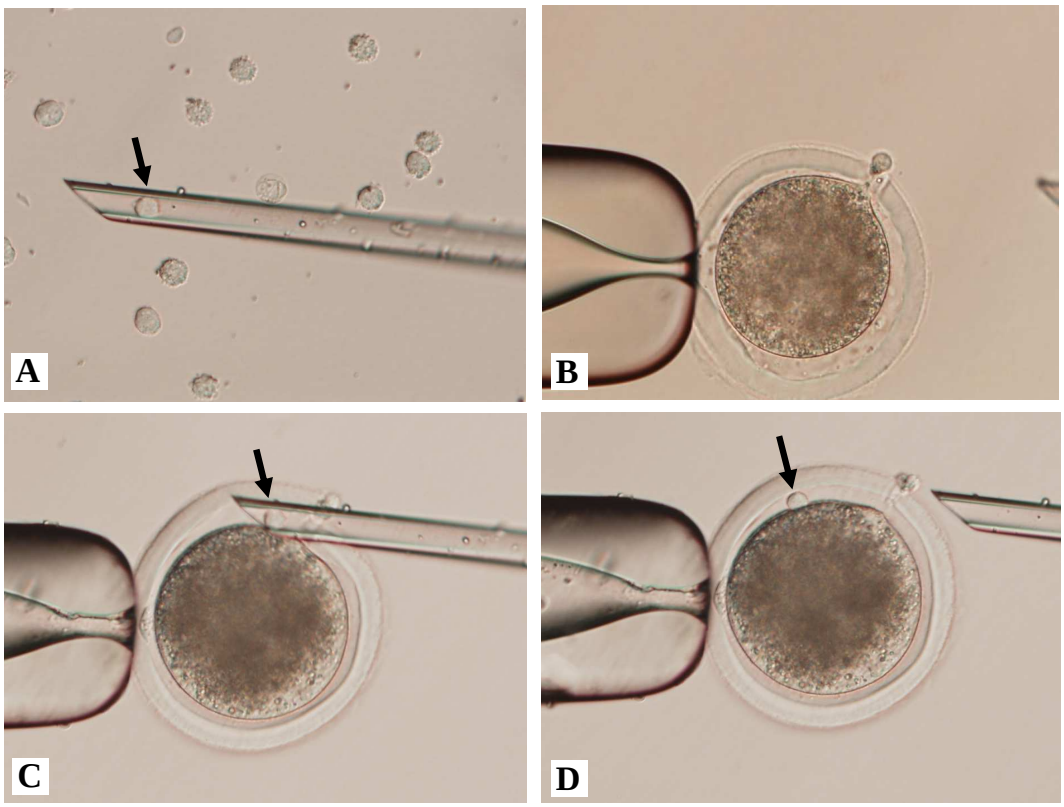

Figure 3.6 Injection procedures. The donor cell was aspirated into injection pipette (A). The donor cell was inserted into the perivilline space of oocyte (B-D). Arrow indicated the donor cell (magnification 200x).

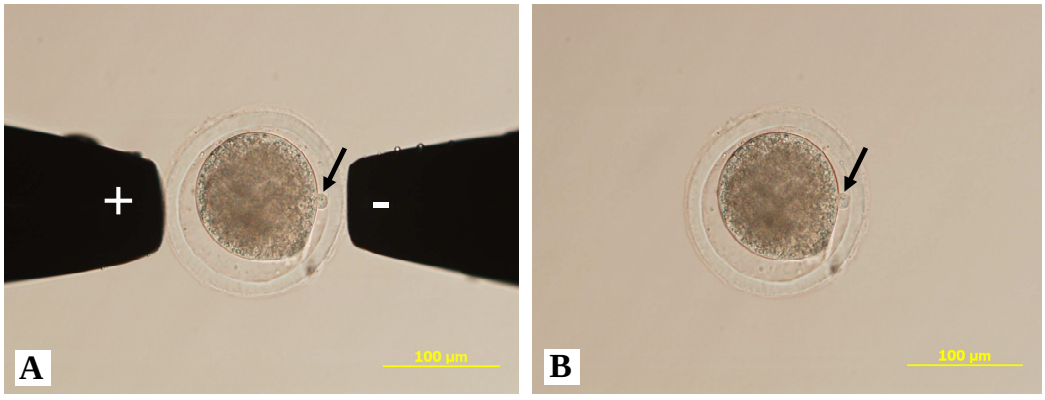

Figure 3.7 Fusion procedures. Donor cell-cytoplast couplet was place between the tips of fusion electrodes (A). Ten minutes after fusion (B, magnification 200x).

3.3.4 *In vitro* embryo culture

The reconstructed embryos were cultured in mSOFaa medium (20 embryos/100 µl) at 38.5°C under a 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 cells in mSOFaa medium at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 5 days. Half volume of mSOFaa medium was replaced daily and the development of embryos was also recorded.

3.3.5 TSA treatment

After fusion, oocytes were separated into two groups (TSA-treated and non TSA-treated). For TSA-treated group, oocytes were placed in Emcare® holding medium supplemented with 50 nM TSA (Sigma, T-8552) for 45 min and after that, oocytes were activated and cultured in medium supplemented with 50 nM TSA up to 10 h (Kishigami et al., 2006). For the non TSA-treated group, oocytes were activated and cultured with the same condition except without TSA supplementation.

3.3.6 Differential staining of blastocysts

Blastocysts at 7 days after cultured were counter-stained to distinguish cells of trophectoderm (TE) and inner cell mass (ICM) as modified from previous report (Suteevun et al., 2006). Briefly, zona pellucida of blastocysts were removed by 0.5% protease (Sigma, P-8811) and washed in mDPBS supplemented with 0.1% PVP. The zona-free blastocysts were incubated in 10% rabbit anti-bovine spleenocyte antibodies for bovine embryos or 100% rabbit anti-gaur fibroblast antibodies for gaur embryos for 30 min. Both antibodies were generated in our laboratory followed the protocol of Iwasaki and colleagues (1990). After incubation, blastocysts were transferred into a mixture of 10% guinea pig complement (Sigma, S-1639), 10 µg/ml propidium iodide

(Sigma, P-4170) and 10 µg/ml Hoechst 33258 (Sigma, B-2883) for 30 min. The blastocysts were mounted with glycerol (Merck, 4094) on glass slide and covered with cover slide. The ICM cells (blue) and TE cells (red) were counted under ultraviolet light provide by a 100W high-pressure mercury burner (Olympus, model BH2-RFL-T3) for fluorescence microscopy (Figure 3.8).

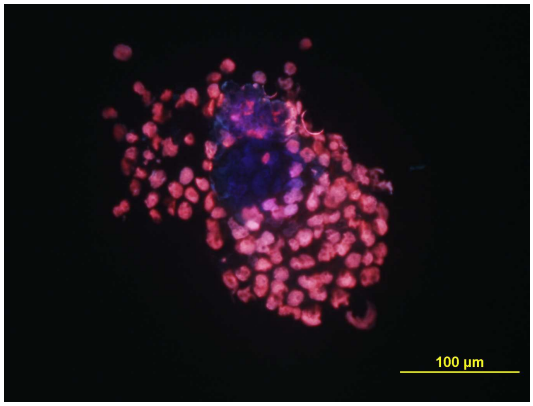

Figure 3.8 Fluorescence image of a blastocyst. Red represents Propidium iodide stain of trophectoderm cells and blue represents Hoechst stained inner cell mass cells (magnification 200x).

3.3.7 Experimental design

The effect of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos derived from male and female fibroblasts was examined. Reconstructed embryos from each treatment group were randomly activated and cultured in medium with 50 nM TSA (TSA-treated) or without TSA (non TSA-treated) supplementation. After 10 h, embryos were transferred to mSOFaa medium without TSA supplementation. Four replicates were performed for each treatment group. Cleavage and developmental rates to eight cell stage were evaluated at 2 days after embryo culture. Percentages of morular and blastocyst were evaluated

at 5 and 7 days after embryo culture, respectively. For assessment of the TE and ICM cells, ten blastocysts at 7 days after embryo culture from each group were examined. The rates of fusion, cleavage, eight cell, morula and blastocyst as well as the TE and ICM cell number of blastocyst from each donor cell type after TSA-treated and non TSA-treated were compared.

3.3.8 Statistical analysis

Statistical analysis of data was evaluated by Completely Randomized Design (CRD) with Statistical Analysis System (SAS Inst. INC., Cary, N.C., USA). Analysis of Variance (ANOVA) and comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed.

3.4 Results

3.4.1 Fusion rates of reconstructed bovine and gaur fibroblasts with bovine enucleated oocytes

The male and female bovine and gaur fibroblasts were fused with enucleated bovine oocytes with the same parameter. After fusion, reconstructed oocytes were washed and incubated in Emcare® holding medium with or without TSA supplementation for 45 min. There was no significant difference in fusion rate among treatment groups ($P>0.05$, Table 3.1). The fusion rate was ranging between 85-90%. The results in table 3.1 indicated that gaur and bovine fibroblasts had similar ability to fuse with enucleated bovine oocyte. TSA treatment, sex and species of donor cell did not affect the fusion rate.

3.4.2 Effects of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos

The rates of cleavage, development to 8 cell and morula stage of bovine SCNT and gaur iSCNT cloned embryos were similar in either TSA-treated or non TSA-treated, male or female fibroblasts. The blastocyst rate of bovine SCNT embryos treated with 50 nM TSA for 10 h was higher than the non TSA-treated embryos ($P<0.05$, Table 3.1). TSA treatment did not enhance the blastocyst rate of gaur iSCNT embryos compared with the non TSA-treated gaur iSCNT embryos. There was no significant difference in developmental potential between male and female cloned embryos.

3.4.3 Effects of TSA treatment on TE and ICM cell of bovine SCNT and gaur iSCNT blastocysts

The cell numbers of TE and ICM of bovine SCNT and gaur iSCNT blastocysts from male and female fibroblasts of TSA-treated or non TSA-treated group were examined. There was no significant difference in the cell number of TE and ICM cells among treatment groups ($P>0.05$, Table 3.2). Moreover, similar proportion of TE: ICM were found in all treatment groups. Gaur iSCNT blastocysts had the same quality as of bovine SCNT. TSA treatment, sex and species of donor cell did not affect the quality of cloned blastocyst.

Table 3.1 Effects of TSA treatment on pre-implantation development of bovine SCNT and gaur iSCNT embryos.

Donor cell type	TSA treatment	Fused (%)	Cultured	Cleaved (%)	No. (%) embryo developed to		
					8-Cell	Morula	Blastocyst
Male bovine	-	206/231 (89.2)	198	193 (97.5)	143 (72.2)	84 (42.4)	63 (31.8)[b]
	+	240/272 (88.2)	237	222 (93.7)	176 (74.3)	121 (51.1)	103 (43.5)[a]
Female bovine	-	187/216 (86.6)	187	175 (93.6)	132 (70.6)	82 (43.9)	63 (33.7)[b]
	+	216/241 (89.6)	210	201 (95.7)	169 (80.5)	108 (51.4)	92 (43.8)[a]
Male gaur	-	197/225 (87.6)	196	187 (95.4)	162 (82.7)	92 (46.9)	65 (33.2)[b]
	+	213/245 (86.9)	210	200 (95.2)	162 (77.1)	89 (42.4)	70 (33.3)[b]
Female gaur	-	187/220 (85.0)	184	180 (97.8)	142 (77.2)	84 (45.7)	69 (37.5)[b]
	+	210/232 (90.5)	210	200 (95.2)	165 (78.6)	100 (47.6)	78 (37.1)[b]

Different superscripts within column indicate significant differences ($P < 0.05$).

Table 3.2 Effects of TSA treatment on bovine SCNT and gaur iSCNT blastocyst quality.

Donor cell type	TSA treatment	Mean (± S.E.M.) number of cells in blastocyst		
		TE	ICM	TE: ICM
Male bovine	-	93 ± 3.5	31 ± 1.3	3.01 ± 0.08
	+	94 ± 3.0	31 ± 1.1	3.04 ± 0.06
Female bovine	-	94 ± 3.3	30 ± 1.0	3.09 ± 0.03
	+	92 ± 3.5	29 ± 1.0	3.14 ± 0.03
Male gaur	-	93 ± 3.7	30 ± 1.7	3.08 ± 0.08
	+	85 ± 2.3	28 ± 0.6	3.03 ± 0.07
Female gaur	-	91 ± 3.7	29 ± 1.2	3.11 ± 0.06
	+	91 ± 3.6	30 ± 1.1	3.05 ± 0.09

The data was not statistically difference ($P>0.05$).

Ten blastocysts were examined for each treatment.

3.5 Discussions

This study demonstrated that the developments to blastocysts of bovine SCNT embryos increased when treated with 50 nM TSA for 10 h after fusion whereas no differences were observed in gaur iSCNT embryos. Moreover, there was not significant difference on blastocyst quality among bovine SCNT and gaur iSCNT embryos either TSA-treated or non TSA -treated.

Histone plays an important role in chromatin structure and transcriptional regulation through modifications of the histone amino-terminus tail including acetylation, methylation, phosphorylation and ubiquitinylation (Wee et al., 2006). Histone acetylation is involved in gene expression, cellular function and DNA replication. Histone acetylation and deacetylation are catalyzed by specific enzyme HAT and HDAC. TSA is a histone deacetylase inhibitor, binds directly to the catalytic site of HDAC, resulting in the inhibition of HDAC to remove the acetyl groups of lysine residues clustered near the amino terminus of core histone and the transcriptional process remains activated. The effect of TSA treatment had been studied in mouse (Kishigami et al., 2006; Rybouckin et al., 2006), bovine (Enright et al., 2003; Shi et al., 2003), pig (Zhang et al., 2007), rabbit (Meng et al., 2009) and rat (Mizumoto et al., 2008) SCNT embryos. Treatment of bovine donor cells with TSA before SCNT increased the development of cloned embryos to the blastocyst stage (Enright et al., 2003). Similarly, Wee et al. (2007) found that cloned embryos with TSA-treated donor cell showed a higher developmental competence than those with normal cells and IVF embryos. In contrast, Shi et al. (2003) examined the treatment of fetal fibroblasts with sodium butyrate (NaBu), another HDAC inhibitor. They found that NaBu can increase the rate of blastocyst by two-fold, however, TSA had no

positive effect on the development of cloned embryos. After that, Kishigami and colleagues (2006) reported that cloned mouse embryos treated with 5-50 nM TSA for 10 h after oocyte activation had two to five-fold increases in blastocyst rate depend on donor cell type. They used tail tip cells, spleen cells, neural stem cells and cumulus cells as donor cells. But no beneficial effect was obtained when embryonic stem cells were used as donor cell. In addition, they found that TSA treatment can increase the success rates of outbred strain mouse cloning (Kishigami et al., 2006; Kishigami et al., 2007). Thus, 50 nM TSA for 10 h, an effective protocol in mouse cloning (Kishigami et al., 2006) were applied in this study. However, the TSA treatment began immediately after fusion because the nuclear reprogramming in cloned embryo started soon after donor nuclei fused into recipient cytoplasm (Gao et al., 2007).

This study indicated that TSA treatment can increase the percentage of blastocyst in bovine SCNT embryos. Similarly, 50 nM TSA treatment increased the blastocyst of cloned bovine embryos (Ding et al., 2008; Iwamoto et al., 2007). On the other hand, Akagi and colleagues (2007) and Iager and colleagues (2008) showed that 50 nM TSA treatment did not affect the development to blastocyst of bovine cloned embryos. The conflicting result may be due to the differences in timing of TSA treatment, the cloning protocol and the condition of embryo culture.

The cell numbers of TE, ICM and the proportion of TE:ICM from bovine SCNT and gaur iSCNT blastocysts were not significant difference in either TSA-treated or non TSA-treated embryos. TSA treatment did not change the cell number and cell allocation of TE and ICM in cloned blastocyst. Another report in mice cloning, cloned embryos treated with TSA for 8 to 12 h had higher blastocyst rate and full term fetuses were obtained (Tsuji et al., 2009). TSA treatment improved

blastocyst rate and quality of the blastocysts but did not increase full-term development of cloned mouse embryos (Rybouchkin et al., 2006). The blastocyst rate of pig cloned embryos increased with TSA but the total cell numbers of blastocyst were not difference compared with the control. However, piglets were obtained after embryo transfer (Zhang et al., 2007; Li et al., 2008). Moreover, Yamanaka and colleagues (2009) reported that TSA treatment could enhance blastocyst rate and total cell number of pig cloned embryos. Shi and colleagues (2008) found that the blastocyst rate of rabbit cloned embryo from TSA-treated group was higher than that of non TSA-treated embryos. On the other hand, TSA treatment did not increase the blastocyst rate, cell number of blastocyst, pregnancy and birth rates when compared with non TSA-treated rabbit SCNT embryos (Meng et al., 2009). From this study, male and female fibroblasts had the same ability to support embryo development of bovine SCNT and gaur iSCNT in either TSA-treated or non TSA-treated embryos. However, Sansinena and colleagues (2005) reported that bovine cytoplasts reconstructed with male banteng fibroblasts had higher cleavage and blastocyst rate than those of female fibroblasts.

Interestingly, TSA had no positive effect on development of gaur iSCNT embryos, however, the blastocys rate of gaur iSCNT were higher than previously reported (Lanza et al., 2000; Mastromonaco et al., 2007). Shi and colleagues (2008) found that there was no significant difference in embryo development of human-rabbit iSCNT embryos after TSA treatment when compared with non TSA-treated embryos. TSA treatment could not improved the blastocyst rate of dog-pig iSCNT embryos when using dewclaw cells but the beneficial effect was found when using tail tip cells as donor cells (Sugimura et al., 2009). The mechanism of nuclear

reprogramming on iSCNT is still unclear. Only increase histone acetylation was not enough for active transcription of silence gene in iSCNT embryos.

TSA can improve pre-implantation development of bovine SCNT embryos but no positive effect on gaur iSCNT embryos. The full-term development of TSA treated embryos and epigenetic reprogramming of iSCNT embryos needs further investigation.

3.6 Conclusion

This experiment indicated that TSA treatment can improve development of bovine SCNT embryos but not gaur iSCNT embryos.

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CHAPTER IV

EFFECT OF TRICHOSTATIN A ON POST-IMPLANTATION DEVELOPMENT OF CLONED BOVINE AND GAUR EMRBYOS

4.1 Abstract

The efficiency of cloning animal by SCNT has been limited due to high fetal loss and neonatal mortality. From previous studies, TSA treatment showed improvement of the cloning efficiency. In this study, the effect of TSA treatment on post-implantation development of bovine SCNT, vitrified/thawed bovine SCNT and gaur iSCNT embryos were examined. The gaur iSCNT embryos were reconstructed by fusing male or female gaur fibroblasts with bovine enucleated oocytes. The bovine SCNT embryos were derived from male bovine fibroblasts using the same procedure as gaur iSCNT. After fusion, the reconstructed embryos were divided into two groups, TSA-treated and non TSA-treated group. For vitrified/thawed bovine SCNT group, the bovine SCNT blastocysts at day 7 of culture from TSA-treated and non TSA-treated groups were vitrified by microdrop technique and warmed by stepwise dilution. The bovine SCNT, vitrified/thawed bovine SCNT and gaur iSCNT embryos from TSA-treated and non TSA-treated groups were non-surgically transferred into seventy three synchronized bovine recipients. The pregnancy diagnosis was done on day 45 after embryo transfer by trans-rectal ultrasound scanning. The pregnancy

statuses were checked monthly until delivery. The results indicated that the pregnancy rates of male and female gaur iSCNT embryos were not significant different in the TSA-treated or the non-TSA treated group ($P>0.05$). The pregnancy rate of gaur iSCNT and vitrified/thawed bovine SCNT embryos were not significant different between TSA-treated and non TSA-treated groups ($P>0.05$). However, the pregnancy rate of TSA-treated bovine SCNT embryos was significantly higher than that of non TSA-treated embryos ($P<0.05$). The fetal developments of TSA-treated and non TSA-treated embryos were no significant different within the same type of embryos ($P>0.05$). However, the fetal development of non TSA-treated embryos from male gaur iSCNT was significantly higher than that of TSA-treated embryos ($P<0.05$). Three cloned bovine calves derived from TSA-treated bovine SCNT embryos were delivered. However, one bovine calf died during birth. One cloned gaur newborn derived from non TSA-treated group of male gaur iSCNT embryo was born by cesarean section but died 12 h after birth due to pulmonary disorder. One out of two recipients carrying TSA-treated female gaur iSCNT embryos had late-term abortion on day 242 and another one recipient had mummified fetus which was cesarean section at day 311 of gestation. Therefore, no gaur newborn was obtained from female gaur iSCNT group. Moreover, twin cloned bovine calves derived from vitrified/thawed embryos of TSA-treated bovine SCNT group were born healthy. DNA microsatellite analysis confirmed that four cloned bovine calves, aborted and mummified gaur fetuses and one cloned gaur newborn were genetically identical to their donor cells. In conclusion, TSA treatment could increase the pregnancy rate of bovine SCNT embryos. No positive effect was found in gaur iSCNT and vitrified/thawed bovine embryos. Besides, TSA treatment cannot improve fetal

development of all types of embryos. Nevertheless, four cloned bovine calves from TSA-treated embryos were born.

4.2 Introduction

Although SCNT has successfully produced offspring in many mammalian species (reviewed by Campbell et al., 2007), cloned embryos have lower pregnancy rate than those from *in vivo* and *in vitro* derived embryos (Booth et al., 2003, Cibelli et al., 2002, Li et al., 2006, Pederson et al., 2005). The viable birth rate of cloned embryos (1-5%) was lower than those from *in vitro* derived embryos (30-60%, Wilmut et al., 2002). Most of the remaining (95-99%) died at various stages of development due to developmental abnormalities (Wells et al., 2004). Moreover, cloned fetuses often have abnormalities correlated with placental problems (placentome malformation and hydroallantois), prolonged gestation, parturition difficulties (high placental and birth weight), high peri- and post-natal death, specific adult phenotypes and short lifespan (Wells et al., 2004). Previous reports found that improper development of placenta may play important role in the fetal abnormalities and low pregnancy rates in bovine SCNT (Stice et al., 1996; Dindot et al., 2004). These failures attributed to the existing epigenetic errors in the donor genome and/or incomplete reprogramming via SCNT (Shimozawa et al., 2002; Wells, 2003). Since the first iSCNT experiments have been reported in 1999 (Dominko et al., 1999), iSCNT has been applied to preserve or rescue endangered species. However, the poor embryo development, low pregnancy and live birth rates have been observed (reviewed by Mastromonaco and King, 2007). Previous studies on gaur iSCNT using bovine oocyte as recipient found the blastocyst formation ranging from 12 to 18%

(Lanza et al., 2000; Mastromonaco et al., 2007). Previous studies on transferring gaur embryos to bovine recipients have resulted in live offspring (Johnston et al., 1994). Although, the cloned gaur was successfully produced (pregnancy rate 25%, birth rate 12.5%, Lanza et al., 2000), the gaur died within two days of birth (Vogel, 2001). Many researchers have tried to improve the efficiency of SCNT by correct the epigenetic reprogramming. Some chemicals have been used to alter the methylation or acetylation patterns of donor chromatin before and after the SCNT procedure such as TSA (Enright et al., 2003; Shi et al., 2003; Rybouhkin et al., 2006; Kishigami et al., 2006), NaBu (Shi et al., 2003), 5-aza-2 deoxycytidine (Enright et al., 2003; Tsuji et al., 2009) and scriptaid (Zhao et al., 2009). Inhibition of histone deacetylase using TSA has been shown to enhance pre-implantation development of SCNT embryos in mouse (Kishigami et al., 2006), bovine (Iwamoto et al., 2007; Ding et al., 2008), pig (Zhang et al., 2007) and rabbit (Shi et al., 2008). To date, the effects of TSA treatment on full-term development have been examined only in mouse (Kishigami et al., 2006; 2007; Tsuji et al., 2009, Maalouf et al., 2009) and rabbit (Meng et al., 2009). Therefore, the effects of TSA treatment on post-implantation development of bovine SCNT, vitrified/thawed bovine SCNT and gaur iSCNT embryos have been evaluated in this study.

4.3 Materials and methods

4.3.1 Embryo production

All blastocysts in this experiment were produced using the method described in Chapter III. Briefly, the male bovine or male or female gaur fibroblasts were transferred to enucleated bovine oocytes. After electrical fusion, the reconstructed

embryos were randomly divided into two groups, TSA-treated and non TSA-treated groups. For TSA-treated group, the embryos were activated by a combination of cytochalasin D and cycloheximide and then cultured in culture medium supplemented with 50 nM TSA. After 10 h of TSA treatment, embryos were cultured in culture medium without TSA supplementation for 7 days. For non TSA-treated group, the embryos were activated and cultured in culture medium without TSA supplementation for 7 days.

4.3.2 Vitrification and warming

The TSA-treated and non TSA-treated of male bovine SCNT embryos were vitrified using microdrop technique. Briefly, the hatching blastocysts (day 7) were washed in TCM199-Hepes (Sigma, H-4034) supplemented with 20% FBS, 7.5% (v/v) ethylene glycol (EG, Sigma, E-9129) and 7.5% (v/v) dimethylsulfoxide (DMSO, Sigma, D-1435) for 3 min and then transferred to vitrification medium, TCM199-Hepes supplemented with 20% FBS, 0.5 M sucrose (Sigma, S-1888), 16.5 % (v/v) EG and 16.5% (v/v) DMSO, for 30 sec. Small drops of the vitrification solution (1-2 µl) containing 5 embryos were directly dropped into liquid nitrogen. After that, the vitrified drops were transferred into cryotube (Nunc, 375418) and kept in liquid nitrogen.

When ready to use, the embryos were thawed by immersion of vitrified drops into 0.6 M sucrose in TCM199-Hepes supplemented with 20% FBS for 5 min at 39°C following stepwise dilution with 0.4 M, 0.2 M and 0 M sucrose for 5 min each. After that, the embryos were washed three times in mSOFaa medium and co-cultured with bovine oviductal epithelial cells under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 12 h before embryo transfer.

4.3.3 Synchronization of recipients

The synchronization program for bovine recipients (2-3 years old) was set up as shown in Figure 4.1. Briefly, an intravaginal progesterone-releasing device (CIDR, Pharmacia & Upjohn Company, Hamilton, New Zealand) was inserted into vagina of recipient for 7 days. Two days before CIDR removal, the recipients received an intramuscular injection of 500 IU equine chlorionic serum gonadotrophin (eCG, Folligon®, Intervet International B.V., Boxmeer, The Netherlands). At the time of CIDR removal, the recipients received an intramuscular injection of 0.75 mg synthetic prostaglandin ($PGF_{2\alpha}$, Iliren®, Intervet International GmbH, Unterschleißheim, Germany). Forty eight hours after CIDR removal, the recipients were checked for standing estrus. The estrus cycle were confirmed by rectal palpation. Then each recipient received an intramuscular injection of 0.25 mg gonadotrophin releasing hormone (GnRH, Fertagyl®, Intervet International B.V.) at 15 h after standing estrus. The embryos were transferred on day 8 post estrus.

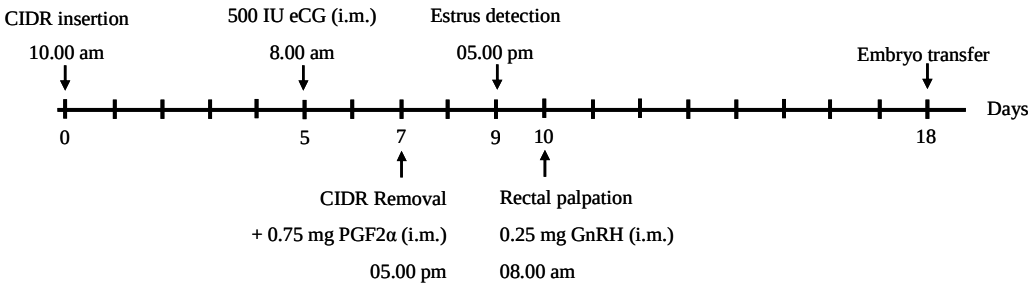

Figure 4.1 Bovine recipients synchronization program.

4.3.4 Embryo transfer (ET)

The bovine SCNT, vitrified/thawed bovine SCNT and gaur iSCNT embryos from TSA-treated and non TSA-treated groups were non-surgically transferred into

synchronized bovine recipients. Briefly, after *in vitro* cultured for 7-8 days, hatching blastocysts were washed three times in Emcare® holding medium. Two or three embryos were loaded into a 0.25-ml ET straw. Afterward, the straw was placed into an ET gun (IMV, France), covered with ET sheath (IMV, France) and sanitary sheath (IMV, France). Then, an ET device was introduced into vagina of the recipient. The embryos were deposited in the ipsilateral uterine horn.

Pregnancy diagnosis was performed at day 45 after embryo transfer by trans-rectal ultrasound scanning. The pregnancy status was rechecked by rectal palpation on day 60, 90, 120, 150, 180, 210, 240 and 270 of gestation.

4.3.5 Sample collection and DNA extraction

Blood samples were collected from cloned bovine calves, cloned gaur newborn and bovine recipients. Tissue samples were biopsied from aborted and mummified gaur fetuses.

Genomic DNA were extracted from blood, tissue samples and fibroblasts of donor cell (as positive control) using Genomic DNA extraction Mini kit (RBC Bioscience, Taiwan, RPC), according to manufacturer's recommendation.

4.3.6 DNA microsatellite analysis

The genotyping of clones and donor cells were performed by polymerase chain reaction (PCR) using bovine DNA microsatellite markers (Table 4.1). Twenty microliters of reaction mixture consisted of 100 ng of DNA sample, 1x PCR buffer (Promega, Madison, WI, USA, M8911), 2 mM $MgCl_2$ (Promega, A3511), 0.2 mM of dNTPs mix (Fermentas, Canada, R0181), 0.5 µM of each primers (Table 4.1) and 0.625 units of Taq polymerase (Promega, M8295). The amplification was carried out as follows: 95°C for 2 min following by 45 cycles of 95°C for 20 sec, annealing

temperature (Table 4.1) for 20 sec, 72°C for 30 sec. The final elongation was at 72°C for 5 min. PCR products were run on a 5% polyacrylamide gel in 1x TBE buffer at 100 V for 50 min. Polyacrylamide gel was stained briefly with 1 mg/ml ethidium bromide (Sigma, E-7637). Then rinse in water for 3 times. The PCR products were visualized under ultraviolet light (ChemiDoc XRS System, Biorad, 170-8070).

4.3.7 Experimental design

The effect of TSA treatment on the post-implantation development of male bovine SCNT, vitrified/thawed male bovine SCNT, male and female gaur iSCNT embryos were examined. Two or three blastocysts from each group were non-surgically transferred to bovine recipients. The pregnancy rate was evaluated on day 45 after embryo transfer by trans-rectal ultrasound scanning. Rectal palpation was done to confirm the pregnancy status on day 60, 90, 120, 150, 180, 210, 240 and 270 of gestation.

4.3.8 Statistical analysis

Statistical analysis of data was evaluated by the non-parametric statistics with Statistical Analysis System. Pearson's χ^2 test was analyzed.

Table 4.1 Bovine DNA microsatellite markers.

Markers	Sequences	Annealing temp. (°C)	References
CSSM66	F: ACACAAATCCTTTCTGCCAGCTGA	52	Barendse et al., 1994
	R: AATTTAATGCACTGAGGAGCTTGG		
ETH3	F: GAACCTGCCTCTCCTGCATTGG	52	Toldo et al., 1993
	R: ACTCTGCCTGTGGCCAAGTAGG		
ETH225	F: GATCACCTTGCCACTATTTCCT	47.5	Steffen et al., 1993
	R: ACATGACAGCCAGCTGCTACT		
HEL9	F: CCCATTCAGTCTTCAGAGGT	55	Kaukinen and Varvio, 1993
	R: CACATCCATGTTCTCACCAC		
INRA005	F: CAATCTGCATGAAGTATAAATAT	40	Vaiman et al., 1992
	R: CTTCAGGCATACCCTACACC		
INRA037	F: GATCCTGCTTATATTTAACCAC	40	Vaiman et al., 1994
	R: AAAATTCCATGGAGAGAGAAAC		

Table 4.1 (Continued).

Markers	Primer sequences	Annealing temp. (°C)	References
INRA063	F: ATTTGCACAAGCTAAATCTAACC	48	Vaiman et al., 1994
	R: AAACCACAGAAATGCTTGGAAG		
MGTG4B	F: GAGCAGCTTCTTTCTTTCTCATCTT	46	Fries et al., 1993
	R: GCTCTTGGAAGCTTATTGTATAAAG		
TGLA53	F: GCTTTCAGAAATAGTTTGCATTCA	43	Barendse et al., 1994
	R: ATCTTCACATGATATTACAGCAGA		
TGLA57	F: GCTTTTTAATCCTCAGCTTGCTG	44	Barendse et al., 1994
	R: GCTTCCAAAACTTTACAATATGTAT		
TGLA126	F: CTAATTTAGAATGAGAGAGGCTTCT	44	Barendse et al., 1994
	R: TTGGTCTCTATTCTCTGAATATTCC		
TGLA263	F: CAAGTGCTGGATACTATCTGAGCA	46	Georges et al., 1995
	R: TTAAAGCATCCTCACCTATATATGC		

4.4 Results

4.4.1 Effects of TSA treatment on post-implantation development of bovine SCNT embryos

Only male embryos from bovine SCNT were examined in this experiment. From Table 4.2, no pregnancy was obtained after transferring eighteen non TSA-treated embryos into seven recipients. On the other hand, six recipients (46.2%) were found pregnant by trans-rectal ultrasound scanning at day 45 after transfer of thirty six TSA-treated embryos into thirteen recipients. The pregnancy rate of TSA-treated embryos was significantly higher than that of non TSA-treated embryos ($P<0.05$). However, three recipients of TSA-treated embryos aborted before day 60, 90 and 120 of gestation. Other three pregnancies (3/13, 23.1%) continued to develope until term. The first cloned bovine calf was naturally born on February 3rd, 2008 (day 275 of gestation; birth weight 35 kg) being healthy with normal appearance (Figure 4.2A). The second cloned bovine calf (Figure 4.2B) was delivered by a caesarean section on February 9th, 2008 (day 274 of gestation; birth weight 42 kg). Unfortunately, perinatal death of the third cloned bovine calf took place, after caesarean section on March 31th, 2008 (day 274 of gestation, birth weight 47 kg). The perinatal dead cloned bovine calf had normal appearance.

Table 4.2 Effects of TSA treatment on post-implantation development of bovine SCNT and gaur iSCNT embryos.

Donor cell	TSA	No. embryos	No. recipients	No. (%) recipients pregnant at days									calving
				45	60	90	120	150	180	210	240	270	
Male bovine	+	36	13	6[a] (46.2)	5 (38.5)	4 (30.8)	3 (23.1)	3 (23.1)	3 (23.1)	3 (23.1)	3 (23.1)	3 (23.1)	3
	-	18	7	0[b]	0	0	0	0	0	0	0	0	0
Male gaur	+	44	18	3 (16.7)	1 (5.6)	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
	-	9	3	1 (33.3)	1 (33.3)	1[a] (33.3)	1[a] (33.3)	1[a] (33.3)	1[a] (33.3)	1[a] (33.3)	1[a] (33.3)	1[a] (33.3)	1[a]
Female gaur	+	50	16	6 (37.5)	2 (12.5)	2 (12.5)	2 (12.5)	2 (12.5)	2 (12.5)	2 (12.5)	2 (12.5)	1 (6.3)	0
	-	3	1	0	0	0	0	0	0	0	0	0	0

Values with different superscripts in same column were significant difference (P<0.05).

Table 4.3 Effects of TSA treatment on post-implantation development of vitrified/thawed bovine SCNT embryos.

Donor cells	TSA	No. embryos	No. recipients	No. (%) recipients pregnant at days									calving
				45	60	90	120	150	180	210	240	270	
Male bovine	+	22	8	3 (37.5)	2 (25)	2 (25)	2 (25)	2 (25)	2 (25)	2 (25)	1 (12.5)	1 (12.5)	2
	-	14	7	2 (28.6)	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)	0	0	0	0	0

Values were not significant difference (P>0.05).

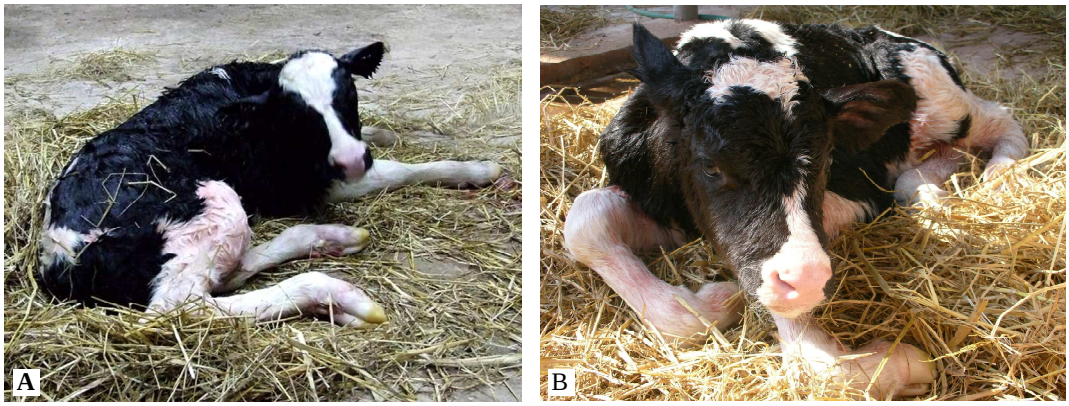

Figure 4.2 Cloned calves derived from TSA-treated bovine SCNT embryos at 30 minutes after birth. The first cloned calf was naturally born on day 275 of gestation (A); the second cloned calf was born by caesarean section on day 274 of gestation (B).

4.4.2 Effect of TSA on post-implantation development of gaur iSCNT embryos

The post-implantation development of gaur iSCNT embryos are shown in Table 4.2. For the male gaur iSCNT embryos, forty four TSA-treated embryos were transferred into eighteen bovine recipients. Nine of non TSA-treated embryos were transferred into three bovine recipients. The ultrasonography at day 45 after embryo transfer showed those three out of eighteen recipients (16.7%) of the TSA-treated group and one out of three recipients (33.3%) of the non TSA-treated group were pregnant. The abortions of all pregnant recipients from the TSA-treated group were found before day 90 of gestation. One recipient of the non TSA-treated group could maintain the pregnancy to term. Although the pregnancy rate and post-implantation development on day 60 were not significant difference between TSA-treated and non TSA-treated embryos, the post-implantation developments on day 90 to term of non

TSA-treated embryos shows significant higher than those of TSA-treated embryos ($P<0.05$). Cloned male gaur newborn was delivered by caesarean section on March 4th, 2008 (day 283 of gestation) with 20 kg birth weight (Figure 4.3A). Although the cloned gaur newborn appeared healthy with normal cardiac rhyme but the tactile hair along the body was not well developed. Three hours after birth, the cloned gaur newborn showed sign of dypsnea and finally died at 12 h after birth. Post-mortem examination revealed no evidences of abnormality, except an accumulation of muco-exudative fluids in the lung and along the respiratory tract. Pulmonary disorder was thought to be the cause of death.

Figure 4.3 Cloned gaur newborn derived from male gaur iSCNT embryo (A). Male gaur donor (B).

For female gaur iSCNT, fifty TSA-treated embryos were transferred to sixteen bovine recipients and three non TSA-treated embryos were transferred to one bovine recipient. The ultrasonography at day 45 after embryo transfer, six out of sixteen recipients (37.5%) of TSA-treated group were found pregnant. No pregnancy was observed in non TSA-treated group. However, there was no significant difference in the pregnancy rate and development to term among these two groups studied

(P>0.05). Four out of six recipients in TSA-treated group aborted before day 60 of gestation and one recipient aborted on day 242 of gestation (Figure 4.4A). Furthermore, mummified fetus was obtained by caesarean section on day 311 of gestation (Figure 4.4B). As a result, no calf was obtained from female gaur iSCNT group.

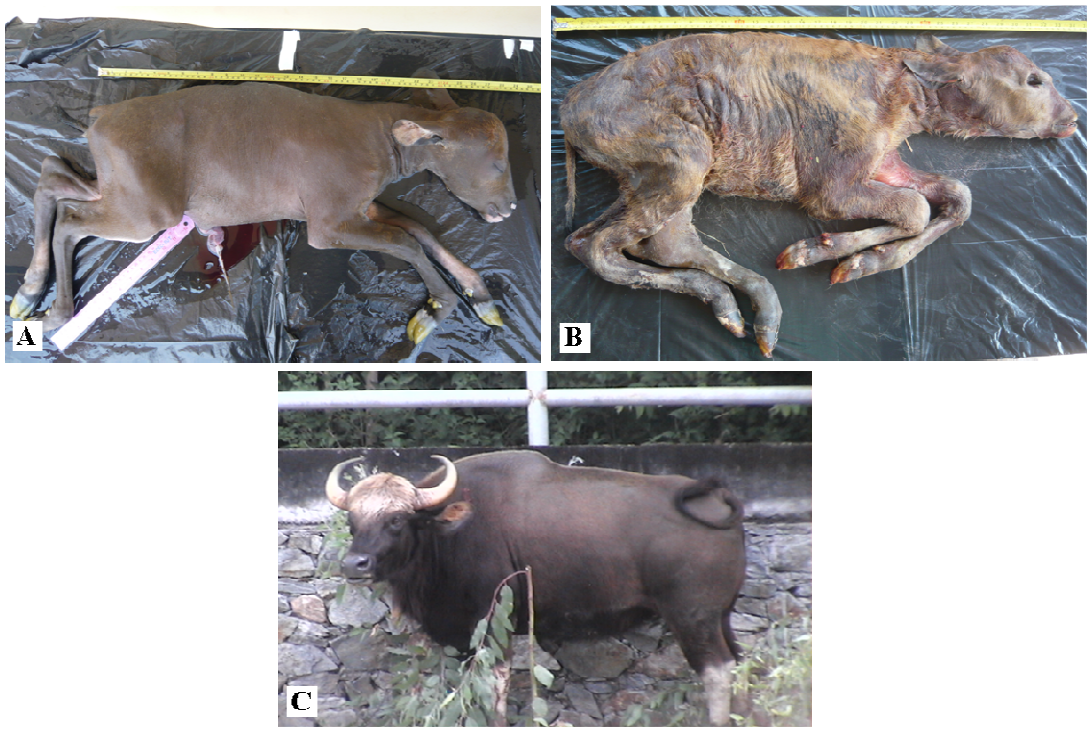

Figure 4.4 Gaur fetuses derived from female gaur iSCNT embryos. The aborted fetus at day 242 of gestation (A); the mummified fetus at day 311 of gestation (B). Female gaur donor (C).

4.4.3 Effects of TSA treatment on post-implantation development of vitrified/thawed male bovine SCNT embryos

Only male bovine embryos were examined in this experiment. From Table 4.3, twenty two vitrified/thawed embryos of TSA-treated group were transferred to eight

recipients and fourteen vitrified/thawed embryos of non TSA-treated group were transferred to seven recipients. The pregnancy rate was not significantly different between TSA-treated (3/8, 37.5%) and non TSA-treated (2/7, 28.6%) groups (P>0.05). In TSA-treated group, two recipients aborted before day 60 and 210 of gestation. Only one recipient could maintain pregnancy to term. Twin cloned bovine calves were born by caesarean section on February 11th, 2008 (day 277 of gestation). Both cloned bovine calves were healthy with birth weight of 25 kg (Figure 4.5). In non TSA-treated group, two pregnant recipients aborted before day 90 and 180 of gestation, respectively. Therefore, no offspring was born from non TSA-treated group.

Figure 4.5 Twin cloned calves derived from vitrified/thawed embryos of TSA-treated bovine SCNT group at 30 minutes after birth (A), one and half hours after birth (B).

4.4.4 DNA microsatellite analysis of cloned bovine calves, cloned gaur newborn, aborted and mummified gaur fetuses

To confirm the genetic identity of the four cloned bovine calves (two bovine calves from fresh embryo group and twin bovine calves from vitrified/thawed embryo

group), twelve bovine DNA microsatellite markers (MGTG4B, TGLA263, TGLA57, ETH225, INRA037, HEL9, TGLA53, CSSM66, ETH3, TGLA126, INRA063, INRA005; Table 4.1) were used for amplification of genomic DNA from the donor cells, the four cloned bovine calves and the three bovine recipients. All markers could distinguish PCR products of the bovine donor from those of three bovine recipients except INRA063 and INRA005 (Figure 4.6). Only the PCR product of the second recipient could be distinguished from the bovine donor by INRA063 whereas INRA005 could not distinguish the PCR product of second recipient from the bovine donor. However, other microsatellite analyses confirmed that all cloned calves were genetically identical to the donor cells and differ from the recipients (Figure 4.6).

To confirm the genetic identity of the cloned gaur newborn, aborted and mummified gaur fetuses, seven bovine DNA microsatellite markers (MGTG4B, TGLA263, TGLA57, ETH225, INRA037, HEL9, TGLA53; Table 4.1) were used for PCR amplification of genomic DNA from the donor cells (male and female gaur fibroblasts), the cloned gaur newborn, aborted and mummified gaur fetuses (Figure 4.7). Amplification failures of mummified gaur fetus template DNA were observed when MGTG4B and TGLA53 primers were used. However, the other primers were able to successfully amplify the gaur and bovine recipient genomic DNA. Analysis of bovine DNA microsatellite markers confirmed that the cloned gaur newborn, aborted and mummified gaur fetuses were genetically identical to their donor cells.

At this point, all cloned bovine calves from fresh and vitrified embryos are growing up normally with good health (Figure 4.8 and 4.9).

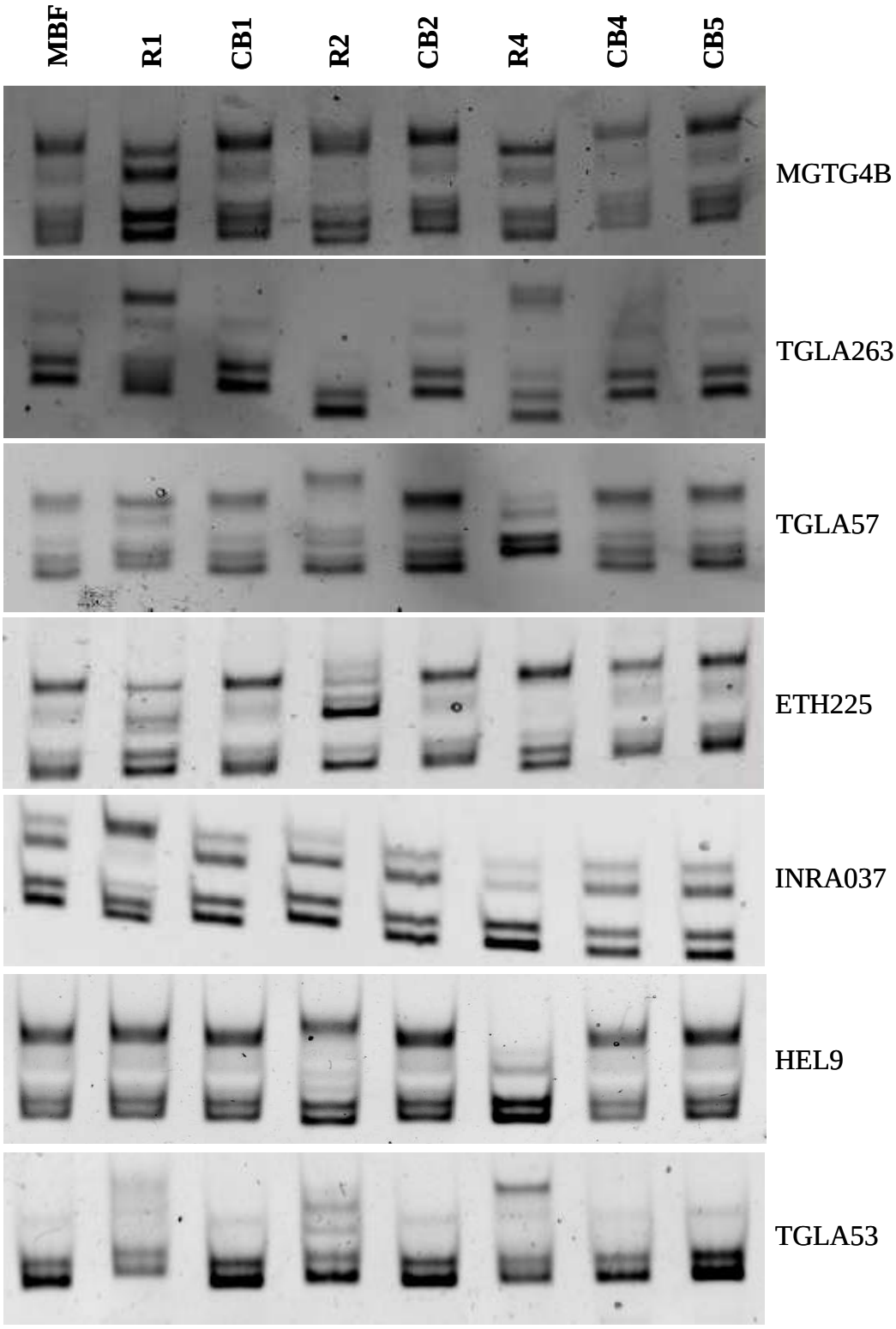

Figure 4.6 DNA microsatellite analysis of the cloned calves.

MBF, male bovine fibroblasts; R, bovine recipient; CB, cloned bovine calf

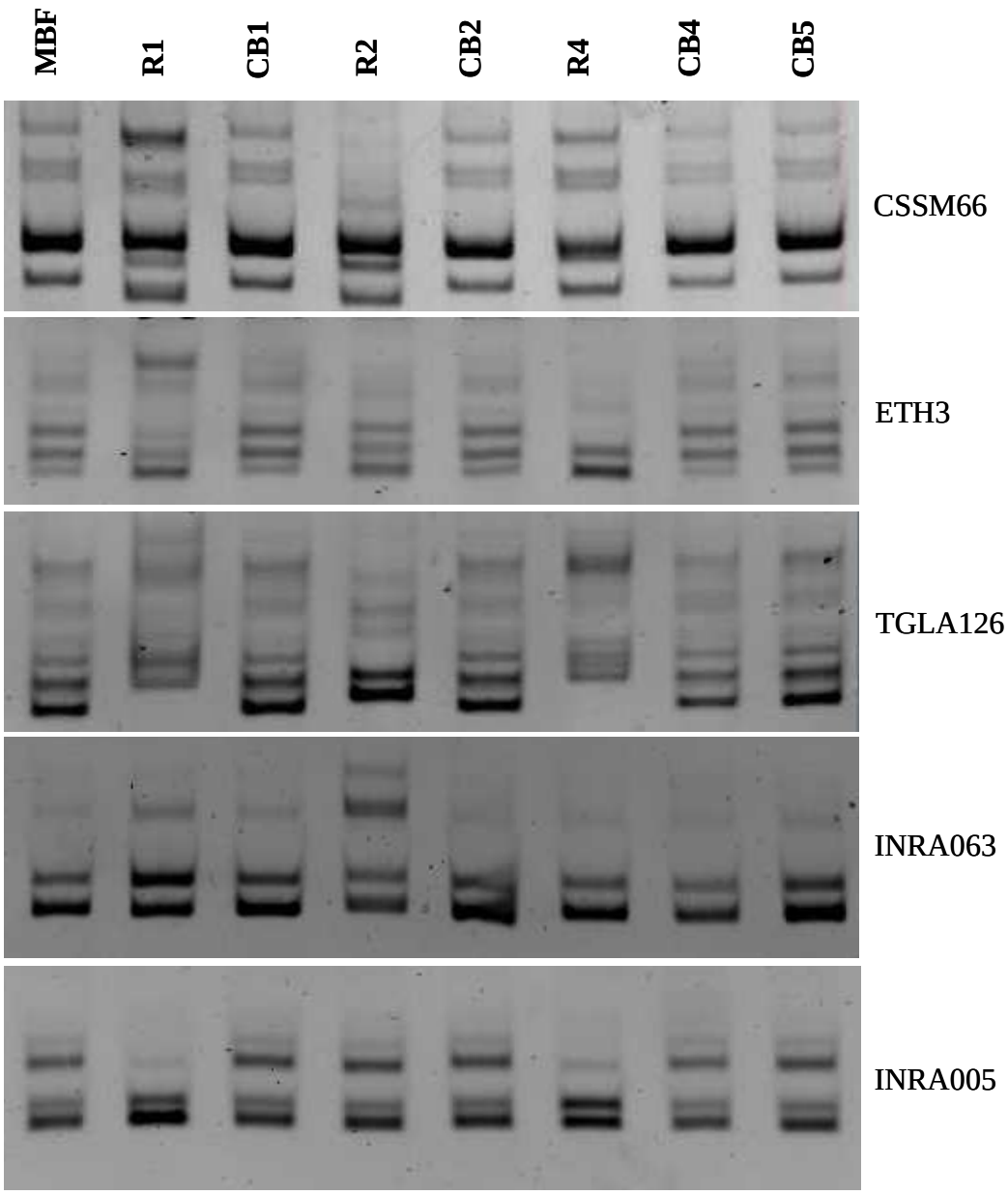

Figure 4.6 (Continued).

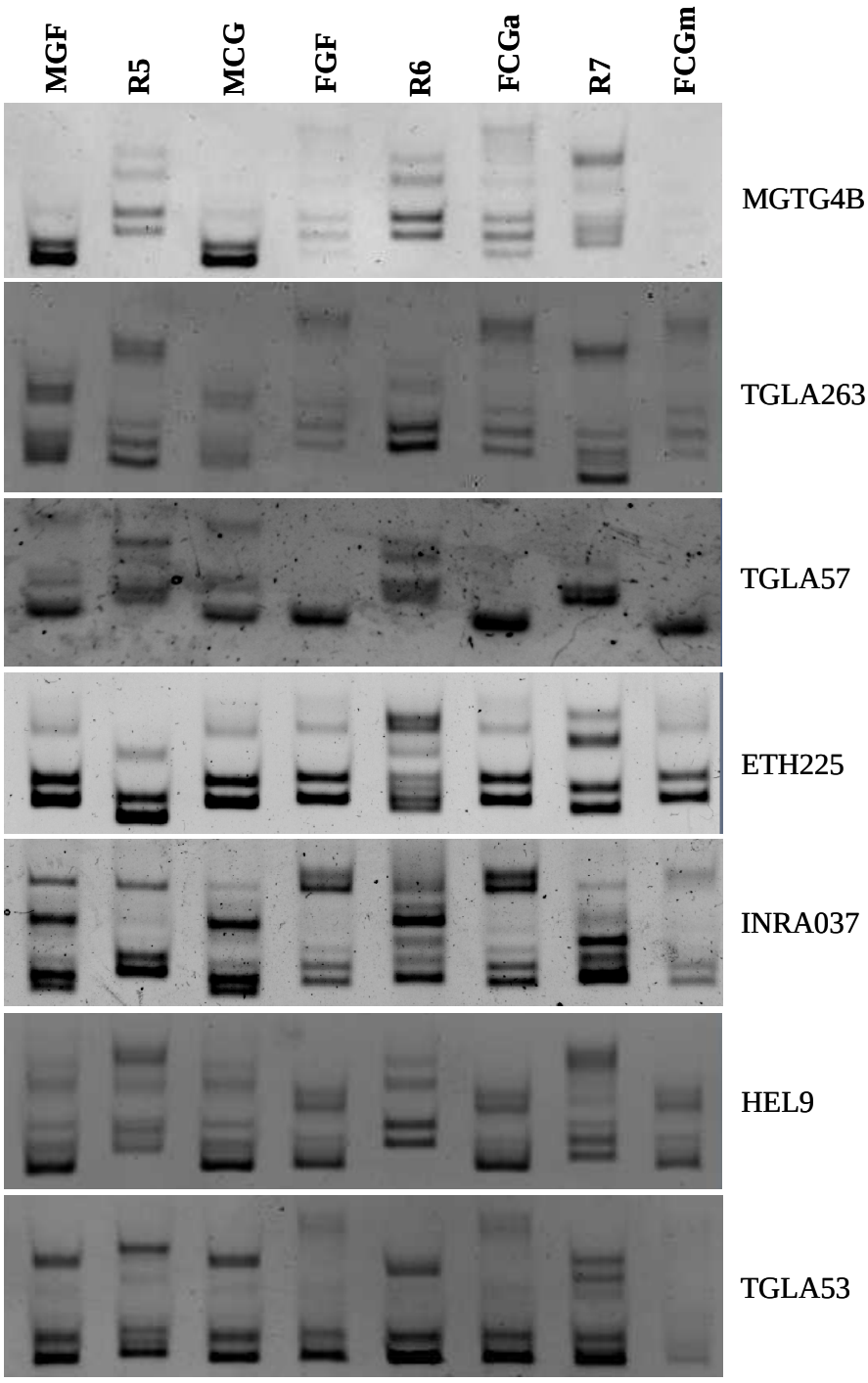

Figure 4.7 DNA microsatellite analysis of cloned gaur. R, bovine recipient; MCG, male cloned gaur; MGF, male gaur fibroblasts; FGF, female gaur fibroblasts; FCGa, female cloned gaur aborted; FCGm, female cloned gaur mummified.

Figure 4.8 Cloned calves from fresh (left) and vitrified/thawed (right) bovine SCNT embryos at 1 month after birth.

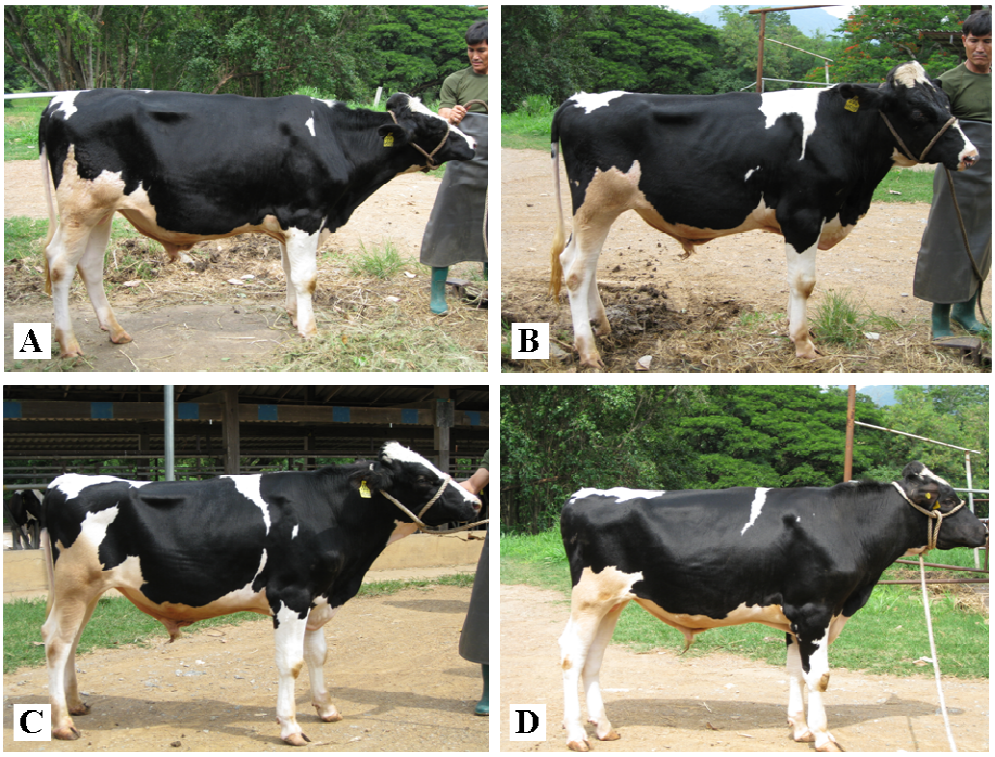

Figure 4.9 Cloned calves derived from fresh (A, B) and vitrified/thawed (C, D) bovine SCNT embryos at sixteen months after birth.

4.5 Discussion

From this study, TSA treatment could increase the pregnancy rate of bovine SCNT embryos, however, no positive effect was observed on gaur iSCNT and vitrified/thawed bovine SCNT embryos. Moreover, TSA treatment could not improve fetal development to term in all embryo types.

This is the first report that examined the effect of TSA treatment on post-implantation development of bovine SCNT, gaur iSCNT and vitrified/thawed bovine SCNT embryos. In this study, the calving rate after transferring TSA-treated bovine SCNT embryos to the recipients (23.1%) was higher than that of previous report (6.8%, Heyman et al., 2002). However, high success rate was reported by Kato and colleagues (1998) who produced eight cloned calves derived from somatic cell of a single adult bovine (80%). The incidence of abortion during the first four months of gestation was found in this study. The fetal losses in the first trimester of pregnancy of bovine SCNT are often associated with aberrant placental development of bovine cloning (Hill et al., 2000; Lonergan et al., 2007; Buczinski et al., 2009). Only the recipients carrying TSA-treated bovine embryos were pregnant. These results indicated that TSA could improve the pregnancy rate of bovine SCNT embryos. However, the effect of TSA treatment on bovine fetal development is still unclear. Previous reports found that TSA treatment could improve post-implantation development of cloned mouse (Kishigami et al., 2006; 2007; Maalouf et al., 2009). After TSA treatment, five-fold increase in the success rate of cloned mouse from cumulus cells has been shown. The cloned pups were healthy without obvious abnormality (Kishigami et al., 2006). Moreover, TSA treatment could improve the success rate of cloning from unclonable strains and all pups appeared normal and

lived for more than 6 months (Kishigami et al., 2007). Maalouf and colleagues (2009) confirmed that TSA treatment had positive effect on full-term development of mouse embryo leading to high rate of live pups. On the other hand, TSA treatment of mouse SCNT embryos for 8 to 12 h increased the blastocyst rate and full-term fetuses were obtained. However, longer TSA exposure (14 and 26 h) reduced the blastocyst rate and no fetus was obtained (Tsuji et al., 2009). In contrast, Meng and colleagues (2009) reported that no differences in the cleavage, blastocyst, and pregnancy rates were found between TSA-treated and non TSA-treated rabbit SCNT embryos. The embryos from both groups can develop to term. However, the TSA treated pups died within an hour to 19 days after birth. Only pups from non TSA-treated embryos grew to adulthood. Therefore, the effects of TSA treatment on cloning efficiency vary depend on species.

In this study, TSA treatment had no positive effect on pregnancy rate of male and female gaur iSCNT embryos. Although, the post-implantation development rate of non TSA-treated male gaur iSCNT embryos was higher than that TSA-treated group, no differences were found in those of female embryos. The fetal losses on the first trimester of gestation were also found similar to bovine SCNT embryos. It might be due to the incompatibility of fetal-maternal interactions between the gaur embryo and the bovine recipient's uterus or abnormal placental development (reviewed by Beyhan et al., 2007). Moreover, the fetal losses were also found in the third trimester of gestation. Similarly, Lanza and colleagues (2000) found that one recipient had late-term abortion on day 202 of gestation. Late-gestation losses in bovine involve in the incomplete reprogramming via SCNT (Heyman et al., 2002) and/or excessive accumulation of allantoic fluid (Wells et al., 1999). The low pregnancy rate and high

abortion incidence could be due to several factors such as iSCNT procedure, abnormal placental development, fetal malnourishment and hypoxia (Hammer et al., 2001). In this study, cloned gaur newborn from non TSA-treated embryos showed respiratory defect and eventually died within 12 h after birth. Previous report found that the live gaur calves were successfully produced after transferred to bovine recipients (Johnston et al., 1994). However, the high mortality rate of calves was found in the first week after birth due to interspecies incompatibilities (Johnston et al., 1994). There was no difference between the sex of the donor cell on post-implantation development in cloned gaur iSCNT embryos. Similarly, Le Bourhis and colleagues (1998) reported that no effect of sex on the pregnancy rate and the calving rate of cloned bovine embryos was observed. A few studies produced live iSCNT offspring successfully after transferring iSCNT embryos such as mouflon (Loi et al., 2001), gaur (Lanza et al., 2000; Vogel, 2001), African wild cat (Gómez et al., 2004), gray wolf (Kim et al., 2007), sand cat (Gómez et al., 2008) and bucardo (Folch et al., 2009) into domestic animal. Unfortunately, only cloned African wild cats and gray wolves are alive today. The cloned gaur survived for only 2 days after birth (Vogel, 2001), the cloned mouflon survived for about 7 months (Trivedi, 2001) and cloned bucardo newborn died a few minutes after birth (Folch et al., 2009).

TSA treatment had no positive effect on post-implantation development of vitrified/thawed bovine SCNT embryos. No differences in pregnancy rate between fresh and vitrified/thawed groups were observed. The pregnancy rate of vitrified/thawed embryos in this study (33.1%) was lower than that of previous report (67.7%, Lonergan et al., 2007). The fetal losses on the first and third trimester of gestation were also found in vitrified/thawed embryos. However, healthy twin cloned

calves were obtained from TSA-treated group. Therefore, this vitrification technique had no detrimental effect on post-implantation development after embryo transfer.

DNA microsatellite analysis confirmed that four cloned bovine calves, aborted and mummified gaur fetuses and one cloned gaur newborn were genetically identical to their own donor cell.

The low pregnancy rate and mortality of fetus on post-implantation stage might be caused by the incomplete reprogramming of important early embryonic genes or/and maternal-fetal incompatibility (reviewed by Beyhan et al., 2007). Thus, better understanding of the molecular and biochemical events during nuclear reprogramming are needed to improve these SCNT and iSCNT procedures.

4.6 Conclusion

TSA treatment could increase the pregnancy rate of bovine SCNT embryos, however, no positive effect was found on fetal development after day 45 of gestation to term but TSA treatment could not enhance post-implantation development of gaur iSCNT nor the vitrified/thawed bovine SCNT embryos.

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CHAPTER V

OVERALL CONCLUSIONS

SCNT has successfully produced offspring of domestic and laboratory animal species. This technique has been applied and adapted to endangered species using oocyte and recipient from domestic animal. This adapted technique is called iSCNT. However, the overall efficiencies of these techniques remain low due to incomplete reprogramming and several other factors. Previous studies found that TSA could enhance cloning efficiency in several species (Kishigami et al., 2006; Iwamoto et al., 2007; Zhang et al., 2007; Ding et al., 2008; Shi et al., 2008). However, this is the first report on the effects of TSA treatment on full-term development of bovine SCNT and gaur iSCNT embryos.

No difference was found in the rates of fusion, cleavage, development to 8-cell and morula stages of bovine SCNT and gaur iSCNT embryos either in the TSA-treated or non TSA-treated group. TSA treatment could increase blastocyst rate of bovine SCNT embryos but did not increase those of gaur iSCNT embryos. Moreover, similar blastocyst qualities were found in all groups. There was no difference on developmental potential of embryos derived from male or female gaur donor cells.

TSA treatment increased the pregnancy rate of bovine SCNT embryos. However, no difference was observed in gaur iSCNT embryos. TSA treatment could not enhance fetal development to term in neither bovine SCNT nor gaur iSCNT embryos. The male and female gaur iSCNT embryos had similar post-implantation

developmental potential. Moreover, TSA had no beneficial effect on vitrified/thawed bovine SCNT embryos in term of post-implantation development. Nevertheless, three cloned bovine calves were obtained from TSA-treated bovine SCNT group but one bovine calf died during birth. The cloned gaur newborn from non TSA-treated group of male gaur iSCNT embryos was delivered by cesarean section at day 283 of gestation. However, the gaur died at 12 h after birth with pulmonary disorder. Furthermore, twin cloned bovine calves from vitrified/thawed group of TSA-treated bovine SCNT embryos were born with good health. DNA microsatellite analysis confirmed that four cloned bovine calves, one aborted and one mummified gaur fetuses, and one cloned gaur newborn were genetically identical to the donor cells. Moreover, four cloned bovine calves grew up and are still alive today.

TSA could improve the pre-implantation development and pregnancy rate of bovine SCNT embryos but had no beneficial effect on full-term development of gaur iSCNT embryos.

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BIOGRAPHY

Ms. Kanokwan Srirattana was born on February 24, 1981 in Bangkok, Thailand. She graduated with a bachelor degree of Science in Biotechnology from the Faculty of Engineering and Industrial Technology, Silpakorn University in Year 2002. After graduation, she worked as research assistance and scientist in the department of Biotechnology, Faculty of Engineering and Industrial Technology at Silpakorn University. At that time, she learns the technique of molecular biology and biochemical technology. Three years later, she applied to study Master degree course in School of Biotechnology, Institute of Agricultural technology, Suranaree University of Technology with Asst. Prof. Dr. Rangsun Parnpai. While studying, she received assistant researcher's scholarship from National Center for Genetic Engineering and Biotechnology and Suranaree University of Technology. She also received a scholarship from the Japan Society for the Promotion of Science - National Research Council of Thailand scientific cooperation program for training in *in vitro* production, manipulation and cryopreservation of bovine embryos at National Livestock Breeding center and mitochondria DNA analysis in interspecies cloned embryo at National Institute of Livestock and Grassland Science, Japan for three months. The results from some part of this study have been presented as poster presentation at the 35th Annual Conference of the International Embryo Transfer Society in San Diego, California, USA, January 3-7, 2009 and oral presentation at the 47th Kasetsart University Annual Conference in Thailand, March 18-20, 2009.