Epigenetics ของตัวอ่อนกระบือปลักโคลนนิ่งและลูกอ่อนและลูกโคโคลนนิ่ง

นางสาวทัศนีย์ สุทีวรรณ

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EPIGENETICS OF CLONED SWAMP BUFFALO EMBRYOS, CLONED CATTLE FETUSES AND CALVES

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ปัจจุบันถึงแม้จะมีรายงานความสำเร็จในการผลิตสัตว์ที่ลอกเลียนพันธุกรรมสัตว์ต้นแบบได้สำเร็จในหลายสปีชีส์โดยเทคโนโลยีโคลนนิ่งแต่เทคโนโลยีนี้ยังคงต้องการการพัฒนาให้มีประสิทธิภาพสูงขึ้น เนื่องจากปัญหาความผิดปกติที่พบได้ในสัตว์โคลนนิ่ง รวมทั้งการแท้งของสัตว์โคลนนิ่งในอัตราสูงระหว่างการตั้งครรภ์จากรายงานการศึกษาพบว่าความผิดปกติเหล่านี้เกี่ยวเนื่องมาจากการเปลี่ยนแปลงในกลไกควบคุมการแสดงออกของยีนซึ่งเกี่ยวข้องในการเจริญเติบโตที่เรียกว่า ‘Epigenetics’ ที่มีต่างจากสัตว์ปกติ ได้แก่ การเติมหมู่เมทิลบนสายดีเอ็นเอ (DNA methylation) การเติมหมู่อะซิติลบนโปรตีนฮีสโตน (histone acetylation) และ รูปแแบบการฝังจำบนสายดีเอ็นเอ (gene imprinting)

กระบือปลัก (Swamp buffalo) เป็นสัตว์ที่พบได้ในประเทศไทย และแถบเอเชียตะวันออกเฉียงใต้ ซึ่งพบว่าการโคลนนิ่งกระบือปลักประสบปัญหาเดียวกันกับการโคลนนิ่งสัตว์ในสปีชีส์อื่น เราจึงทำการศึกษากลไกการควบคุมการแสดงออกของยีน (epigenetics) ได้แก่ รูปแบบ DNA methylation และ histone acetylation ในตัวอ่อน (embryo) กระบือปลักระยะก่อนฝังตัวด้วยเทคนิค immunostaining และตรวจสอบระดับการแสดงออกของยีนที่ควบคุมเอนไซม์ซึ่งใช้ในการเกิด DNA methylation ได้แก่ เอนไซม์ DNA methyltransferase (*DNMTs*) ชนิด 1, 3A และ 3B และ histone acetylation ได้แก่ เอนไซม์ histone (de)acetyltransferase (*HDAC* และ *HAT*) ชนิด 1 ในตัวอ่อนกระบือปลักระยะก่อนฝังตัว จากการทดลองพบว่าตัวอ่อนกระบือปลักโคลนนิ่งในระยะนี้มีการลดระดับเมทิลบนสายดีเอ็นเอ (DNA demethylation) ที่ผิดปกติ นอกจากนี้พบว่าการเจริญเติบโตของตัวอ่อนระยะนี้ แปรผันตามระดับความสัมพันธ์ของ DNA methylation และ histone acetylation ด้วย ผลการศึกษาระดับการแสดงออกของยีนควบคุมการสร้าง DNA methylation และ histone acetylation พบว่าตัวอ่อนกระบือปลักโคลนนิ่งมีระดับ *DNMT1* สูงกว่าปกติในระยะแรก ระดับเอนไซม์ *DNMT3A* และ *3B* สูงกว่าปกติในตัวอ่อนระยะบลาสโตซิส และ ระดับเอนไซม์ *HDAC1* และ *HAT1* สูงกว่าปกติเล็กน้อยในตัวอ่อนโคลนทุกระยะด้วย

นอกจากนี้ ได้ทำการศึกษารูปแบบการแสดงออกของยีนฝังจำ (imprinted gene) โดยศึกษายีน *IGF2r* จากลูกอ่อนระยะฝังตัว (fetus) ในช่วงอายุวันที่ 25, 45 และ 75 หลังการตั้งครรภ์ จากลูกอ่อนโคลนนิ่งที่ได้จากการแท้ง จากสัตว์โคลนนิ่งซึ่งตายภายในระยะเวลาอันสั้น

คลอด และ สัตว์โคลนนิ่งที่มีสุขภาพสมบูรณ์ เราได้ศึกษารูปแบบของการเลือกแสดงออกของยีนที่ฝังจำ (allellic imprinted gene pattern) โดยใช้เทคนิค SSCP จากการศึกษาพบว่าสัตว์โคลนนิ่งทุกชนิดมีรูปแบบการแสดงออกของยีนฝังจำที่ผิดปกติรวมทั้งในสัตว์โคลนนิ่งที่มีสุขภาพสมบูรณ์ด้วยแต่พบว่าลูกอ่อนโคลนนิ่งที่ได้จากการแท้งมีการแสดงออกของอัลลีลที่ผิดปกติมากอย่างเห็นได้ชัด นอกจากนี้ เราได้ทำการศึกษาระดับการแสดงออกของยีน *IGF2r* ในลูกอ่อนโคลนนิ่งอายุ 25, 45 และ75 วัน และพบว่า ระดับการแสดงออกของยีน *IGF2r* ไม่มีความแตกต่างจากลูกอ่อนปกติในระยะเดียวกัน

สาขาวิชาเทคโนโลยีชีวภาพ

ปีการศึกษา 2548

ลายมือชื่อนักศึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

TATSANEE SUTEEVUN : EPIGENETICS OF CLONED SWAMP BUFFALO EMBRYOS, CLONED CATTLE FETUSES AND CALVES. THESIS ADVISOR : RANGSUN PARNPAI, Ph.D. 117 PP.
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CHROMATIN REMODELING/DNA METHYLATION/EPIGENETICS/GENE IMPRINTING/HISTONE ACETYLATION/NUCLEAR TRANSFER

Nuclear transfer or cloning is a potential technology for the production of genetically identical animals, and cloned offsprings have been reported in many species. However, this technology needs more improvement due to the high rate of loss that has been observed during gestation and the abnormalities that have been found in cloned animals. Epigenetic alterations such as DNA methylation, histone acetylation and genetic imprinting, lead to improper control of gene expression during the developmental period and have been suspected to be the cause of the myriad abnormalities observed in clones.

Swamp buffalo, a multipurpose animal that is widely found in South East Asia and Thailand, has also been successfully cloned with blastocyst formation reported but unfortunately low cloned birth rates have been obtained. We study first here the epigenetic characteristic of cloned and IVF swamp buffalo embryos during preimplantation development. DNA methylation and histone acetylation are the key mechanisms examined in our study. Global DNA methylation and histone acetylation were observed using immunostaining and expression levels of genes that control DNA methylation; *DNA methyltransferase 1*, *3A* and *3B*, and histone acetylation; *histone* throughout developmental stages. To study imprinted genes; the expression pattern of

IGF2r gene was examined in fetal cloned calves during gestation periods at day 25, 45 and 75 of gestation and aborted fetuses and clones calves derived from death shortly after birth and normal living clones. Allelic imprinted gene expression patterns were identified using SSCP. Real time RT-PCR was performed to study the *IGF2r* expression levels in cloned and control fetuses at days 25, 45 and 75 of gestation.

Aberrant global demethylation patterns were observed in cloned swamp buffalo embryos during the preimplantation developmental stage. Although, histone acetylation was not expressed as an aberrant global pattern during this period, improper interactions between DNA methylation and histone acetylation was obviously marked in cloned embryos at the 8-cell stage, at which high rates of developmental failure are observed in clones. The mRNA expression patterns of genes responsible for these mechanisms, *DNMT1*, *3A*, *3B*, *HAT1* and *HDAC1* showed comparable patterns between cloned and IVF preimplantation embryos. Clones showed higher mRNA levels of *DNMT1* at an early stage, whereas, hyper-expression of *DNMT3A* and *3B* were illustrated at the blastocyst stage as compared to IVF embryos. Consistent to the results of *de novo* DNA methylation gene expression, the *HAT1* and *HDAC1* mRNA levels displayed higher levels in cloned embryos. For imprinted gene observations in cloned cattle, improper allelic expression patterns were found in many organs of aborted cloned fetuses. However the tissue derived from healthy living cloned also showed the slightly different allelic expression pattern when compared to those derived *in vivo*. The result of *IGF2r* gene expression displays comparable expression levels in most organs of interest between cloned and control fetuses derived at day 25, 45 and 75 of gestation.

We summarize that the aberrant DNA demethylation pattern in cloned swamp

buffalo embryos during the preimplantation stage may be the cause of developmental failure as has been reported in other species. We suggest that the loss of optimal interaction between histone acetylation and DNA methylation may relate to development malfunction during the early embryonic stages. Our investigations in different cattle tissues also support that the fidelity of allelic imprinting gene expression patterns relates to normal fetal development.

School of Biotechnology

Academic Year 2005

Student's Signature Tatsanee Sakigusa

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LIST OF ABBREVIATIONS

3’UTR	=	3' Untranslated Region
bp	=	base pair
CT	=	cycle threshold
cDNA	=	complementary DNA
d	=	day
DNA	=	deoxyribonucleic acid
g	=	gram
h	=	hour
H3K9	=	histone3 lysine9
IgG	=	gamma immunoglobulin
IVF	=	*in vitro* fertilization
MII	=	metaphase II
min	=	minute
ml	=	milliliter
mM	=	millimolar
NT	=	nuclear transfer
PCR	=	polymerase chain reaction
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
SEM	=	standard error means
V	=	volts

LIST OF ABBREVIATIONS (Continued)

μg	=	microgram
μM	=	micromolar
μl	=	microlitre
μsec	=	microsecond

CHAPTER I

INTRODUCTION

1.1 Introduction

Swamp buffalo (*Bubalus bubalis*) and cattle (*Bos taurus* and *Bos indicus*) are important multi-purpose livestock used in Thailand for agricultural production and also in many countries depend heavily on their production of meat and milk in addition to their value as laboring farm animals.

Swamp buffalo (*B. bubalis*) can be found from the Philippines to as far as India. However, the buffalo population in many countries has markedly decreased because of an annual negative growth rate during the past 10 years. According to the Department of Livestock Development report (2000), Thailand, which has the largest buffalo population, has now shown a 3.4% reduction over the last decade. The Philippines and Malaysia also indicate an annual negative growth rate of 1.3% and 2.3%, respectively. In Thailand, six million swamp buffalos were reported in 1981 and that number has fallen to 1.2 million in 1999 and numbers less than eight hundred thousand currently. It was predicted that not only could buffalo be endangered in Thailand, but they are also at risk for global extinction because of the annual worldwide decline. The reduction of swamp buffalo is partly due to the slow rate of maturation and low rate of rebreeding after calving. Although, the assisted reproductive technologies; artificial insemination and *in vitro* fertilization

(Pavasuthipaisit et al., 1992) have been used to assist buffalo reproduction but its achievement is still very low in raising the number of swamp buffalo because of the difficultly detecting heat and the short period of ovulation. Somatic cell nuclear transfer technology is known to be an efficient strategy to preserve endangered animals through donor cell cryopreservation. The somatic cell nuclear transfer has been reported in various species such as sheep (Wilmut et al., 1997), mouse (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999), cattle (Galli et al., 1999), buffalo (Parnpai et al., 1999), gaur (Vogel, 2001), goat (Keefer et al., 2002), cat (Shin et al., 2002), horse (Choi et al., 2002), mule (Woods et al., 2003), rat (Zhou et al., 2003) and pig (Polejaeva et al., 2000). However, the nuclear transfer technique needs more understanding and technical improvement because very few of the reconstructed embryos succeed in developing to term resulting in fetal loss throughout gestation. Although, nuclear transfer swamp buffalo blastocyst have been reported since 1999 (Parnpai et al., 1999), no scientific information in terms of successful pregnancies has been reported in this species. An intrincsic disorder such as incompleted nuclear reprogramming during early developmental stages has been suggested to be the cause of many abnormalities observed in cloned swamp buffalo embryos. Thus, the epigenetic reprogramming regulating gene expression during the early developmental stages in cloned swamp buffalo embryo should be defined.

Epigenetic modifications are the processes that influence the function of genes without affecting the genetic sequence. These include global changes in DNA methylation and histone acetylation as well as imprinted gene expression, which are involved in transcriptional regulation. Some cloned animals die shortly after birth, and some encounter various abnormalities such as chronic pulmonary hypertension,

circulatory distress, placental edema and LOS (large offspring syndrome) (Han et al., 2003; Reik et al., 2003). Incomplete nuclear reprogramming, such as genetic imprinting, is considered to be the cause of these abnormalities during cloned fetal development. Among all of the species that nuclear transfer has been performed, cattle show the most efficiency and highest number of live births. However, a high rate of fetal loss and abnormalities have been commonly observed in this cloned species (Hill et al., 2000). Aberrant gene expression patterns have been observed in clones during development (Wrenzycki et al., 2004). Thus, more advanced information on epigenetic mechanisms should be pursued in this species.

Here, we hypothesized that cloned embryos may exhibit characteristics of aberrant epigenetic reprogramming during the preimplantation stages. Our preliminary experiments in cloned swamp buffalo embryos were designed to examine global DNA methylation and histone acetylation levels using immunochemistry throughout the preimplantation developmental stages, which would correlate with aberrant epigenetic reprogramming thought to be manifested after nuclear transfer. Moreover, we defined the temporal expression levels and patterns of five chromatin remodeling genes, DNA methyltransferase (*DNMTs*), histone acetylase (*HAT*) and deacetylases (*HDAC*), which are responsible in epigenetic modifications, using real time RT-PCR in single metaphase II oocytes and throughout embryonic preimplantation stages.

To study post implantation developmental stages, the allelic expression of imprinted genes was examined in clones; fetuses at day 25, 45 and 75 of gestation, aborted fetuses, calves that died shortly after birth and living clones as compared to conventionally bred calves. We hypothesized that the aborted fetuses and dead cloned animals may show abnormalities in the allelic expression of imprinted genes and

living clones would be similar to control animals. These experiments provide the characteristic allelic imprinted gene expression for these groups studied. Hence, we expected to find obvious clues to the observed abnormalities, which are crucial to the success or failure of cloned animal development in terms of epigenetic reprogramming.

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CHAPTER II

REVIEW OF LITERATURES

2.1 Preimplantation development in mammals

Preimplantation embryos undergo several morphological changes and cellular proliferation throughout development in a step specific manner. This period is controlled by an intrinsic mechanism, which has an exact pattern and a variety of modifications, in order to support embryonic developmental reliably. Over 5,000 genes have been found to be specifically expressed only during the preimplantation period (Ko et al., 2000). Three out of four of the 15,700 genes which are expressed throughout development, show very low expression but are nesscessay for normal embryonic devlopment (Wrenzycki et al., 2005). This demonstrates the sensitivity of the restricted programing during embryonic development.

During the early development of all species the zygotic genome is inactive. The stored maternal mRNA is used as the source of cellular activity in the maternal-zygotic transition (MZT) period, during which the zygotic genome becomes active. The MZT period occurs in a species dependent manner. In bovine embryos, zygotic genome activation occurs at the 8-16 cell stages, whereas it occurs at the 4-cell stage in humans and the 2-cell stage in mice (Shi et al., 2003; Telford et al., 1990). This period acts as a check point for the completion and realiability of the embryonic genome. Many embryos fail to develop at this period. After the MZT period protein synthesis depends on transcription of the zygotic genome. The fidelity of embryonic genome activation influences normal

genome. The fidelity of embryonic genome activation influences normal embryonic development. At the blastocyst stage, the cells differentiate into 2 lineages; the inner cell mass (ICM) and trophoblast cells (TE), which develop into embryos and extraembryonic tissues, respectively (Morgan et al., 2005).

The process of determining gene activity and the cell lineages are essential to embryonic development and differentation. The phenomena which regulates this determining process is known to be controlled by epigenetic mechanisms (Reik et al., 2003).

2.2 Epigenetics

Over 15,000 genes are marked for expression throughout murine development (Ko et al., 2000). Many of these genes are expressed only during the preimplantation period and are then shut down in somatic cells by epigenetic regulations of specific genes expression patterns (Ehrlich, 1982; Imamura et al., 2001). This implicates the crucial role that epigenetics plays during embryonic development. During preimplantation, the fidelity of the expression levels and the timing of these genes expression critically influence normal embryonic development and differentiation. The vast majority of factors that switch on and off gene activity are epigenetic (Li, 2002). Epigenetics influence gene activity without affecting the DNA sequence. Epigenetics can also be modified by the environment and transmitted to progeny. Two major mechanisms of epigenetic activity are DNA methylation and histone modifications.

DNA methylation is the major epigenetic mechanism, which can interact with and modulate the other regulatory mechanisms (Li, 2002). Methylation plays a role in MEPC), which results in the blocking of the binding of transcription factors on the DNA. Another mechanism of transcriptional regulation by methylation is the association of methylated

DNA with the deacetylation of histones which leads to the condensation of the DNA into a chromatin conformation. This interrupts the binding of transcription factors on the DNA, resulting in gene repression. DNA methylation also interacts with other histone modifications to modulate gene repression and heterochromatic regions (Reik et al., 2003).

Histone modifications are another epigenetic mechanism, which directly effects gene activity through the modification of histone proteins, including acetylation, methylation and phosphorylation. Histone modifications are a post-translational modification, which plays an important role in regulating gene activity through chromatin conformation. The addition or removal of specific modifications at marked positions on the DNA confer the specific function of the gene, which is governed by the conformation of the DNA. It has been realized that these modifications are dynamic during development and vary among different tissues. Accordingly, acetylation of histones tends to destabilize chromatin structure, it causes nucleosomes to loosen, leading to gene expression. Removing acetylation results in gene inactivation (Li, 2002). Most phosphorylation and methylation modifications shut off genes and the surrounding area, however, modifications at some specific positions regulate for different functions.

2.3 DNA methylation

DNA methylation is a process whereby methyl groups bind to the carbon at position 5 of the pyrimidine ring of cytosine bases in mammalian DNA. The primary function of DNA methylation is for host defense system; protecting the cell from foreign DNA introduction (Kisseljova et al., 1998). In eukaryotes, there are two types of normal methylation processes. First, active methylation (*de novo* methylation) involves the rearrangement of methylation patterns during the embryogenesis process and in adult cells (Laird, 1997). Second, passive methylation, which adds a methyl group onto the DNA

strand after DNA replication (Monk, 1990). The specific mechanism by which this dynamic genome methylation occurs is unknown. However, the mechanism is involved with a group of enzymes: DNA methyltransferase (*DNMTs*) (Bestor, 2000). These enzymes have been classified into 5 types; *DNMT1*, *DNMT2*, *DNMT3A*, *DNMT3B* and *DNMT 3L*, but only 3 of them are considered to be the main catalytic enzymes of the DNA methylation mechanism *in vivo*; *DNMT1*, *DNMT3A* and *DNMT3B.*

DNMT1 is the major methyltransferase enzyme used in somatic cells, which is predominantly involved in passive DNA methylation by maintaining methylation through mitosis, DNA replication and in the DNA repair processes of the cell (Leonhardt et al., 1992). Characteristically, *DNMT1* has a 5 to 30 fold preference for adding methyl groups to hemimethylated CpG sites, which are established by *DNMT3A* and *DNMT3B* (Yoder et al., 1997). The DNA methyltransferase enzyme in cattle is encoded by the *DNMT1* gene. This enzyme employs the splicing isoform, *DNMT1o*, which is important for embryonic development. This variant isoform can be detectable during the preimplantation period (Howell et al., 2001; Ratnam et al., 2002). After fertilization, DNA is passively demethylated, passed through DNA replication except for some specific regions such as imprinted DNA and centromeric regions. In cattle, *DNMT1o* is excluded from the embryonic nucleus within the first few cleavages divisions and is allowed to enter the embryonic nucleus for short period during the 8-cell to 16-cell stage before disappearing again (Howell et al., 2001; Ratnam et al., 2002). In the mouse, *DNMT1o* is replaced by *DNMT1* at the implantation stage (Ratnam et al., 2002) and *DNMT1* is allowed to reenter the nucleus after implantation (Howell et al., 2001; Ratnam et al., 2002). The fluctuating pattern of this enzyme may be because it is involved in maintaining imprinted genes during the preimplantation period (Howell et al., 2001). Disruption of the *DNMT1* gene effects genome-wide DNA demethylation and causes embryonic lethality after gastrulation (Li et al., 1993). Knockout *DNMT1* (-/-) murine embryos show biallelic expression of most

imprinted genes, suggesting that *DNMT1* is involved in controlling the monoallelic expression of imprinted genes (Li et al., 1992). Cattle IVF embryos show a subsequently decreasing expression pattern of *DNMT1* throughout preimplantation development (Golding and Westhusin, 2003).

DNA methyltransferase enzymes type 3 alpha *(DNMT3A)* and 3 beta *(DNMT3B)* are involved in *de novo* methylation (active methylation), and are expressed at high levels during embryogenesis. *DNMT3A* and *3B* have a similar function in genome-wide *de novo* methylation, which is necessary for proper embryonic development, after passive demethylation, following fertilization. Although, *DNMT3A* and *3B* have a similar function, their catalytic domain shows that this occurs through a different mechanism. *DNMT3A* shows methylation activity on the flanking site of pyrimidines rather than purines, *DNMT3B* does not show this preference. *DNMT3A* cannot replace *DNMT3B* in function because it is less efficient than *DNMT3B* at methylating CpG rich DNA such as centromeric minor satellite repeats which play a role in chromosome stability (Okano et al., 1999). However, DNA methylation by *DNMT3A* can be controlled more accurately. Thus, *DNMT3A* and *DNMT3B* are both needed for normal development. An investigation by Watanabe and colleagues (2002) revealed that *DNMT3A* and *DNMT3B* have a different timing of gene expression in the mouse during the implantation period, preferring *DNMT3A* in the later stages but *DNMT3B* during the early stages. This implies that these two proteins function differently during the early stage of embryogenesis. A deficiency of *DNMT3A* and *3B* leads to hypomethylation and embryonic lethality. Keneda and colleagues (2004) demonstrated that *DNMT3A* plays a key role in paternal and maternal imprinting of genes. Deficiency of *DNMT3B* has been reported in ICF (immunodeficiency, centromeric instability, facial abnormalities) syndrome, which is caused by hypomethylated pericentromeric satellite regions on specific chromosomes (Miniou et al., 1997). There is evidence that the *de novo* methylation function of *DNMT3A* and *3B* play an important role

during the preimplantation developmental period but not appear to be involved in murine preimplantation development (Okano et al., 1999; Watanabe et al., 2002). Proper expression of *DNMT3A* and *3B* are crucial for normal development. The *DNMT3A* and *3B* expression patterns in bovine, have only been observed in the blastocyst stage and are described to have high expression levels of both genes at this stage (Wrenzycki et al., 2005).

2.4 Histone acetylation

The chromosome is the comprehensive structure of packed chromatin, which is packaged by the basic repetitive unit of chromatin; the nucleosome. Nucleosomes contain DNA and histone proteins packaged into core histones. Core histone proteins are composed of four subunits; H2a, H2b, H3 and H4. Each has a histone tail, which is a long N-terminal. The post-translational modifications of histone tails provide an epigenetic marking system to regulate gene expression (Turner, 2000). Among these, histone acetylation is the most widely studied. Acetylation leads to chromatin loosening and gene transcription. Histone acetylation is achieved by the histone acetylase (*HATs*) enzymes, which add acetyl groups to specific lysine residues to neutralize the positive charges on the amino-terminus of the histone protein, which leads to chromatin decondensation and transcriptional activation. Conversly, histone deacetylation promotes chromatin condensation, resulting in blocked gene transcription through the action of histone deacetylase (*HDACs*) enzymes which can remove the acetyl group from the histones causing chromatin condensation and gene repression .

Over twenty acetyltransferases catalyze the reaction by which acetyl groups associate with histone tails (Katan-Khaykovich and Struhl, 2002). The remarkable family of *HATs* belongs to the *GNAT* superfamily, which is involved in transcriptional initiation.

This superfamily includes the *GCN5*, *PCAF* and *HAT1*, which are divided into 2 types, type A and B. *HATs* Type A; such as *GCN5*, are found in the nucleus, where they play a role in gene activation. *HATs* Type B are found in cytoplasm and function to acetylate nascent histones in the cytoplasm, except for *HAT1*, which can shuttle to the nucleus and play a functional role in transcriptional expression and chromatic assembly (Grunstein, 1997). Contrary to the *HAT* function, the histone deacetylases (*HDACs*) enzymes are responsible for removing acetyl groups from histone proteins in nucleosome, leading to the repression of gene activity. *HDACs* are divided into 3 classes; class I is composed of *HDAC* 1, 2, 3 and 8, which are generally localized to the nucleus and directly regulate specific gene transcription and differentiation (McGraw et al., 2003). The *HDAC1* enzyme was reported to have the strongest expression signal in the class I group during embryogenesis. *HDAC* class II, unlike class I, is cytoplasmic and is shuttled to the nucleus when they are needed. Class II contains *HDAC* 4, 5, 6, 7, 9 and 10. Class III enzymes contains 7 members; *SIR*1-7, which do not localize in the nucleus. Class III *HDACs* are homologues of yeast, which are found in human. There is evidence that histone deacetylation is tightly linked to DNA methylation. Fuks et al. (2001) demonstrated that *HDAC* interacts with *DNMT 3A* at a cysteine-rich region; ATRX-homology domain, and they play a role in transcriptional repression.

The expression of histone acetylation and deacetylation enzymes were observed throughout preimplantation cattle embryonic development by McGraw et al. (2003) and demonstrated the low but detectable levels of the *HDAC1* and *HAT1* genes during the early stages. The expression of these genes was shown to be at the highest level during the blastocyst stage and much higher expression levels of *HDAC1* than *HAT1* were observed**.**

2.5 Genomic imprinting

Genomic imprinting is an epigenetic phenomenon characterized by parent-of-origin-specific gene repression. One of the two parental alleles is suppressed and the other is expressed normally. DNA methylation is the key molecular mechanism of this allelic suppression. Most imprinted genes are genes regulating development. Thus the proper allelic expression and the level of imprinted gene expression is very important for the development.

Parental imprinting must withstand genome-wide demethylation at the primordial germ cell stage following the imprints establishment during gametogenesis and is maintained stabily through embryogenesis and development (Hajkova et al., 2002; Kafri et al., 1992). Most imprinted genes organize in clusters and contain differentially methylated regions (DMR) which can have repeats of over a thousand CpG dinucleotides and are known as CpG islands. Generally, CpG islands are unmethylated and are usually found in house-keeping genes, with the exception of imprinted genes. The CpG islands on imprinted genes are normally methylated, which make the DMR subject to epigenetic regulation of the imprinted genes activity (Reik and Walter, 2001; Tilghman, 1999). Most DMRs control the gene expression.

So far, 73 imprinted gene have been found in mice (Yang et al., 2005), but only fews of them have been proven to be imprinted in cattle; the *IGF2, IGF2r, H19* and *Nnat* genes. Among different species, imprinted gene expression patterns have variation. Some imprinted genes are not imprinted in primates, whereas they may be highly conserved with regards to epigenetic regulation in other species (Killian et al., 2001). Among those genes, the *IGF2* and *IGF2r* genes are well studied and show a comparative expression pattern among species (Humpherys et al., 2001; Yang et al., 2005).

Insulin like growth factor 2 receptor (*IGF2r*), is a maternally expressed coding-protien, encoded by the *IGF2r* gene. This gene is composed of the unmethylated and active

promoter upstream and a methylated CpG island downstream which, inhibits the maternal antisense strand expression. To the contrary, the paternal allele confers the methylated and inactive promoter and unmethylated CpG island, thus, this leads to a silent state (Surani, 2001). The *IGF2r* gene expresses the receptor for insulin growth factor 2, which induces fetal growth. Aberrant gene expression can cause the accumulation of fetal growth factor, leading to the occurance of Large Offspring Syndrome (LOS) in sheep and cattle.

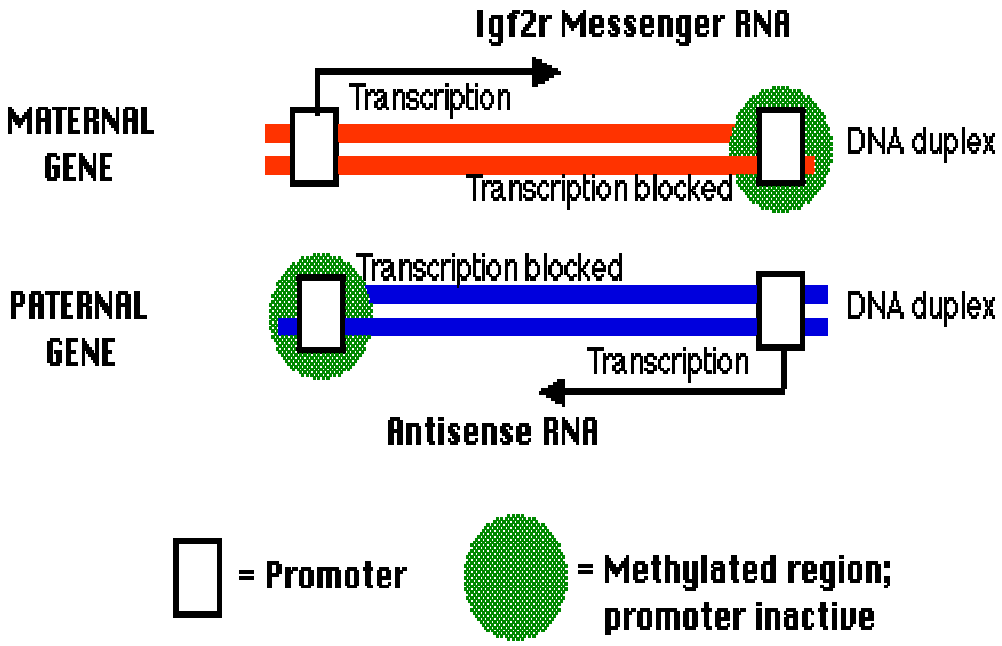

Figure 2.1 The *IGF2r* gene, a maternally expressed protein-coding gene, contains the upstream promoter to activate maternal allele expression and the downstream CpG island acts as the transcription inhibitor for the paternal allele (Imprintedgen, 2005).

2.6 Epigenetic reprogramming in preimplantation development

Reprogramming involves the removal of epigenetic marks from the nuclear DNA and confers another set of epigenetic mark into the DNA (Reik et al., 2001; Surani, 2001). Nuclear reprogramming induces a toti- or pluri-potent state, complete developmental gene expression and lineage cell division during the embryonic stages. Nuclear reprogramming occurs through two events. First, complete reprogramming takes place in the primordial germ cell and second, partial reprogramming, takes place after fertilization.

Different species show different timing of reprogramming by fertilization but the same pattern is conserved. After fertilization, protamines, which are present heavily in the sperm nucleus, are replaced by histones, which can be easily modified. Histones in the sperm nucleus are highly acetylated and the male nucleus undergoes a rapid, genome-wide active demethylation immediately following fertilization (Santos et al., 2002). Contrary, maternal genome is not change until the passive DNA methylation occurs at first cleavage division (Morgan et al., 2005). However, reprogramming at this step is partial, the specific region, such as gene imprinting and heterochromatic region, are not demethylated.

During the preimplantation stages, the global DNA methylation is reduced at the first few cleavage divisions in order to unlock genes from the methylated state and start essential developmental gene expression. Although, the reprogramming pattern is conserved in mammals, the precise timing of expression is different and depends on the species. In mice, DNA demethylation starts at the first division and is stabilized at implantation. In bovine, the same demethylation period as mice has been observed, and de novo methylation occurs at the 8-16 cell stage (Dean et al., 2001). At the morula stage, nuclear reprogramming determines the start of the program for differentiation. Cells are differentiated into two types, ICM and TE, of which the ICM is more highly methylated than the TE.

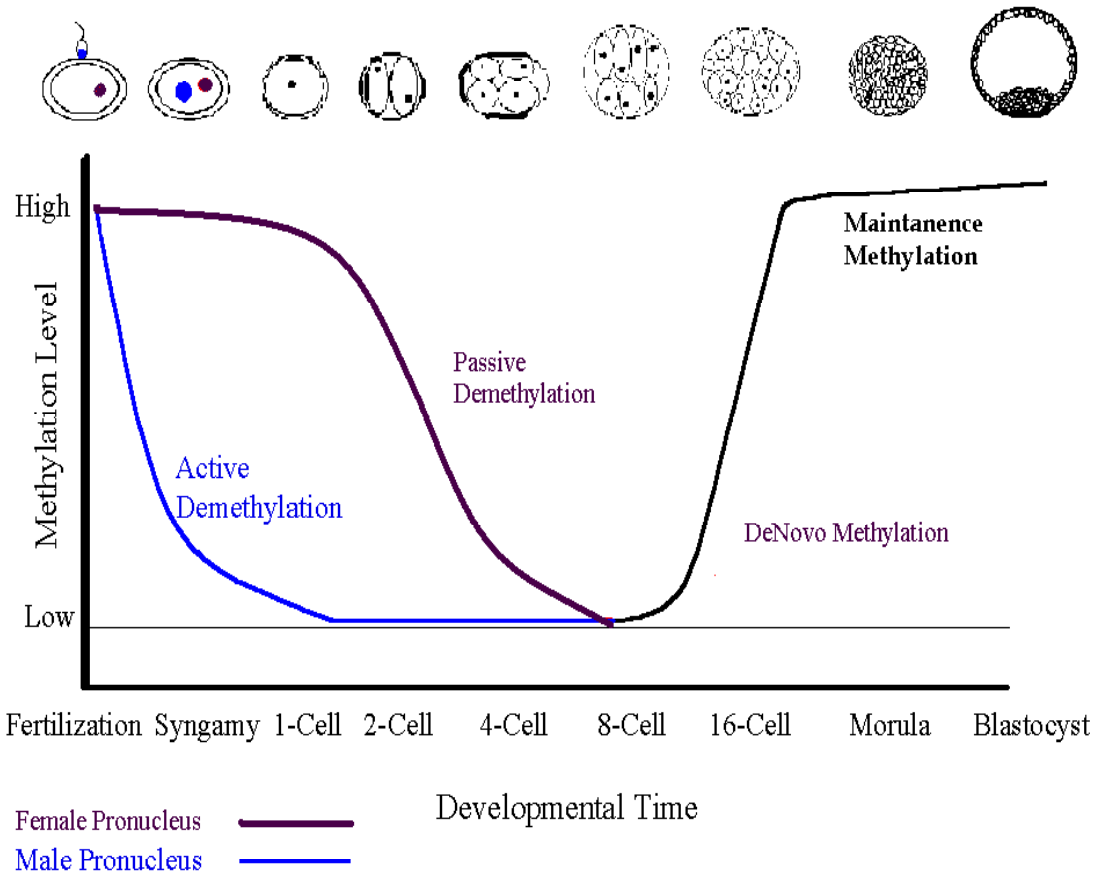

Figure 2.2 Epigenetic reprogramming by DNA methylation during bovine preimplantation development. A schematic reprogramming diagram (Reik et al., 2001) shows immediate active demethylation in the sperm nucleus after fertilization, whereas, the female genome is passively demethylated via cleavage divisions. The embryonic nuclei are reset to the different developmental program by *de novo* methylation at the eight to sixteen-cell stages.

2.7 Nuclear transfer

Somatic cell nuclear transfer (SCNT) is one of the most provacative technological achivements of the last decade. This is a potent technique to reprogram the differentiated cell to a totipotent state. Nuclear transfer takes the genetic material from a donor cell nucleus and places it in an enucleated oocyte where it is reprogrammed via mechanisms that nowaday remain entirely unknown. Electricity and chemical reagents are used to activate the nuclear activity and the nuclear reprogramming of the zygotic genome.

SCNT became well known with the birth of the cloned sheep, Dolly, reported by Wilmut et al, (1997) . In 1998 the birth of a cloned mouse was reported by (Wakayama et al., 1998) and soon the cloning of other species was extensivly reported such as in cattle (Kato et al., 1998), gaur (Vogel, 2001), cats (Shin et al., 2002), horse (Choi et al., 2002), rats (Zhou et al., 2003), mule (Woods et al., 2003) and pigs (Polejaeva et al., 2000), using a variety of fetal and adult donor cell types (Hill et al., 2000; Kato et al., 1998; Keefer et al., 2001; Park et al., 2001; Polejaeva et al., 2000; Yin et al., 2002).

Not only SCNT is a valuable technique to produce genetically identical animals with excellent characteristics but it can be used for many other purposes, such as preserving endangered animals and medical therapy. Many studies have been reported which use SCNT for genetic rescue, such as in the mouflon, as by report of Loi et al, in 2001 (2001). To preserve buffalo species, fetal buffalo fibroblasts were tranferred into bovine oocytes to produce blastocysts as reported by Parnpai et al. (1999) and Kitiyanant et al. (2001). There are studies introducing giant panda nuclei into rabbit oocytes and zygotic transfer to cat and rabbit reproductive tracts (Chen et al., 2002) and Yang et al. (2003) reported blastocyst formation by transferring rhesus monkey fibroblast nuclei to rabbit oocytes.

Although, blastocyst formation and live clones have been achieved in many species, the efficiency of nuclear transfer is still very low as compared to conventional breeding and

current IVF technology. The low efficiency is due to a high percentage of abortion events during gestation, death shortly after birth of cloned animals and abnormality in live clones (Garry et al., 1996; Kato et al., 1998). These abnormalities have been assumed to be caused by incomplete epigenetic reprogramming in clones, which suggests that imperfect nuclear transfer techniques and *in vitro* culture systems are used.

2.8 Nuclear transfer and Epigenetics

Cloned embryos and animals are often examples of incomplete reprogramming usually involving aberrant gene expression. Some genes which encode metabolic enzymes have normal expression whereas some essential embryonic developmental genes are abnormally expressed in cloned embryos (Daniels et al., 2000). Abnormalities in the gene expression of cloned embryos are generally not caused by the primary structure of the DNA strand (such as genetic mutation). Rather, it more likely results from inefficient activation or expression of genes that are involved in regulatory systems (Boiani et al., 2002). Epigenetic modifications are suspected to be the cause of this aberrance in the development of cloned mammals by nuclear transfer (Kang et al., 2001a; 2001b; 2001c). This altered gene expression may be due to incomplete epigenetic reprogramming of donor genomic DNA (Han et al., 2003). Nuclear reprogramming by nuclear transfer involves chromatin reorganization, which directs gene regulation.

DNA methylation and histone acetylation are epigenetic modifications that are suspected to be affected in cloned embryos and animals. DNA methylation can cause transcriptional silencing and regulates gene activity during development.

Kang et al. (2001b) showed that the DNA methylation patterns of cloned bovine embryos contain higher methylation levels than *in vivo* embryos. Investigations of DNA methylation patterns in cloned rat and mouse embryos have identified, they have a similar

pattern to that of the donor cell, which is consistent with the studies by Bouc'his et al. (2001), that have shown the failure of demethylation of cloned embryos after the 2-cell stage, leading to high level of DNA methylation throughout preimplantation embryonic development. Cezar et al. (2003) observed the difference of DNA methylation patterns between dead cloned and naturally mated bovine fetuses. Different embryonic DNA methylation patterns have been observed among animal species (Kang et al., 2001b). In cloned pig embryos, DNA methylation has been found to have a similar pattern to the control pattern at all embryonic developmental stages. The explanation is that process may be more efficient in cloned pig embryos than bovine embryos. DNA methylation patterns have never been reported in cloned buffalo embryos. The investigation of the methylation patterns in cloned buffalo embryos is very interesting in order to provide the information of cloned buffalo development.

Another epigenetic regulation, histone acetylation is required for the development of cloned bovine embryos (Santos et al., 2003). Histone acetylation plays a critical role in chromatin remodeling and can reactivate nuclear reprogramming in the somatic donor cell. Enright et al. (2003a) reported that the histone status is remodeled in the developmental process of nuclear transfer and the increase of histone acetylation activity improves the *in vitro* development of cloned bovine embryos (Enright et al., 2003b).

In clones of many species, the most common abnormal phenotypes are perinatal death, placental abnormality and animal overgrowth (De Sousa et al., 2001; Tamashiro et al., 2000; Tanaka et al., 2001). These phenotypes consequently occur to alteration of imprinting gene expression, indicating that these abnormalities may be caused by aberrant imprinted gene expression. During normal preimplantation development, imprinted genes must resist the genome-wide demethylation event, however, variations in imprinting gene expression are still found in cloned embryos and are associated with fetal growth (Solter, 2000). These anomalies may be caused by either the *in vitro* manipulation, cultivation of

oocytes and donor cells or incomplete donor nuclear reprogramming (Feil, 2001; Khosla et al., 2001).

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CHAPTER III

EPIGENETIC CHARACTERISTIC OF CLONED AND IN VITRO FERTILIZED SWAMP BUFFALO EMBRYOS

3.1 Abstract

Swamp buffalos are endangered due to reproductive problems and this is a cause for concern because many countries heavily depend on its product. Nuclear transfer is potential strategy for use in endangered animal preservation. The technology succeeded in the blastocyst development and prevention of the swamp buffalo oocytes depletion using the oocyte cryopreservation. However, the development to term of cloned swamp buffalo has very low success rate. The same problem was found in clones of other animal species. An abnormal epigenetic mechanism is suspected to be cause of the developmental failure. DNA methylation and histone acetylation are key players in epigenetic modification, which display dynamic variable patterns during the embryonic development. The knowledge of aberrant epigenetic modifications is the base for solving these developmental problems and improving the reproductive technology in the swamp buffalo. Here we have studied for the first time, the relation of the preimplantation embryonic development and global DNA methylation and histone acetylation patterns in cloned and IVF swamp buffalo embryos using immunochemistry and confocal microscopy analysis. We revealed the aberrant demethylation pattern in early preimplantation

stage of swamp buffalo embryos and high variation of DNA methylationlevels among nuclei derived from cloned embryos. In addition, we compared the DNA methylation and histone acetylation interaction in the SCNT and IVF swamp buffalo throughout the embryonic development and suggest the correlation of these two epigenetic mechanisms may crucial to embryonic development in early preimplantation periods.

3.2 Introduction

The swamp buffalo (*Bubalus bubalis)* is a multi-purpose animal. Many countries heavily depend on buffalos' meat, milk, and labor. Thus, the importance of the swamp buffalo is equal to cattle in many regions of the world. However, the buffalo population in many countries has drastically reduced owing to its very low birth rate. Because of this, the swamp buffalo may be endangered and could potentially become extinct. Although assisted reproductive technologies, such as, artificial insemination and *in vitro* fertilization, were used to increase the number of live births, the success of buffalo production is still low due to various reproductive problems such as silent estrus, seasonal anoestrus, long post-partum anestrous period, late onset of puberty and low conception rates (Nandi et al., 2002).

Somatic cell nuclear transfer (SCNT) is a potentially useful strategy for preventing the extinction of an animal species because the somatic donor cells are easily accessible in large quantities and can be cryopreserved for long periods of time. In 1999, Parnpai et al. (1999) reported successful creation of swamp buffalo cloned blastocysts. cloned swamp buffalo was born and died a few days after birth. This low efficiency seems to be a common trait of SCNT in all species in which live cloned

animals have been obtained. The success rate of SCNT, however, has increased significantly in species such as the cattle due to the large number of studies conducted. There have been very few studies on the buffalo due to the limited population distribution of this species.

It is known that nuclear transfer can reprogram a differentiated somatic cell to a totipotent state because cloned animals are born with all tissue types (Kikyo et al., 2000; Wade and Kikyo, 2002). However, incomplete nuclear reprogramming is believed to be the cause of developmental failure in cloned animals. Nuclear reprogramming is brought about by epigenetic mechanisms, such as DNA methylation and histone acetylation, which do not involve changes in the DNA sequences. The maintenance of the correct DNA methylation level is important for embryonic development (Li et al., 1992; Okano et al., 1999). There is evidence that aberrant DNA methylation leads to loss of imprinting (Li, 2002), which play essential role in fetal development. Furthermore, DNA methylation cooperates with histone deacetylation and heterochromatic proteins to cause the heterochromatin configuration and global gene silencing.

DNA methylation has been studied in cloned embryos from numerous species. With the exception of the pig, all species studied so far showed DNA hypermethylation (Dean et al., 2001; Kang et al., 2001). Additionally, aberrant gene expression was also reported in cloned embryos (Daniels et al., 2001; Humpherys et al., 2002; Schultz, 1993; Wrenzycki et al., 2001). The latter observation may be related to DNA methylation which can directly and indirectly block transcription and lead to subsequent fetal abortion, abnormalities developmental in cloned animals.

Histone acetylation neutralizes the histone positive charge through binding of

the acetyl group on lysine residue at histone N-terminus and allows chromatin decondensation and gene activation (Jaskelioff and Peterson, 2003; Kikyo et al., 2000; Wade and Kikyo, 2002). Enright et al. (2003) indicated that histone acetylation patterns in somatic cells can be remodeled in culture. Santos et al. (2003) studied the histone acetylation at H3K9 in bovine preimplantation embryos, and revealed that the nuclear transfer embryos displayed hyperacetylation at all embryonic stages examined.

The assessment of the epigenetic status in cloned embryos of different species is valuable in order to learn more about its role in early mammalian development and determine its relation to low cloning efficiency. This information can be used to improve the nuclear transfer efficiency. In the present study, we report for the first time DNA methylation and histone acetylation patterns in preimplantation swamp buffalo embryos in comparison to IVF embryos.

3.3 Materials and Methods

3.3.1 Donor cell preparation

Ear skin was biopsied from a swamp buffalo at a local abattoir and kept in modified Dulbeccco's phosphate buffer saline at 4 °C during transportation to the laboratory. Skin tissues were removed from cartilage and cut into small pieces (about 1X1 mm^2) before being placed in 60 mm culture dish (Nunc GmbH & Co. KG, Kamstrup, Denmark) and covered with a glass slide. Five milliliters of alpha modified Eagle's medium (α MEM, Sigma, St. Louis, MO, USA) plus 10% fetal bovine serum (FBS) was added to the dish and the tissue was cultured under a humidified atmosphere of 5% CO_2 in air at 37.0°C. Fibroblast outgrowth from the ear skin tissue

was harvested and seeded in 25 cm^2 culture flask (Nunc) in αMEM plus 10% FBS. To preserve these cells, skin fibroblasts were frozen with 10% DMSO in αMEM plus 20% FBS at the third cell culture passage and stored in liquid nitrogen. For NT, frozen-thawed fibroblasts were cultured to 4 - 8 passages in αMEM plus 10% FBS and used as nuclear donor cells. The sub-confluence donor cells were re-suspended in Emcare medium (ICP bio, Auckland, New Zealand) before injection without serum starvation.

3.3.2 Somatic cell nuclear transfer

Swamp buffalo oocytes derived from abattoir-collected ovaries were matured *in vitro* (Parnpai et al., 1999) cells were mechanically removed by repeat pipetting using a fine-tip pipette in 0.2% hyaluronidase and were subsequently washed 5 times in Emcare medium. For enucleation, matured oocytes were placed in culture medium containing 5 μg/ml cytochalasin B (Sigma) for 15 min. The zona pellucida above the first polar body was cut with a glass needle and a small volume (about 5 to 10%) of cytoplasm was extruded. Complete enucleation was confirmed by staining with Hoechst 33342 (Sigma). To transfer the donor cells, individual fibroblasts were injected into the perivitelline space of the enucleated oocytes, and fusion was initiated by placing the couplets into Zimmerman fusion medium (Zimmermann and Vienken, 1982) followed by electrical stimulation using two DC pulses at 26 V, for 17 μsec using SUT F-1 (Suranaree University of Technology). The reconstructed embryos were activated in 7% ethanol for 5 min and then cultured in modified synthetic oviductal fluid culture media (mSOF) (Gardner et al., 1994) containing 3 mg/ml BSA (Sigma), 1.25 μg/ml cytochalasin D (Sigma) and 10 μg/ml cycloheximide (Sigma) for

5 h. The activated embryos were cultured in 100 μl droplets of mSOF under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C for 2 d. The 8-cell stage embryos were selected and co-cultured in 100 μl droplets of mSOF with bovine oviductal epithelial cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5°C for another 5 d. Embryos at 2-cell, 4-cell, 8-cell, morula and blastocyst stages were collected at 22, 28, 34, 108 and 132 h post-activation, respectively. All embryos at the 2-, 4-, 8-cell, morula and blastocyst stages were fixed in 4% paraformaldehyde and stored at 4^oC until further analysis.

3.3.3 In vitro fertilization (IVF)

In order to control for variation from genetic differences, buffalo semen from the same bull was used for the production of the IVF embryos. The frozen semen was thawed at 37^oC and washed twice by centrifugation at 500 g for 7 min in Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) without BSA but supplemented with 10 mM caffeine (Sigma). The pellet was resuspended in caffeine-BO at the concentration of 8 x 10^6 sperm/ml and then diluted with an equal volume of BO medium supplemented with 20 mg/ml BSA and 20 μg/ml heparin (Sigma). The cumulus oocyte complexes (COC) after 22 h of maturation, were transferred into 100 μl droplets of sperm suspension under mineral oil (Sigma) and incubated under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C for 2 d. The 8-cell stage embryos were selected and co-cultured in 100 μl droplets of mSOF with BOEC under humidified atmosphere of 5% CO_2 in air at 38.5°C for another 5 d. Embryo collection and fixation were as described for SCNT embryos.

3.3.4 Immunostaining of preimplantation buffalo embryos

The fixed embryos were washed in phosphate-buffer saline (PBS) before permeabilization in 0.5% Triton X-100. Embryonic DNA was denatured by incubation in 4N HCl for 1 h at 37^{o}C and then blocked to prevent non-specific binding in PBS containing 2% BSA (PBS-BSA 2%) for 1 h. The embryos were stained with a 1:50 dilution of primary mouse monoclonal antibody against 5-methyl cytosine (5-MC, Eurogentech, San Diego, CA) and a 1:50 dilution of primary rabbit monoclonal antibody against acetylated histone H3 lysine 18 (H3K18, Cell Signaling-Technologies Inc., Boston, MA). The embryos were subsequently incubated in fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (1:100; Jackson ImmunoResearch, PA) and in Texas Red isothiocyanate (TRITC)-conjugated anti-rabbit IgG (1:100; Jackson ImmunoResearch), respectively. Individual embryos were mounted with 50% glycerol on slides.

3.3.5 Confocal microscopy

The immunostained embryos were observed with confocal microscopy (TCSSP2 True scanning; Leica Microsystems, Heidelberg, Germany). DNA methylation and histone acetylation on H3K18 were analyzed and quantified by manually outlining a limited area of each individual nucleus at the brightest focus plane for the emission light of FITC and TRITC fluorescence. The emission intensities of all individual nuclei of embryos at the 2-, 4- and 8-cell stages, and 20 nuclei per morula and 30 nuclei per blastocyst were recorded. The arbitrary fluorescence intensity of each nucleus in an embryo was used to represent the DNA methylation/histone acetylation of that nucleus and the level of global DNA

methylation/histone acetylation in an embryo was represented by the sum of all nuclei in that embryo. The experiments were performed in four replicates with 3 embryos per stage and per replicate. Appropriate controls for auto-fluorescence and non-specific binding by the secondary antibodies were included.

3.3.6 Blastocyst counter stain and cell count

To distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), embryos were counter-stained. Briefly, the zonae pellucidae of blastocysts at days 6.5 post-activation/insemination were removed by 0.5% protease (Sigma) and washed in SOF medium. The zona-free blastocysts were incubated in 10% rabbit anti-buffalo spleenocyte antibodies generated in our lab (Iwasaki et al., 1990) for 45 min before subsequently transferred into the mixture of 10% guinea pig complement (Sigma), 75 μg/ml propidium iodide (PI; Sigma) and 100 μg/ml Hoechst 33258 (Bis-benzimide; Sigma) for 45 min. The ICM cells (blue) and TE cells (red) were counted under UV light fluorescence microscopy.

3.3.7 Statistical analysis

Data analyses for the differences in embryonic development, DNA methylation and histone acetylation, as assessed by relative amounts of arbitrary FITC and TRITC emission signals, respectively, were carried out by ANOVA using the general linear model (GLM) procedure in the Statistical Analysis System (SAS version 9.0, SAS Inc., Cary, NC). The main effects were embryo type (SCNT or IVF), stage of embryo development and their interactions. The predicted difference (PDIFF) function of the GLM procedure was used to compare Least Squares Means among the developmental

stages between embryo types. A *P*-value of <0.05 was considered significant. To achieve normal distribution and homogeneity of variance, the data for histone acetylation were log10 transformed before analyses. To determine the correlation between DNA methylation and histone acetylation, the Pearson correlation analysis was performed in SAS.

3.4 Results and discussion

3.4.1 Preimplantation developmental of swamp buffalo embryos

Developmental rates of SCNT and IVF swamp buffalo embryos are summarized in Table 1. Stable losses were seen throughout embryo development in both IVF and SCNT groups. In most cases, the developmental rates for embryos derived from SCNT were lower than those for embryos derived from IVF. Significant developmental difference between the two types of embryos was observed at the blastocyst stage ($P<0.05$). Interestingly, the most dramatic loss of SCNT embryos, as much as 42%, occurred during the transition from the 8-cell to the morula stages. In blastocysts, there was 3 times as many cells in TE as in ICM in embryos from both groups. No differences, however, were observed in the numbers of cells in ICM or TE, or the proportion of ICM over total cells between blastocysts from SCNT or IVF.

Table 3.1 Development of preimplantation swamp buffalo embryos derived from nuclear transfer (SCNT) and *in vitro* fertilization (IVF)

Embryo type	Experiment (n)	Fertilized/Fused Embryos	Embryos development rates* (means ± SEM)			No. of cells in blastocyst (means ± SEM)		
			Cleavage	Morula	Blastocyst	ICM	TE	% ICM
SCNT	4	86	0.85±0.13[a]	0.43±0.08[a]	0.35±0.01[a]	25.6 ± 12.0	88.7 ± 29.1	20%
IVF	4	162	0.77±0.13[a]	0.63±0.07[a]	0.51±0.01[b]	29.3± 3.8	97.3 ± 12.4	22%

[a,b] Values within columns with different superscripts are significantly different *($P \leq 0.05$).*

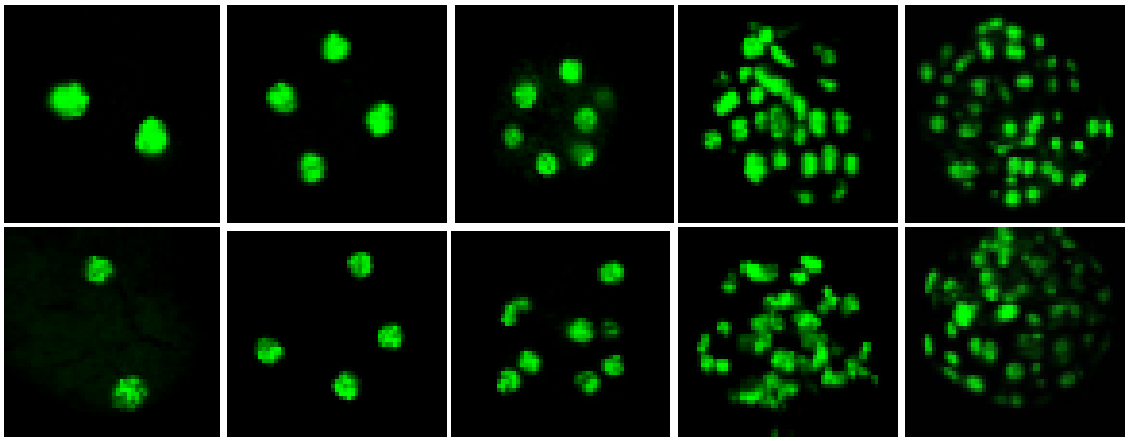
*The cleavage rates were calculated based on the numbers of fertilized/fused embryos. The rates of developmental to morula or blastocyst were calculated based on the number of cleaved embryos.

3.4.2 DNA methylation in preimplantation swamp buffalo embryos

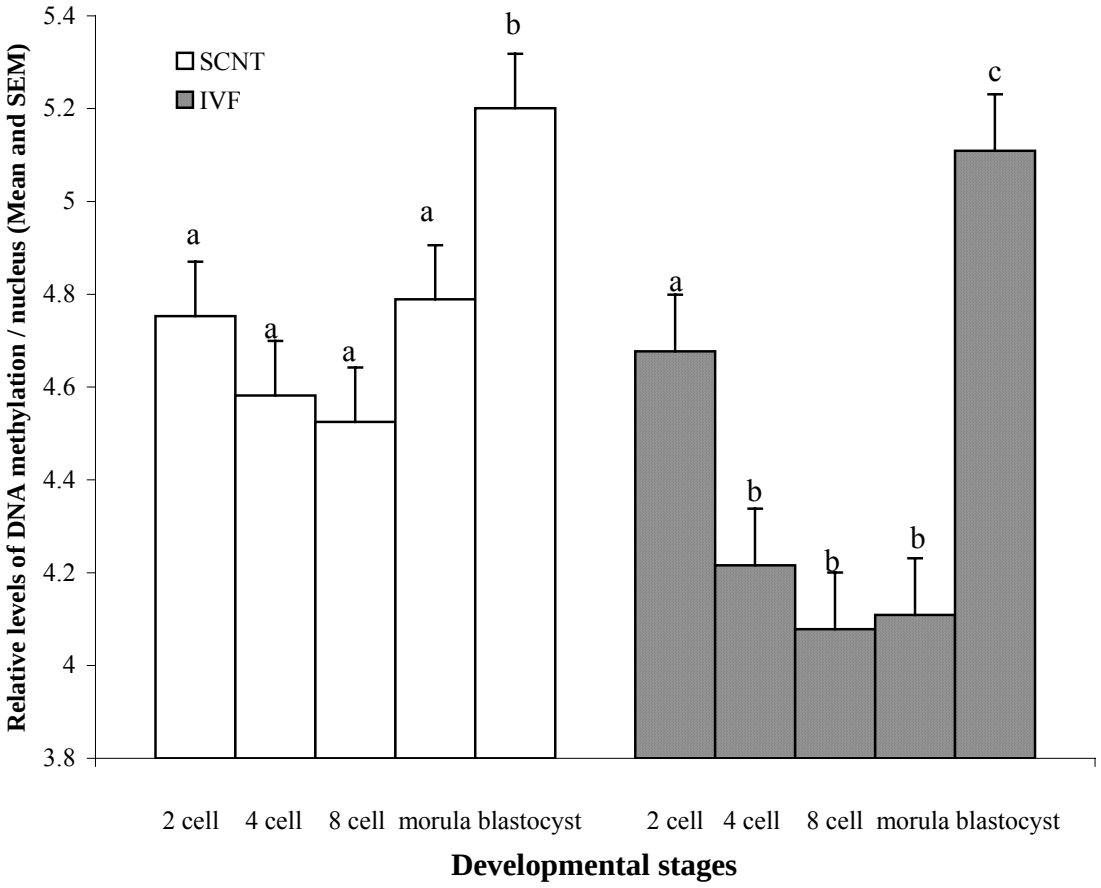
Representative images of SCNT and IVF preimplantation embryos immunostained for DNA methylation are shown in Fig. 1A. To compare with previously published data, we obtained and analyzed data for global DNA methylation of the entire embryo because these data were the only criterion used in studies of methylation status of cloned embryos in other species. Additionally, we also obtained and analyzed data for individual nuclei in each embryo because the DNA methylation status in a particular nucleus is independent of that in another nucleus of the same embryo, and mosaic DNA methylation of different nuclei in cloned embryos have been observed (Dean et al., 2001). DNA methylation of each nucleus, as represented by the means of arbitrary fluorescence intensities of nuclei of SCNT and IVF embryos at various embryonic developmental stages are shown in Fig. 1B. In both types of embryos, the relative levels of DNA methylation per nuclei decreased from the 2-cell stage until the 8-cell stage, when they reached the lowest level. Then the levels of DNA methylation started to increase at morula stage and reaching the highest level at the blastocyst stage. Between embryo types, those derived from SCNT had higher relative levels of DNA methylation/ nucleus at all stages examined ($P<0.05$). Additionally, DNA methylation levels of nuclei from SCNT embryos were much more variable than those in IVF embryos, from the 4-cell to blastocyst stage in SCNT as compared to IVF embryos. This demonstrates the highly heterogenic nature of DNA methylation in cloned embryos.

The relative levels of global methylation for each embryo, as represented by the sum of arbitrary fluorescence intensities of all nuclei in the embryo (Fig. 1C), were also significantly higher in embryos derived from SCNT than those from IVF at 4-cell, 8-cell and morula stages ($P<0.05$).

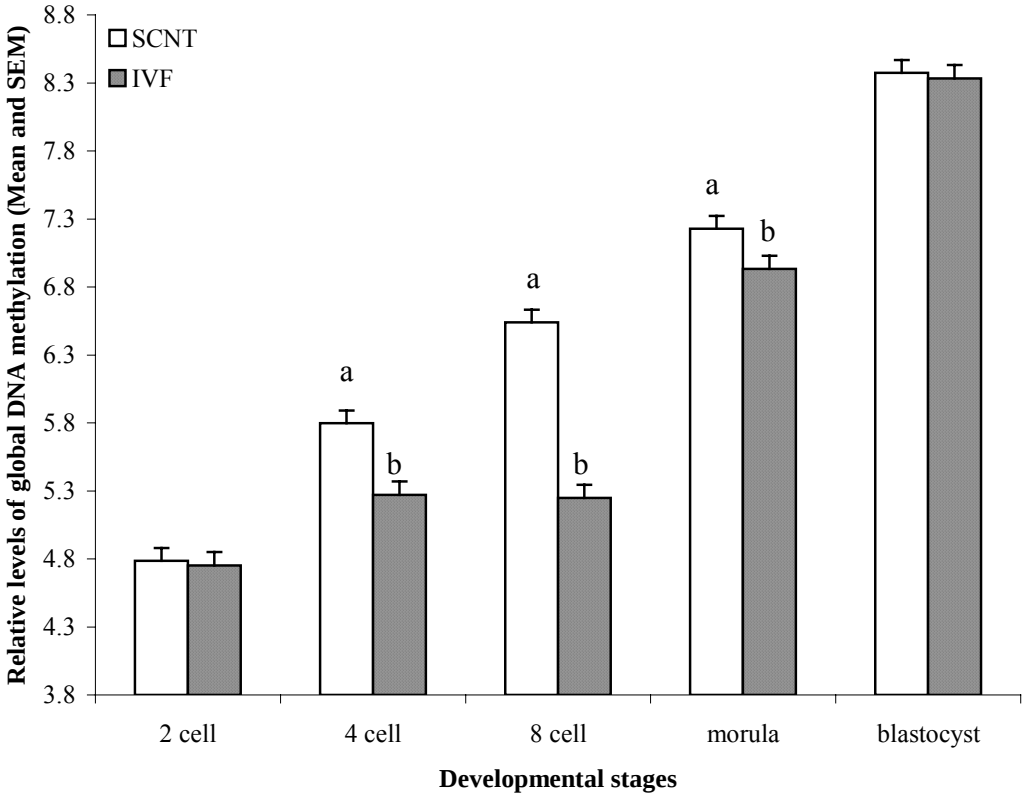
Figure 3.1 DNA methylation in the nuclear transfer and IVF embryos.



(A) FITC intensities in the nuclear transfer and IVF embryos. Representative images of SCNT (top row) and IVF (bottom row) swamp buffalo embryos at the 2-cell to the blastocyst stages stained for 5-methylcytosine



(B). Comparison of DNA methylation level in SCNT and IVF embryos using averaged mean intensities. Relative levels of DNA methylation/nucleus in SCNT (white bars) and IVF embryos (black bars). Different letters (a, b, c) depict significant differences in relative levels of DNA methylation across developmental stages within each embryo type.

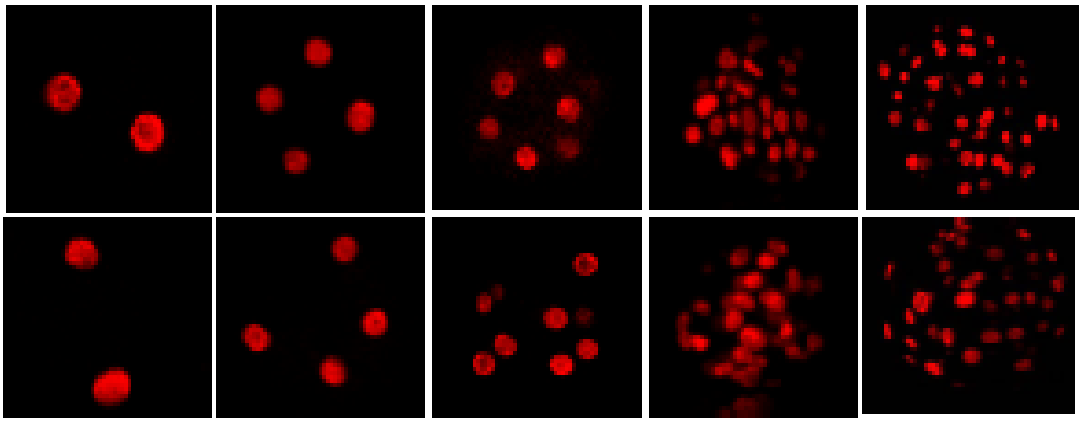


(C) Comparison of DNA methylation level in the SCNT and IVF embryos using sum of intensities. Relative levels of global DNA methylation in SCNT (white bars) and IVF embryos (black bars). Different letters (a, b) depict significant differences in relative levels of global DNA methylation between embryo types at the same developmental stage.

3.4.3 Histone acetylation in preimplantation swamp buffalo embryo

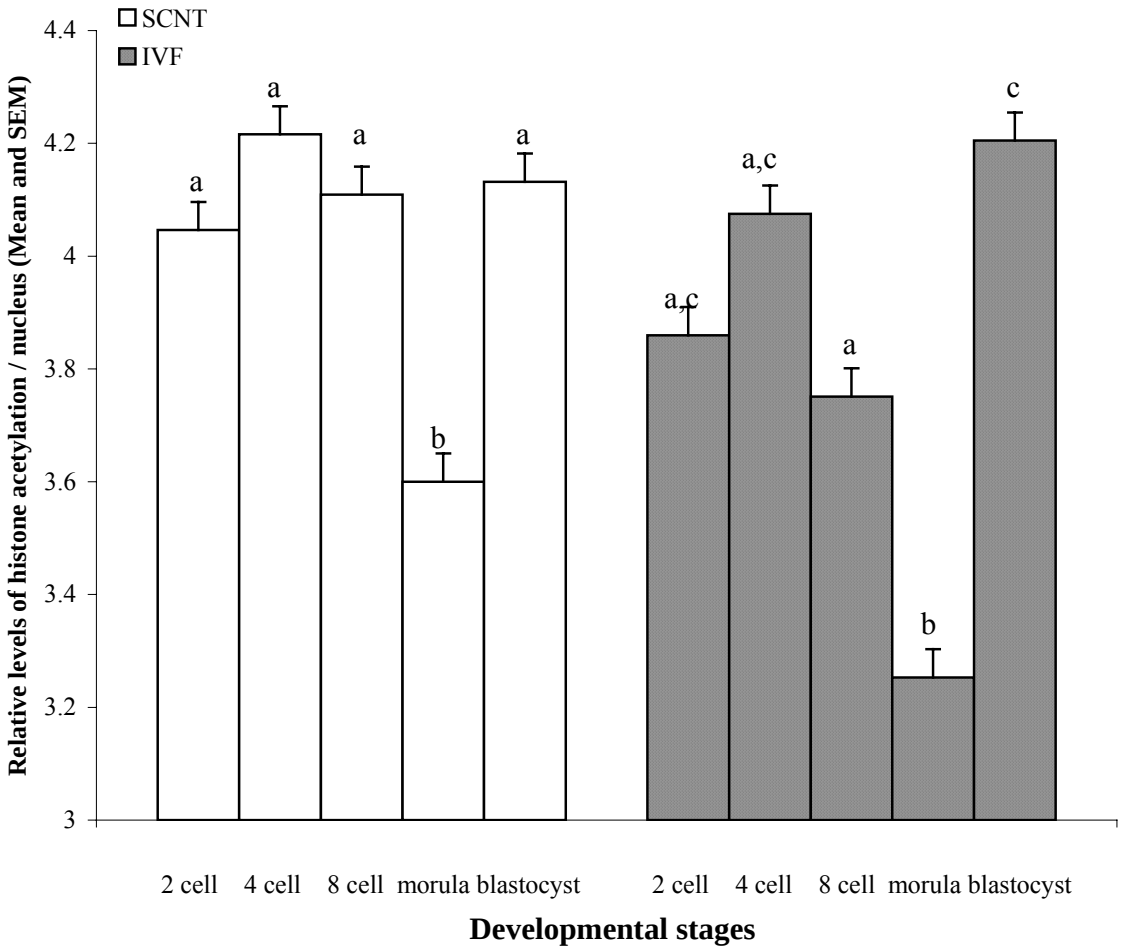
Representative images of SCNT and IVF buffalo embryos immunostained for acetylated histone are shown in Fig. 2A. The mean relative levels of histone acetylation in each nuclei of SCNT and IVF embryos at various stages of embryonic development are shown in Fig. 2B. Overall, we observed similar patterns of changes in the relative levels of histone acetylation in nuclei of SCNT and IVF embryos throughout preimplantation development. Embryos of both types had moderate increases from 2-cell to 4-cell stages, which was followed by decreases to the lowest level at the morula stage. Subsequently, histone acetylation nucleus increased again to the highest level at the blastocyst stage. Similar to the DNA methylation data, the relative levels of histone acetylation of individual nuclei of cloned embryos were also highly variable, from the 4-cell to 8-cell in cloned embryos as compared in IVF embryos. Globally, higher relative levels of histone acetylation were observed in the SCNT embryos than IVF embryos at the 4-cell and 8-cell stages (Fig. 2C; $P<0.05$).

Figure 3.2 TRITC intensities in the nuclear transfer and IVF embryos

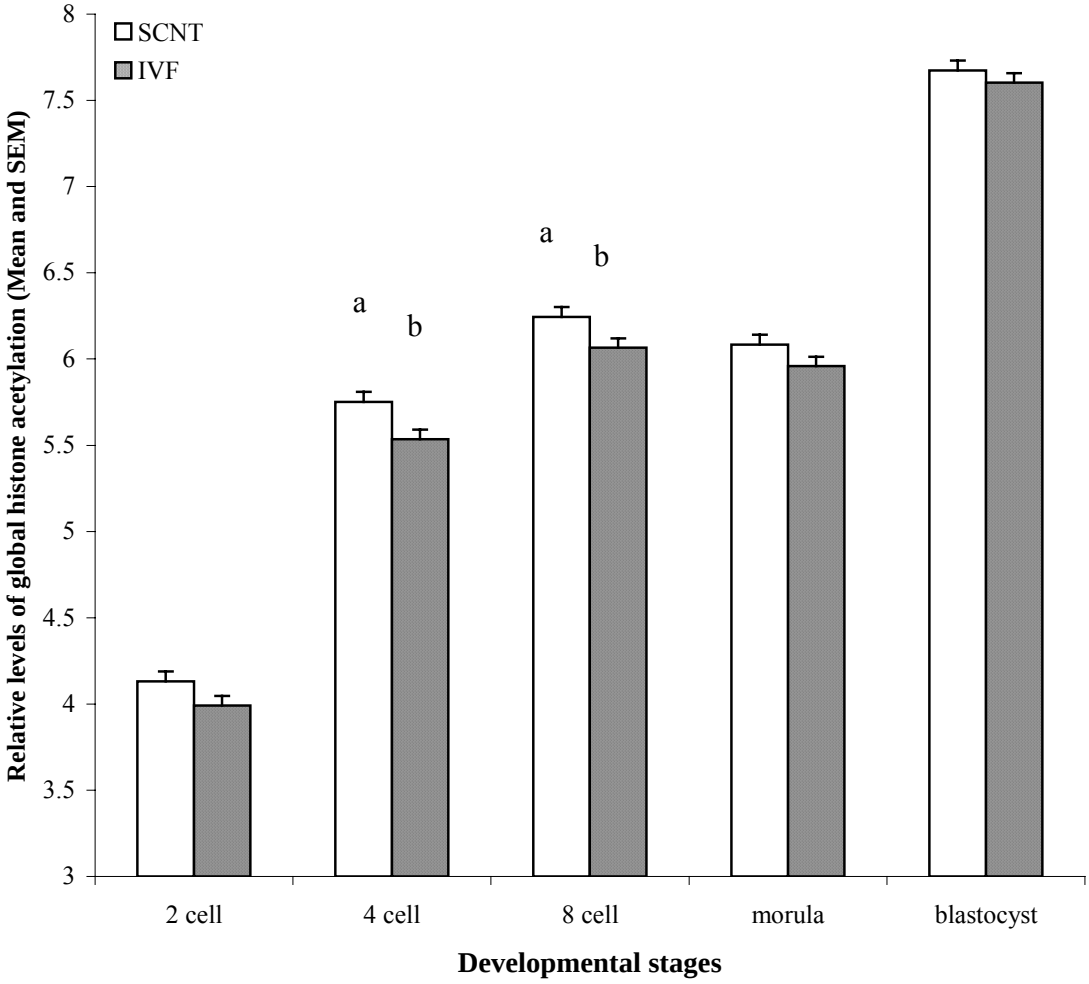


(A) Comparison of histone acetylation level in SCNT and IVF embryos.

Representative images of SCNT (top row) and IVF (bottom row) swamp buffalo embryos at the 2-cell to the blastocyst stages stained for acetylation in histone H3 lysine 18.



(B) Comparison of histone acetylation level in the SCNT and IVF embryos using averaged mean intensities. Relative levels of histone acetylation/nucleus in SCNT (white bars) and IVF embryos (black bars). Different letters (a, b, c) depict significant differences in relative levels of histone acetylation across developmental stages within each embryo type.



(C) **Comparison of histone acetylation level in the SCNT and IVF embryos using sum of mean intensities.** Relative levels of global histone acetylation in SCNT (white bars) and IVF embryos (black bars). Different letters (a, b) depict significant differences in relative levels of global histone acetylation between embryo types at the same developmental stage.

3.4.4 Correlation of relative levels of DNA methylation and histone acetylation during preimplantation development

It was previously reported that in individual genes that are under epigenetic regulations such as those imprinted or X-linked, the levels of DNA methylation and acetylation of H3 and/or H4 are reversely correlated (Kharroubi et al., 2001; Yoshida et al., 1990). We, therefore, analyzed correlations of DNA methylation and histone acetylation in buffalo embryos in the present study. We found significant positive correlations between the relative levels of DNA methylation and histone acetylation in both IVF (r = 0.65) and SCNT embryos (r = 0.87) at the 2-cell stage. At the 4-cell stage, the correlation was significant in SCNT embryos (r = 0.85), but not from IVF embryos (r = 0.46). Interestingly, for embryos at the 8-cell stage, correlations between the global histone acetylation and DNA methylation showed reversed in the two embryo types; i.e., significant positive correlation (r = 0.76) was found in the IVF embryos but not in the SCNT group (r = -0.1). When the correlation in individual embryo were examined at this stage, ten in twelve of the IVF embryos had significant positive correlations (r > 0.75), whereas most of SCNT embryos (9/12) showed low or negative correlations (-0.3 < r < 0.3). At the blastocyst stages, little correlations of global methylated DNA and acetylated histones were found among the IVF or SCNT embryos (r < 0.3).

3.4.5 Discussion

In the present study, we produced preimplantation buffalo embryos from IVF and SCNT and studied their relatively levels of DNA methylation and histone acetylation. We found that during in vitro culture of cloned and IVF buffalo embryos, higher

developmental arrest rate were found in SCNT embryos and this developmental arrest was mostly observed during the period of 8-cell to morula stages. This observation is in agreement with a report by Kittiyanant et al. (2001), who had a 33% blastocyst rate after a decline from a 77% cleavage rate in swamp buffalos embryos during in vitro production. This arrest in embryonic development is correlated with the beginning of de novo DNA methylation mechanism in the IVF embryos at this stage. The sharp decline of the development rate of the SCNT embryos at this stage corresponds with the drastic changes in DNA hypermethylation. Because DNA methylation has been shown to suppress gene expression, it is a plausible explanation that the hypermethylation may cause improper expression of developmentally important genes, which in turn lead to the developmental failure in the SCNT embryo.

Although there is no prior data for comparison in buffalo cloned or IVF embryos, the hypermethylation of DNA in SCNT swamp buffalo embryos and the dynamic changes of DNA methylation during embryo development observed in the present study are consistent with previous work in cloned embryos of the bovine and other species (Beaujean et al., 2004; Dean et al., 2001; Kang et al., 2001). In cloned bovine embryos, it was shown that both higher DNA de novo methylation and insufficient DNA demethylation account for the aberrant hypermethylation of DNA (Dean et al., 2001). It was also shown that higher levels of maternal *DNMT1*, which is responsible for maintaining DNA methylation, were present in cloned bovine embryos (Wrenzycki et al., 2001). Additionally, higher variations of DNA methylation in different cells of each cloned buffalo embryo observed here also indicate that nuclear reprogramming occurs throughout the preimplantation development and is incomplete in many cells of the embryos.

Previously, Enright et al. (2003) reported that the lysine 18 on histone H3 (H3K18) is a common acetylated lysine position and is acetylated throughout the bovine embryonic stages. Therefore, in the present study, we also chose to use the same antibody to detect histone acetylation status and found the same observation in the buffalo embryos. We also found moderate histone hyperacetylation among swamp buffalo SCNT at all preimplantation stages. This agrees with the report by Santos et al. (2003), who reported hyperacetylation of H3K9 in bovine preimplantation cloned embryos. Surprisingly, we observed the lowest histone acetylation level in the morula stage among all preimplantation stages analyzed. It is unclear what significance in embryo development this phenomenon serves and its uniqueness to the buffalo embryos is a possibility.

Previously, the negative correlations between the levels of DNA methylation and histone acetylation were found on chromatins of individual genes subjected to epigenetic relations. In the present study, we analyzed the correlations between these two chromatin modifications on the whole cell and whole embryo basis. Also, for the first time, we studied the correlations of many different stages of preimplantation development, whereas previous studies were limited to the correlations in bovine blastocysts (Enright et al., 2003; Santos et al., 2003), which had both hypermethylation and hyperacetylation. In the present study, we found that buffalo embryos at different stages of development have different correlations between global DNA methylation and histone acetylation, suggesting dramatic changes in chromatin modifications during early embryo development. However, the inconsistent correlations between DNA methylation and histone acetylation at the 4- and 8-cell stages in IVF vs. SCNT embryos indicates that the interaction of these epigenetic mechanisms influences early embryonic development.

3.5 Conclusion

In summary, we generated cloned buffalo embryos and compared their epigenetic status with that of IVF embryos. We found that cloned embryos are not only hypermethylated but also more heterogeneous in both DNA methylation and histone acetylation among different cells of the same embryos than those in IVF embryos. Additionally, the anomalous correlations between DNA methylation and histone acetylation may contribute to the failure of development of cloned embryo.

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CHAPTER IV

ANOMALOUS mRNA LEVELS OF CHROMATIN REMODELING GENES IN SWAMP BUFFALO (*Bubalus bubalis)* CLONED EMBRYOS

4.1 Abstract

The swamp buffalo *(Bubalus bubalis)* is a multi-purpose animal in agriculture that is challenged by extinction due to low reproductive efficiency. Nuclear transfer (NT) has been used to preserve special breeds of buffalo as well as to increase the number of animals. However, cloned buffalo embryos have problems in term development, as in other species. To understand the chromatin remodeling activities in cloned embryos and to improve the NT technology, we examined the expression profiles of five genes involved in DNA and histone modifications, *DNMT1*, *DNMT3A*, *DNMT3B*, *HAT1* and *HDAC1*, in single swamp buffalo metaphase II oocytes, NT and *in vitro* fertilized (IVF) embryos from the 2-cell to the blastocyst stage by quantitative real time RT-PCR. We observed similar expression dynamics for all genes studied in the NT and IVF embryos: relatively constant levels of expression for all genes were found from the MII oocyte up to the 8-cell stage; the levels of mRNA for *HAT1* and *DNMT3B* continued to be stably expressed up to the blastocyst stage; while dramatic increases were seen for *DNMT3A* and *HDAC1*. Alternatively, the levels of *DNMT1*

started to decrease at the 8-cell stage. Despite the similarity in the dynamics of gene expression, dramatic differences in the relative levels of these genes between NT and IVF embryos were observed. The expression levels of all DNA modifying genes were higher in the NT embryos than in the IVF embryos at the 8-cell and blastocyst stages. The genes *HDAC1* and *HAT1* were also expressed significantly higher at the blastocyst stage in the NT embryos. Our results suggest differences in chromatin remodeling between NT and IVF embryos and that lower levels of DNA passive demethylation and higher levels of DNA *de novo* methylation occurred in the NT embryos. These observations are novel in the species of buffalo, and may allude to developmental failure of cloned buffalo embryos due to the transcriptional repression effect of most genes studied here.

4.2 Introduction

In cloned animals, characteristic anomalies such as increased birth weight, placental abnormalities and immune deficiency have been observed in many species (De Sousa et al., 2001; Hill et al., 2000; Tamashiro et al., 2000; Tanaka et al., 2001; Wakayama and Yanagimachi, 1999). Furthermore, abortion rates and neonatal deaths are consistently higher in animals generated by nuclear transfer (NT) than those by *in vitro* fertilization (IVF) and artificial insemination (AI). Incomplete epigenetic regulation is suspected to be the cause of the abnormalities and the low efficiency associated with NT (Daniels et al., 2001; Humpherys et al., 2002; Wrenzycki et al., 2001).

Epigenetic modification involves altering gene expression without changing

the DNA sequence. DNA methylation and histone acetylation are the key mechanisms of this process. DNA methylation has been shown to cause transcriptional silence; thus, the alteration of the methylation status could affect the expression of genes under epigenetic regulation. It has been reported that the DNA of cloned bovine embryos was aberrantly hypermethylated (Bourc'his et al., 2001; Dean et al., 2001). Kang et al. (2001) also demonstrated that cloned bovine embryos display hypermethylated repetitive elements in their genome. Additionally, aberrant methylation patterns were found in cloned aborted fetuses, animals that died soon after birth and in adult animals (Cezar et al., 2003). In preimplantation embryos, the parental genomes are actively and passively demethylated after fertilization. This demethylation is followed by de novo methylation at the 8-cell to 16-cell stage (Dean et al., 2001). The DNA methylation mechanism relies on the catalytic activity of DNA methyltransferases (DNMTs). The gene DNMT1 maintains the methylation pattern during replication; whereas, DNMT3A and 3B are responsible for de novo methylation. The DNA methyltransferases 3A and 3B work together with many related proteins, such as histone deacetylases in silencing gene expression. Another widely studied form of epigenetic modification is the acetylation of the lysine residues at the amino-terminus of histones. This is achieved by histone acetylases (HATs) that cause the acetylation and neutralization of the positive charges on the amino-terminus of the histones, leading to chromatin decondensation and activation of gene transcription. Conversely, histones can be deacetylated by histone deacetylases (HDACs), which cause chromatin condensation and gene repression (Bird, 2002). Enright et al. (2003) and Santos et al. (2003) observed hyperacetylation in preimplantation cloned cattle embryos. In bovine IVF embryo, both HDAC1 and HAT1 genes were detected at the

8-cell stage and *DNMTs*, the lowest level of *DNMT1* was observed at the blastocyst stage. *DNMT3A/B* has only been analyzed and detected in bovine blastocysts (Russell and Betts, 2005; Wrenzycki et al., 2005).

Swamp buffalos (*Bubalus bubalis*) are multi-purpose animals important for agriculture. Many countries depend heavily on buffalos' production for meat and milk, in addition to their value for labor. However, the buffalo population in many countries has markedly decreased due to low reproduction efficiency, such as a long gestation period and late onset of puberty. Nuclear transfer is a potential strategy for preserving endangered buffalo breeds through the cryopreservation of buffalo donor cells. To achieve cloning of the buffalo, swamp buffalo NT was attempted and blastocyst development was obtained in 2001 (Kitiyanant et al., 2001). However, the pregnancy rate after embryo transfer for NT embryos was much lower than that of IVF (Misra et al., 1999). In the case of NT, fetal loss is commonly found throughout gestation suggesting that NT embryos have intrinsic abnormalities that are manifested after transfer, even though they can reach the blastocyst stage (Saikhun et al., 2004). Our own studies, examining DNA methylation and histone acetylation by confocal microscopy, revealed aberrant DNA demethylation and histone acetylation patterns in cloned buffalo embryos as compared to IVF embryos during the preimplantation period. Exploring the underlying mechanism for the abnormal methylation by studying chromatin modifying enzymes will help elucidate whether this is due to abnormal DNA demethylation or *de novo* DNA re-methylation. This may provide insight into the causes of the low efficiency in swamp buffalo NT and enable the modification of existing protocols for greater success. In the present study, we examined the levels of mRNA for five chromatin remodeling genes: *DNMT1*, *DNMT3A*, *DNMT3B, HAT1*

and *HDAC1,* all in single metaphase II oocytes, NT and IVF embryos at various stages of preimplantation development by quantitative real time RT-PCR. We found that the NT embryos contained significantly higher mRNA levels of DNA methylation genes as well as *HDAC1*, all of which have been shown to repress gene transcription.

4.3 Materials and Methods

4.3.1 Donor cell preparation

Ear skin was biopsied from a single swamp buffalo in order to control for genetic variation. The biopsy was kept in modified Dulbeccco's phosphate buffer saline at 4 °C during transportation to the laboratory. Skin tissues were removed from cartilage and cut into small pieces (about 1X1 mm^2) before being placed in 60 mm culture dish (Nunc GmbH & Co. KG, Kamstrup, Denmark) and covered with a glass slide. Five milliliters of alpha modified Eagle's medium (αMEM, Sigma, St. Louis, MO, USA) plus 10% fetal bovine serum (FBS) was added to the dish and the tissue was cultured under a humidified atmosphere of 5% CO_2 in air at 37.0°C. The fibroblast cell outgrowth from the ear skin tissue was harvested and seeded in 25 cm^2 culture flask (Nunc) in αMEM plus 10% FBS. To preserve these cells, ear fibroblasts were frozen with 10% DMSO in αMEM plus 20% FBS at the third cell culture passage and stored in liquid nitrogen. For NT, frozen-thawed fibroblasts were cultured 4 to 8 passages in αMEM plus 10% FBS and used as nuclear donor cells. No serum starvation was performed and the sub-confluence donor cells were re-suspended in Emcare medium (ICP bio, Auckland, New Zealand) before injection.

4.3.2. Somatic Cell Nuclear Transfer

Swamp buffalo oocytes derived from abattoir-collected ovaries were matured in *in vitro* maturation medium (Parnpai et al., 1999) for 22 h. The cumulus cells were mechanically removed by repeat pipetting using a fine-tip pipette in 0.2% hyaluronidase and were subsequently washed 5 times in Emcare medium. For enucleation, matured oocytes were placed in culture medium containing 5 μg/ml cytochalasin B (Sigma) for 15 min. The zonae pellucidae above the first polar body was cut with a glass needle and a small volume (about 5 to 10%) of cytoplasm was extruded. Complete enucleation was confirmed by staining with Hoechst 33342 (Sigma). To transfer the donor cells, individual fibroblasts were injected into the perivitelline space of the enucleated oocytes, and fusion was initiated by placing the couplets into Zimmerman fusion medium (Zimmermann and Vienken, 1982) followed by electrical stimulation using two DC pulses at 26 V, for 17 μsec using SUT F-1 (Suranaree University of Technology). The reconstructed embryos were activated for 5 min in 7% ethanol and then cultured in modified synthetic oviductal fluid culture media (mSOF) (Gardner et al., 1994) containing 3 mg/ml BSA (Sigma), 1.25 μg/ml cytochalasin D (Sigma) and 10 μg/ml cycloheximide (Sigma) for 5 h. The activated embryos were cultured in 100 μl droplets of mSOF under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C for 2 d. The 8-cell stage embryos were selected and co-cultured in 100 μl droplets of mSOF with bovine oviductal epithelial cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5°C for another 5 d. Embryos at 2-cell, 4-cell, 8-cell, morula and blastocyst stages were collected at 22, 28, 34, 108 and 132 h post-activation, respectively.

4.3.3 In vitro fertilization (IVF)

In order to control for variation from genetic differences, buffalo semen from the same bull was used for the production of the IVF embryos. The frozen semen was thawed at 37^{o}C and washed twice by centrifugation at 500 g for 7 min with Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) without BSA but supplemented with 10 mM caffeine (caffeine-BO; Sigma). The pellet was resuspended in caffeine-BO at the concentration of 8 x 10^6 sperm/ml and then diluted with an equal volume of BO medium supplemented with 20 mg/ml BSA and 20 μg/ml heparin (Sigma). The cumulus oocyte complexes (COC) after 22 h in *in vitro* maturation medium, were transferred into 100 μl droplets of sperm suspension under mineral oil (Sigma) and incubated under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C for 2 d. The 8-cell stage embryos were selected and co-cultured in 100 μl droplets of mSOF with BOEC under humidified atmosphere of 5% CO_2 in air at 38.5°C for another 5 d. The 2-cell, 4-cell, 8-cell, morula and blastocyst embryos were collected at approximately 28, 34, 40, 114 and 138 h in culture, post-insemination, respectively.

4.3.4 Embryo staining and cell count

To distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), embryos were counter-stained. Briefly, the zonae pellucidae of blastocysts at days 6.5 post-activation/insemination were removed by 0.5% protease (Sigma) and washed in SOF medium. The zonae-free blastocysts were incubated in 10% rabbit anti-buffalo spleenocyte antibodies generated in our lab (Iwasaki et al., 1990) for 45 min before subsequently transferred into the mixture of 10% guinea pig complement (Sigma), 75

μg/ml propidium iodide (PI; Sigma) and 100 μg/ml Hoechst 33258 (Bis-benzimide; Sigma) for 45 min. The ICM cells (blue) and TE cells (red) were counted under UV light fluorescence microscopy.

4.3.5 Real time reverse transcription (RT)-PCR

The sequences of the five genes of interest for buffalo were not available in the GenBank; in order to design specific primers for the buffalo, we first amplified fragments of these genes from buffalo genomic DNA using primers designed from cattle sequences of the same genes. The PCR products were then sequenced and the new sequences were used to design buffalo primers (Table 4.1). Six MII oocytes, NT and IVF embryos in every stage of preimplantation development were analyzed individually in the study. Total RNA from single oocytes and embryos was extracted using TRIzol reagent (Invitrogen Life Technologies, CA, USA) with linear acrylamide (Ambion Inc., Austin, TX, USA) followed by RNase-free DNase treatment to remove any possible genomic DNA contamination. Reverse transcription was carried out at 50^{o}C for 1 h with Superscript III (Invitrogen). Individual cDNA samples were diluted based on the results of a pilot study that measured the cycle threshold (CT) for each target gene at each developmental stage. The total volume of 25 μlreal time RT-PCR reaction mixture contained 5 μl of cDNA, 12.5 μl of SYBR green master mix (Applied Biosystems Inc., CA, USA) and 0.3 μM of forward and reverse primers. All oocytes and embryos were analyzed in triplicate for every gene. The specificity of the real time RT-PCR product was proven by melting curve analysis. To compare the relative levels of gene expression in NT and IVF embryos, the comparative cycle (CT) method, also known as the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), was applied

using the following formula: the relative amount of mRNA for the target gene = $2^{-\Delta\Delta CT}$, where CT = threshold cycle for the target gene amplification, $\Delta CT = CT_{\text{target gene}} - CT_{\text{endogenous reference}}$, and $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$. The endogenous reference gene chosen for this study was histone H2A (*H2A*) because it was reported to have a constant expression level throughout preimplantation development (Robert et al., 2002). The calibrator was a mixture of RNA from organs and blastocysts and was included in each real-time amplification. Validation of the amplification efficiency of target genes and the endogenous reference gene was completed before using the $2^{-\Delta\Delta CT}$ method for quantification. Briefly, different dilutions of cDNA were amplified by real time RT-PCR using the gene specific primers. The corresponding ΔCT values were plotted against the log of each cDNA amount and the data were plotted using linear regression analysis. The values of the slopes of the validation curves for each gene was less than 0.1, indicating that the same amplification efficiency was obtained for all samples with both high and low amounts of the cDNA. All validation and quantification methods were suggested by the manufacturer of the real time amplification kit (Applied Biosystems Inc.). The CT values were adjusted based on cDNA dilution and the 1/CT values were used to examine the temporal pattern of gene expression throughout preimplantation development.

4.3.6 Statistical analysis

Data analyses for the differences in embryonic development and relative mRNA levels for each gene were carried out by ANOVA using the general linear model (GLM) procedure in the Statistical Analysis Systems (SAS, version 9.0, SAS Inc., Cary, NC). For embryo development, the percentages of cleavage and blastocyst

development were arc sine transformed and analyzed by one-way ANOVA with the main effect as the embryo type (IVF vs. NT). For the comparison of the relative mRNA levels for each gene, two-way ANOVA was utilized with the main effects as embryo type and developmental stage; and the interaction term as the embryo type X developmental stages. The differences between relative levels of gene expression in NT and IVF embryos were log10 transformed and the Least Square Means method in GLM was used for multiple comparisons across developmental stages within each embryo type. A probability value of $P \leq 0.05$ was considered significant. The expression patterns of *HDAC1* and *DNMT3A* were determined the correlation by the Pearson analysis in SAS.

Table 4.1 Swamp buffalo primer sequences used in real time RT-PCR

Gene	Accession number for cattle	Buffalo primer sequences 5' - 3'	Product Size (bp)	Melting Temperature (^{o}C)
DNMT1	AY173048	forward- GAGGGCTACCTGGCTAAAGTC reverse- CATTCGCTTCCCGACTGAAA	88	78
DNMT3A	AY271299	forward- CGAGGTGTGTGAGGACTCCAT reverse- ACGTCCCCGACGTACATGA	93	79
DNMT3B	AY224713	forward- AGCATGAGGGCAACATCAAAT reverse- CACCAATCACCAAGTCAAATG	98	77
HAT1	BT021536	forward- CTTCAGACCTTTTTGATGTGGTTTATT reverse- GCGTAGCTCCATCCTTATTATACTTCTC	112	85
HDAC1	AY504948	forward- GCACTGGGCTGGAACATCTC reverse- GGGATTGACGACGAGTCCTATG	98	79
H2A	M37585	forward- TTTGTGGATGTGTGGAATGAC reverse- TTCGTGGAGATGAAGAATTGG	95	76

4.4 Results and discussion

4.4.1 Development of IVF and NT swamp buffalo embryos

The developmental rates of the swamp buffalo embryos derived from the IVF and NT are summarized in Table 4.2. No significant differences were observed in development rates from cleavage to morula between NT and IVF embryos. However, a higher blastocyst rate *(P<0.05)* was obtained for IVF embryos. Similar numbers of cells in ICM (~30cells/embryo) and TE (~90 cells/embryo) were found in both NT and IVF blastocysts.

4.4.2 Levels of mRNA for chromatin remodeling genes

In the present study the mRNA levels of five chromatin remodeling genes were examined throughout preimplantation development. The endogenous reference gene, *H2A*, displayed a constant mRNA level from the oocyte up to the 8-cell stage. The mRNA levels of *H2A* increased with embryonic development after the 8-cell stage until reaching its highest level at the blastocyst stage (Figure 4.1). Due to the variable expression of *H2A* during development, the $2^{-\Delta\Delta CT}$ method could not be used for comparisons across different stages of development within each embryo type. Therefore, we only compared statistically the relative levels of mRNA between embryo types at the same stage of development and temporal changes within each embryo type were evaluated based on 1/CT values.

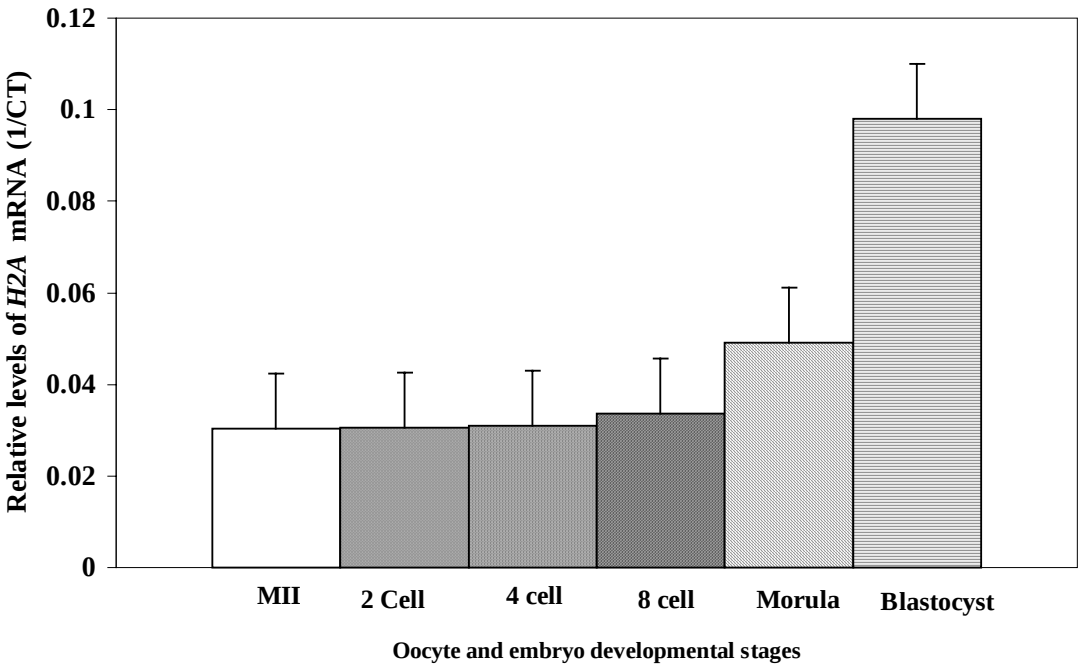
Table 4.2 Development of preimplantation swamp buffalo embryos derived from NT and IVF

Embryo type	Experiment (n)	Fertilized/Fused Embryos	Embryo development (mean ± SEM)			No. of cells in blastocysts (mean ± SEM)		
			Cleavage	Morula	Blastocyst	ICM	TE	% ICM
NT	3	206	0.90±0.06[a]	0.74±0.03[a]	0.34±0.03[a]	25.6±5.21	88.7±10.8	20%
IVF	3	199	0.76±0.06[a]	0.67±0.04[a]	0.50±0.03[b]	29.2±2.09	97.3±4.15	22%

Values within column with the different superscripts are significantly different *($P \leq 0.05$)*.

The cleavage rate was calculated based on the number of fertilized/fused embryos. The developmental rates to morula and blastocyst were calculated based on the number of cleaved embryos.

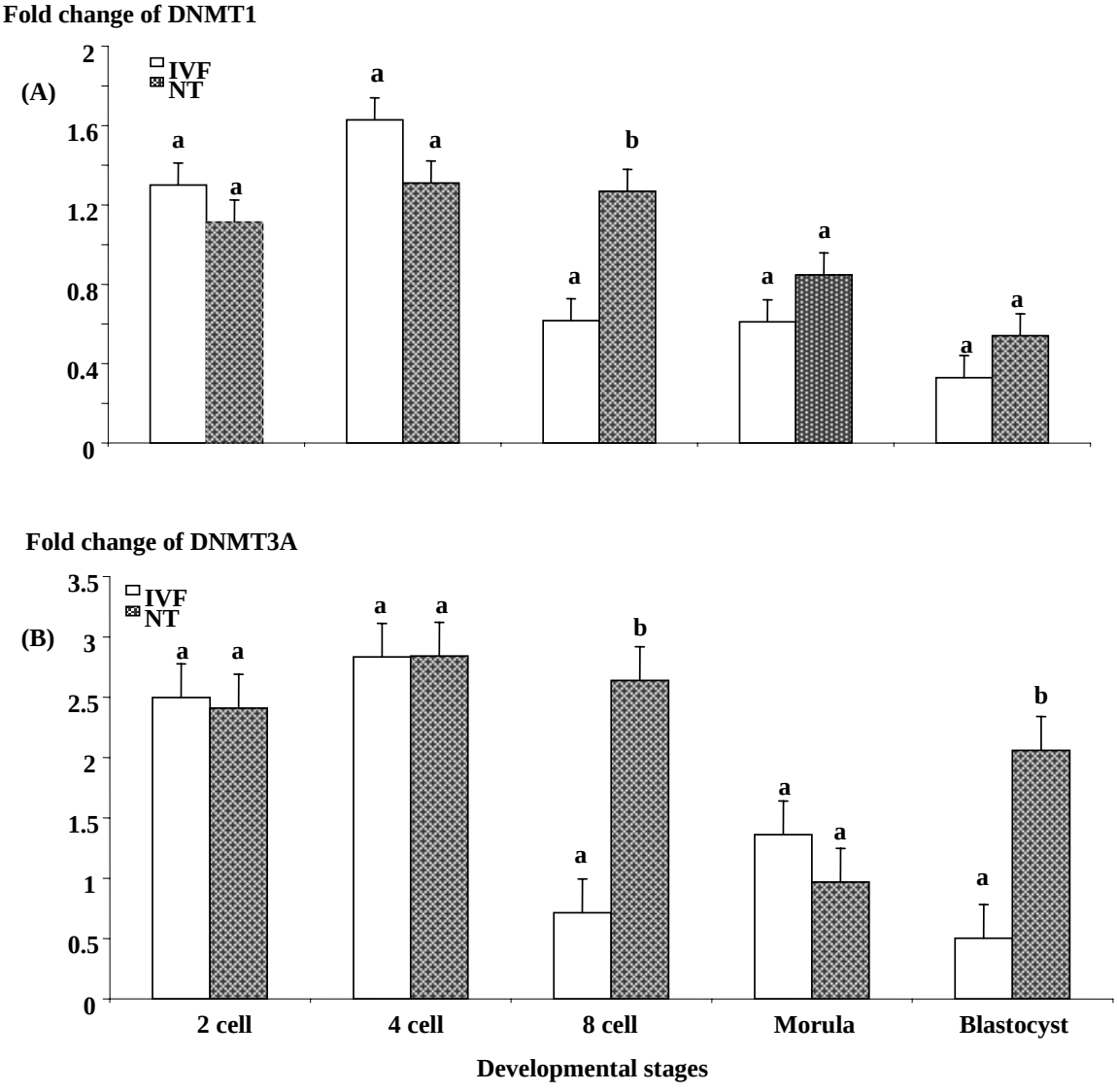
Figure 4.1 The relative levels of *H2A* expression in oocyte and IVF embryos at different developmental stages.



The relative levels of mRNA for the passive DNA methylation gene, *DNMT1*, were fairly constant from the MII oocyte stage up to the 4-cell stage. However, *DNMT1*

started to decrease at the 8-cell stage and reached its lowest levels at the blastocyst stage. Despite the similar trends in the levels of *DNMT1* in NT and IVF embryos, *DNMT1* appeared to always be higher in the NT embryos and this became significant at the 8-cell stage and nearly so at the blastocyst stage (P=0.06; Figure 2A). In contrast, the *de novo* DNA methylation genes, *DNMT3A* and *3B,* showed nearly the reverse change after cleavage development compared to that of *DNMT1*. The levels of mRNA for both genes increased from the morula stage and reached the highest level at the blastocyst stage. Significantly higher levels of both genes were found at the 8-cell and blastocyst stages in the NT as compared to the IVF embryos *(*P<0.05; Figures 2B, 2C). Similar patterns of temporal expression were also observed in the mRNA levels of the histone modifying genes, *HAT1* and *HDAC1,* during cleavage development. They were relatively unchanged from the MII oocytes to the 8-cell stage in both IVF and NT embryos. Subsequently, both genes showed a dramatic increase in expression and reached their highest expression level at the blastocyst stage. When gene expression levels were compared between the NT and IVF embryos, significantly higher levels for both genes (P<0.05 and P<0.01, respectively) were found in NT embryos at the blastocyst stage (Figures 3A, 3B). We also observed significantly (P<0.05) higher levels of *HDAC1* at the morula stage in the NT embryos. Interestingly, a high correlation (r=0.99) was found in the expression levels between *HDAC1* and *DNMT3A* throughout the developmental stages examined.

Figure 4.2 Comparison the levels of mRNA for the DNA methylation genes



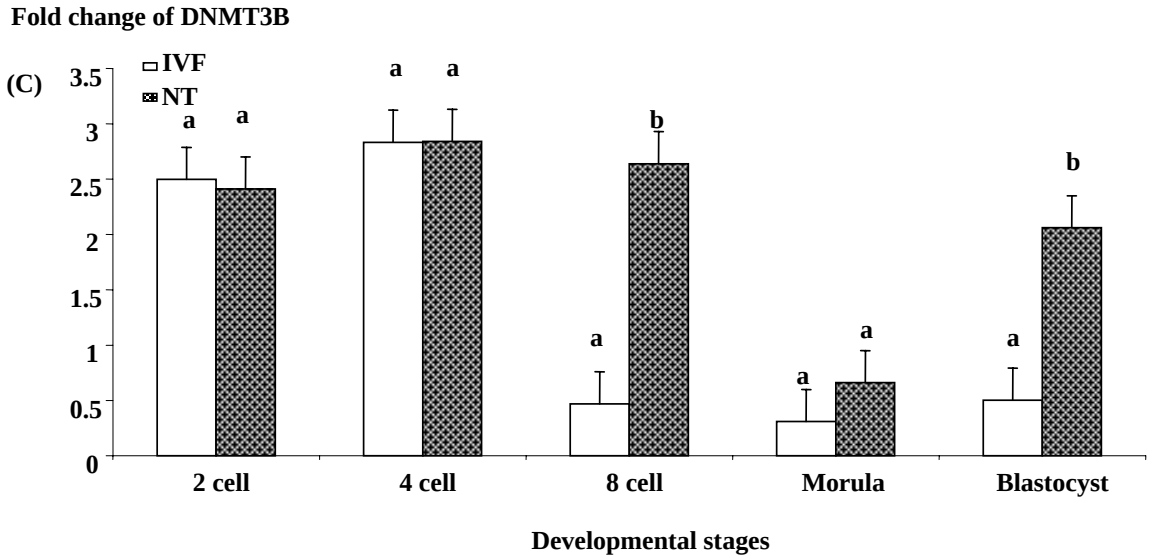
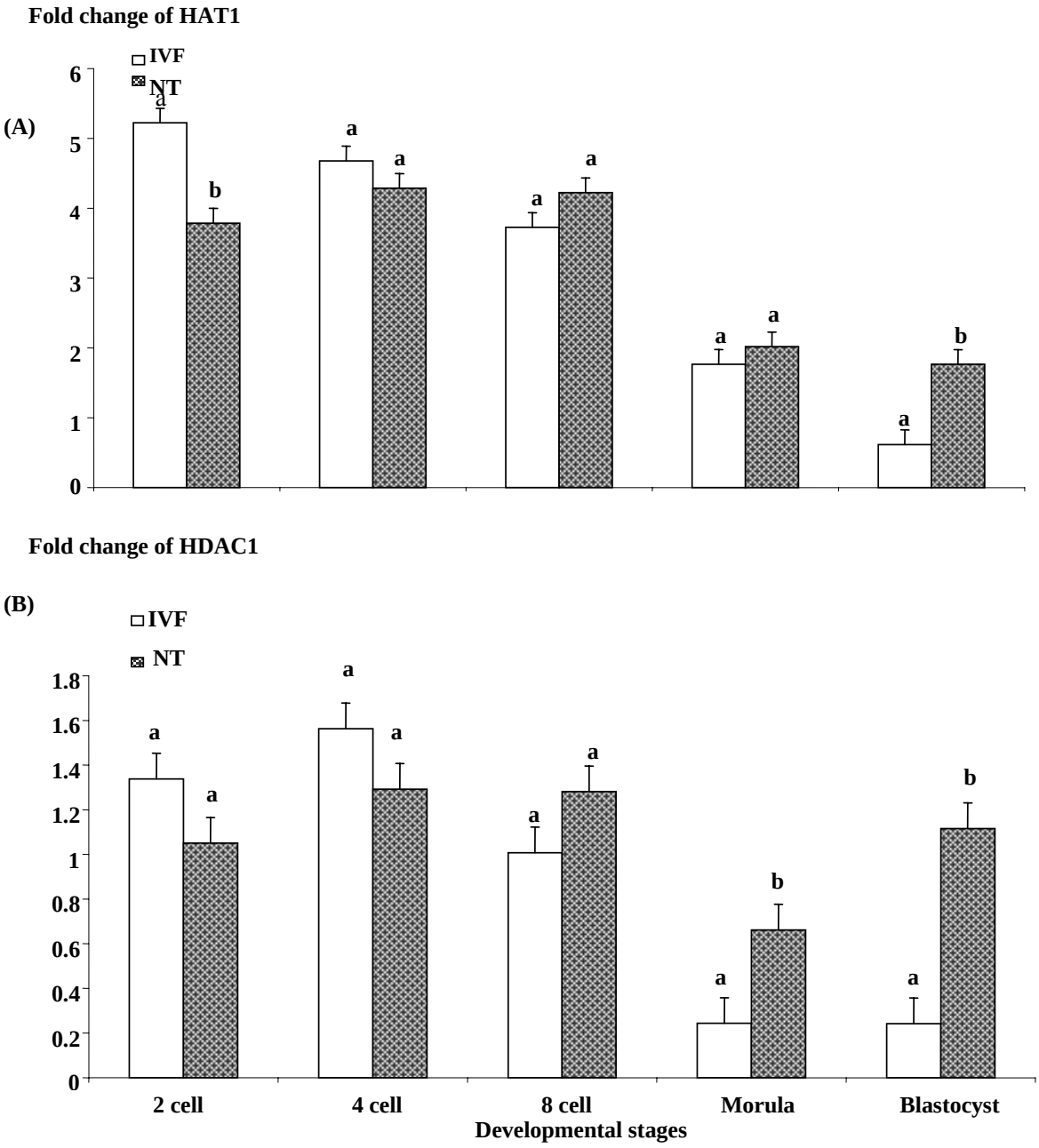


Figure 4.2 shows the comparison of the fold change in the levels of mRNA for the DNA methyltransferase genes between IVF (white bars) and NT embryos (black bars) at different stages of embryo development: (A) *DNMT1*, (B) *DNMT3A* and (C) *DNMT3B*. The fold change is the difference between the embryos and the calibrator. Bars with different superscripts within a particular stage of development are significantly different ($P<0.05$).

Figure 4.3 Comparison the levels of mRNA for the histone modification genes



Comparison of mRNA levels for the histone modification genes between IVF (white bars) and NT embryos (black bars) at different developmental stages: (A) *HDAC 1*and (B) *HAT1*. The fold change is the difference between the embryos and the calibrator. Bars with different superscripts within stage are significantly different ($P<0.05$).

4.4.3 Discussion

In the present study, we produced IVF and NT swamp buffalo embryos and documented for the first time, the dynamics of expression of five chromatin remodeling genes in these embryos. The efficiency of producing NT embryos and the cell numbers of NT and IVF embryos separated into the two lineages, ICM and TE, are comparable to or higher than those reported previously in the same species (Atabay et al., 2004; Saikhun et al., 2004). In our study, we found approximately 90 cells in the TE and an additional ~30 cells in the ICM. Although no data on the cell numbers for *in vivo* buffalo embryos are available for comparison, the number of cells in the ICM of the swamp buffalo NT and IVF embryos are low compared to those of cattle blastocysts (Van Soom et al., 1997), possibly due to inefficient culture conditions. The significantly lower developmental rate of NT embryos after cleavage development indicates that problems with nuclear reprogramming may have occurred. To understand the epigenetic changes during NT embryos' preimplantation development, we studied five genes responsible for DNA methylation and histone acetylation modifications.

We found that NT embryos contained higher levels of mRNA for *DNMT1* (8-cell embryos) and for *DNMT3A/3B* (blastocysts). Taken together, the increased levels of all DNA methylation genes studied here, suggest that the NT embryos have higher levels of enzymatic activities not only for maintaining existing methylation, but also for *de novo* DNA methylation. These data provide an underlying mechanism for the global hypermethylation in the buffalo NT embryos observed by our lab. While these findings are novel in the species of buffalo, they agree with observations in cattle NT embryos, which were found to be aberrantly hypermethylated and also to contain

higher levels of mRNA for the *DNMTs* (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001).

The enzyme *HDAC1* is responsible for deacetylating histones, which causes transcriptional repression. Interestingly, our results demonstrated a strong positive correlation (r=0.99) between *HDAC1* and *DNMT3A,* due to a similarity of expression patterns for these two genes at all stages, suggesting a coordinated regulation of these two genes and a connection of their functions. This observation is consistent with the reports of Fuks et al. (2001) and Datta et al. (2003), who found that *DNMT3A* is associated with *HDAC1* and forms a protein complex involved in transcriptional repression. Because both *HDAC1* and *DNMT3A* act to inhibit transcription, the observation that the mRNA for these two genes was elevated in the NT embryos at the blastocyst stage, may indicate reduced transcription levels which could lead to further developmental abnormalities.

The gene *HAT1* is a type B histone acetylase. It functions to acetylate non-specific nascent histone targets and is involved in chromatin assembly (Roth and Allis, 1996; Ruiz-Garcia et al., 1998). To date, the expression of *HAT1* and *HDAC1* has only been studied in bovine IVF embryos (Enright et al., 2005), neither has been studied in NT embryos. Thus, our study represents the first characterization of histone acetylation capabilities of NT embryos in all species cloned. It has been reported that global levels of histone acetylation were higher in NT than IVF embryos in cattle (Enright et al., 2005; McGraw et al., 2003). It is not surprising that higher expression of *HAT1* was found in the buffalo NT embryos studied here.

We found that *H2A*, which was used as a reference gene in our study, had constant expression at early developmental stages, and this was followed by increased

expression at the morula and blastocyst stages. This finding is inconsistent with the study of Robert et al. (2002), who measured the expression of many housekeeping genes in bovine embryos. They found *H2A* was the only gene which had relative constant expression during preimplantation development; thus, identifying it as the best internal reference for early embryos. The discrepancy between our study and that of Robert et al. (2002) could be due to their use of pooled embryos or because of species differences. Because *H2A* expression increased at the blastocyst stage in our study, we only used it when comparing NT and IVF embryos at the same developmental stage, hence, all comparisons are valid. A housekeeping gene that can serve as an ideal internal control for gene expression studies across early preimplantation development has yet to be identified.

Another interesting finding of the present study may indicate the timing of maternal-zygotic transition (MZT), which is still unclear in buffalo embryos. The MZT is the period where maternal mRNA is replaced by embryonic mRNA. In this study, we found that the NT embryos had a lower blastocyst rate than the IVF embryos. However, this did not result from a lower cleavage rate of the NT embryos. In fact, the NT embryos had a higher, although not significant, cleavage rate than the IVF embryos. These data indicate that the development of NT embryos up to the 8-cell stage may be sufficiently supported by the maternal components of the oocytes. Upon the depletion and degradation of maternal mRNAs, the NT embryos had lower further developmental rates, which may be due to an incomplete activation of the embryonic genome. We suggest that the 8-cell stage, as is in the case for cattle, may be when MZT occurs in the swamp buffalo. Additionally, our real time RT-PCR results also support this assumption as critical changes, either increases or decreases,

occurred in gene expression at the 8-cell stage for all genes studied. Future studies examining this critical event in swamp buffalo embryos are needed to confirm this hypothesis.

4.5 Conclusions

In summary, our results showed relatively constant levels of expression of all chromatin remodeling genes studied up to the 8-cell stage. Interestingly, higher expression levels of all genes at the blastocyst stage and all DNA modifying genes at the 8-cell stage were found in the NT embryos as compared to the IVF embryos. These data provide the underlying mechanism for the abnormal hypermethylation in cloned embryos, which could cause transcriptional repression and may be one of the reasons for the developmental failure of cloned embryos at later stages.

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CHAPTER V

THE EFFECT OF SOMATIC CELL NUCLEAR TRANSFER ON THE IMPRINTED GENE *IGF2r* EXPRESSION

5.1 Abstract

Cloned animals often suffer from loss of development during gestation and many abnormalities typically classified as Large Offspring Syndrome (LOS). Cattle are an interesting species to study according to their highest rate of success in nuclear transfer among all of the species cloned to date. The imprinted insulin like growth factor receptor (*IGF2r*; mannose-6-phosphate) gene was chosen to investigate aspects of fetal growth and development in cloned cattle. Here we analyzed the *IGF2r* expression patterns in identically genetic clones of several age groups; day 25, day 45, and day 75 fetuses as well as spontaneously aborted fetuses, calves that died shortly after birth and healthy cloned calves using single stranded conformational polymorphism (SSCP) gel electrophoresis. Variable pattern of *IGF2r* allelic expression in major organs such as brain, cotyledon, heart, liver, spleen and intercotyledon using a G/A transition in the 3'UTR of *IGF2r* was discovered. The expression levels of *IGF2r* by real time RT-PCR was found to be highly variable among the clone groups. We conclude that proper *IGF2r* expression is necessary for

survival to term, but is most likely not a cause of early fetal lethality or an indicator of postnatal fitness.

2 Introduction

Imprinted genes are expressed in a parent-of-origin specific manner and mostly function as fetal growth regulators (Hall, 1990; Moore and Haig, 1991). Imprinted genes are established during gametogenesis and maintained throughout embryogenesis before undergoing erasure of gene imprinting marker and parent-of-origin specific re-establishment in the primordial germ cells. All imprinted genes contain epigenetically established differentially methylated regions (DMRs), which play a role in the imprinting process. Many species differences have been found among mammals with different placental types, probably due to the fact that imprinted genes regulate fetal growth and the placenta is the maternal-fetal interface of nutrient transfer.

To date, more than 73 imprinted genes have been identified in mice and/or human, many of which have been proven to be orthologous in other species. A highly conserved imprinted gene between mice and cattle is the insulin like growth factor 2 receptor (*IGF2r*) gene (Killian et al., 2001). The *IGF2r* gene is a maternally expressed imprinted gene and functions to suppress fetal growth by acting as a scavenger receptor of the potent fetal mitogen *IGF2*. *IGF2r* expression is regulated by DMRs, an antisense transcript, *AIR* and a promoter restricted histone code in humans and mice.

Imprinting is usually studied using genetic markers that can distinguish between the maternal and paternal alleles. Single nucleotide polymorphisms (SNPs)

are single base changes or insertion/deletions in the DNA sequence. SNPs have been widely used in imprinting studies because of their abundance in the mammalian genome and because they are inherited in a traditional Mendelian pattern.

Large Offspring Syndrome (LOS) and loss of development to term have beenobserved in many cloned species (Hill et al., 2000; Tamashiro et al., 2000; Tanaka et al., 2001; Wakayama and Yanagimachi, 1999). These defects are likely to occur in a systemic fashion that directly affects fetal growth and survival. Somatic cell nuclear transfer deprives the imprinted genes of their natural pattern of passing through the gonad to correctly establish epigenetic parent-of-origin specific imprinting patterns. Incomplete epigenetic reprogramming of reconstructed embryos has been implicated in the high incidence of morbidity and mortality of cloned animals.

Due to these reasons disruptions in imprinted genes have been seen as likely candidates for the cause of LOS and abnormal imprinted gene expression patterns have indeed been reported in cloned mice (Humpherys et al., 2002; Inoue et al., 2002) and cattle (Yang et al., 2005). Thus, due to the conserved nature, epigenetic regulation and direct involvement in fetal growth, *IGF2r* was one of the best candidates available to study the fidelity of epigenetic reprogramming through allelic expression and expression levels in cloned cattle (Ferguson-Smith et al., 1991).

In the present study we aimed to 1) establish a through pattern of *IGF2r* allelic expression and expression levels throughout the development of the placenta by examining day 25 trophectoderm (before implantation), day 45 trophectoderm (after implantation) and day 75 cotyledon and intercotyledon (after the differentiation of placental structures) of control and age matched cloned fetuses as well as the cotyledon and intercotyledon of healthy, live cloned cattle, 2) examine *IGF2r* allelic

expression patterns in day 75 cloned and control fetuses and the allelic expression patterns in prenatal fetal tissues from controls and spontaneously aborted cloned calves and calves that died shortly after birth and healthy live clones including the brain, heart, spleen and liver, and 3) determine the expression of the *IGF2r* antisense transcript, *AIR* in cloned cattle.

5.3 Materials and methods

5.3.1 Tissue samples, extraction of genomic DNA and total RNA

Control organ/tissue samples were obtained from slaughterhouse cattle and cattle generated from embryo transfer and artificial insemination (Thailand and Ireland respectively). Six day 25, seven day 45 and seven day 75 cloned cattle samples were generated using skin fibroblasts from an elite Holstein-Friesian dairy cow. Cloned cattle samples obtained from three aborted, four cloned calves and six living clones were generated from donor cells derived from a 13 year-old beef animal. Organ/tissue samples that were collected include trophectoderm, cotyledon, intercotyledon, brain, heart, spleen, and liver. The samples were used for heterozygosity screening of a SNP in the 3'UTR of *IGF2r,* allelic expression analysis by single strand conformational polymorphism gel electrophoresis (SSCP) and expression level analysis by real time RT-PCR. All organ samples were frozen immediately after collection and stored at -80ºC until analysis. Genomic DNA was extracted from blood and organ samples using the DNeasy kit from Qiagen (Valencia, CA) and total RNA was extracted from frozen organ samples using the RNeasy kit (Qiagen). The extracted RNA was treated with RNase-free DNase to remove any possible contaminating genomic DNA. The

Institutional Animal Care and Use Committee at the University of Connecticut and an equivalent organization at the Institute of Agricultural Technology (Thailand) and University College Dublin approved all procedures involving the use of animals.

5.3.2 Genotyping for heterozygosity of a SNP of bovine *IGF2r*

Genomic DNA was amplified using PCR primers designed to flank a SNP within the 3'UTR of the cattle *IGF2* receptor gene; *reverse* 5'-AGAAGCCTTAATTTGCACA-3' and *forward* 5'-AGCCAAACAAGAGTACAAA-3'. PCR was performed using 100 ng of genomic DNA with 0.6 pmol/µl of each primer with Taq DNA polymerase (Invitrogen life technologies) in a total volume of 25 µl. The following amplification conditions were used: an initial denaturation step of 94^{o}C for 3 min followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 20 sec, and extension at 72^{o}C for 50 sec, final extension at 72^{o}C for 7 min. PCR products were analyzed on a 1% agarose gel before screening for heterozygosity by SSCP. The SSCP was performed by denaturing 15µl of PCR product in 30 µl denaturing buffer (final concentrations 95% deionized formamide, 20 mM EDTA, and 0.05% each xylene cyanol and bromophenol blue) at 95^{o}C for 10 minutes and snap cooled on ice for 10 minutes. Denatured PCR products were immediately loaded into a 12% polyacrylamide gel. Gels were run at 4^{o}C C for 20 h. The voltage was set the same as the base pair number of the product, for instance a 250 bp product was run at 250 volts. Each polyacrylamide gel was stained with the silver staining method. Conditions were as follows, final concentrations are; 1. Fixing: 5 minutes in 200 ml 10% ethanol and 0.5% acetic acid, 2. Washing: once in 10% ethanol, 3. Staining: 1 hour in 200 ml 0.1% AgNo3 in 10% ethanol, 4. Washing: twice in 10% ethanol, 5.

Developing: 15-20 minutes in 200 ml 1.55 NaOH, 0.01% NaBH4 and 0.15% formaldehyde in 10% ethanol. The gels were dried and archived.

5.3.3 Allele specific expression of the imprinted bovine *IGF2r* gene

For all samples identified as heterozygous for the *IGF2r* SNP, total RNA was extracted following the manufacturers protocol of the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen) to remove any contaminating DNA. One step RT-PCR was performed with 10 ng/µl total RNA, 0.6 pmol/µl of *IGF2r* primer pair and 2 µl of one-step RT-PCR enzyme mix (Qiagen) in a total volume of 25 µl. RNA was reverse transcribed using the following conditions; 50^{o}C for 30 min and 95^{o}C 15 min before PCR amplification using the following conditions; 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 20 sec, and extension at 72^{o}C for 50 sec, final extension at 72^{o}C for 7 min. The RT-PCR products were analyzed for allelic expression by SSCP.

5.3.4 Real time reverse transcription (RT)-PCR of bovine *IGF2r*

Total RNA from day 25, day 45 and day 75 cloned and control animals was extracted using the RNeasy kit (Qiagen, USA) and reverse transcription was carried out at 50^{o}C for 1 h with Superscript III (Invitrogen). The real time RT-PCR reaction mixture contained 5 µl of cDNA, 12.5 µl of SYBR green master mix (Applied Biosystems Inc., CA, USA) and 0.3 µm of forward and reverse primers in a total volume of 25 µl. All tissues were analyzed in triplicate for every gene. The specificity of the real time RT-PCR product was proven by a melting curve analysis.

To compare the relative levels of gene expression in cloned and control tissues, the comparative cycle (CT) method, also known as the $2^{-\Delta\Delta CT}$ method was applied using the following formula: the relative amount of mRNA for the target gene = $2^{-\Delta\Delta CT}$, where CT = threshold cycle for the target gene amplification, $\Delta CT = CT_{\text{target gene}} - CT_{\text{endogenous reference}}$, and $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$. The endogenous reference gene chosen for this study was beta-actin gene. The calibrator was a mixture of RNA from liver and lung tissue and was included in each real time amplification. Validation of the amplification efficiency of target genes and the endogenous reference gene was completed before using the $2^{-\Delta\Delta CT}$ method for quantification. Briefly, different dilutions of cDNA were amplified by real time RT-PCR using the gene specific primers. The corresponding ΔCT values were plotted against the log of each cDNA amount and the data were plotted using linear regression analysis. The values of the slopes of the validation curves for each gene was less than 0.1, indicating that the same amplification efficiency was obtained for all samples with both high and low amounts of the cDNA. All validation and quantification methods used were suggested by the manufacturer of the real time amplification kit (Applied Biosystems Inc.).

5.3.5 Expression of the bovine *IGF2r* antisense transcript, AIR

Genomic DNA was amplified using PCR primers designed to span the intron region between *IGF2r* exons 1 and 2, thereby ensuring only the antisense transcript *AIR* would be amplified; *forward* 5'-AGCCAAACAAGAGTACAAA-3' and *reverse* 5'-CGAGACCCCACCAGACTAGAC-3'. PCR was performed using 100 ng of

genomic DNA with 0.6 pmol/µl of each primer with Taq DNA polymerase (Invitrogen life technologies) in a total volume of 25 µl. The following amplification conditions were used: an initial denaturation step of 94 ^{o}C for 3 min was followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 20 sec, and extension at 72^{o}C.

5.3.6 Statistical analysis

Relative differences in the mRNA levels among different organs from cloned and control animal tissues were analyzed using the Kruskal-Wallis subroutine of the Statistical Analysis System (SAS) and a probability value of $P \leq 0.05$ was considered to be significant.

5.4 Results and discussion

5.4.1 Genotyping for heterozygosity of SNP in the 3' UTR of bovine *IGF2r*

All day 25, day 45, day 75, aborted, dead and living clones and age matched control cattle were screened for heterozygosity of an SNP in the 3'UTR of the *IGF2r* gene. A specific band of 298 bp using the cattle *IGF2r* primers was amplified and is shown on a 1% agarose gel (Fig.5.1). The PCR products were sequenced and a single nucleotide polymorphism; instead adenosine to guanosine (G to A transition), was observed (Fig.5.2). The heterozygous and homozygous patterns are shown in (Fig.5.3) by SSCP. All of the cloned samples were heterozygous for the G/A transition as well as controls for each age group.

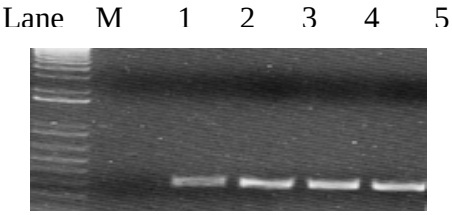

Figure 5.1. The 298 bp PCR product derived from amplification of 3'UTR *IGF2r* cattle gene. Lane M: 100 bp ladder maker, lane 1: negative control and lane 2-5: PCR product from cattle genomic DNA.

5.4.2 Allele specific expression of the imprinted *IGF2r* gene

The allelic expression of *IGF2r* in the trophectoderm of day 25, 45 and 75 cloned and control fetuses were demonstrated in Table 5.1. Control fetuses showed somewhat monoallelic with some leakage of the imprinted allele. *IGF2r* expression was also found to be somewhat monoallelic with some leakage of the imprinted allele in the organs and tissues of day 75 fetuses and prenatal calves. The allelic expression of *IGF2r* in the trophectoderm of day 25 and day 45 cloned fetuses was shown to be somewhat monoallelic with some leakage of the imprinted allele. We found variable expression patterns in the allelic expression of *IGF2r* in the organs and tissues of day 75 and spontaneously aborted cloned fetuses. Most of the organs and tissues of day 75 cloned fetuses were shown to have slightly monoallelic expression with some leakage of the imprinted allele however, two cloned fetuses (day 75) were found to have very

nearly monoallelic expression as well as allele switching in the spleen. Spontaneously aborted cloned calves were found to have the most variable allelic expression patterns out of all the age groups studied.

The allelic expression of *IGF2r* in many organs and tissues of the aborted, decease and healthy clones and control calves were demonstrated in Table 5.2. They were found to have monoallelic expression (no leakage) with a high incidence of allele switching, most notably in an aborted cloned fetus were monoallelic expression and switching to the paternal allele occurred in the cotyledon, heart and liver and monoallelic expression of the maternal allele in the brain and intercotyledon. Clones that died shortly after birth and the cotyledons and intercotyledons of healthy living clones mostly showed normal somewhat monoallelic expression with leakage of the imprinted allele.

Table 5.1 Allelic expression pattern of *IGF2r* gene in cloned and control fetuses at day 25, 45 and 75 of gestation.

	Trophecto -derm	**Heart**	**Brain**	**liver**	**Spleen**	**Cotyle -don**	**Intercoty- don**
D25 Control	G/a	-	-	-	-	-	-
D25Control	G/a	-	-	-	-	-	-
D25 control	G/a	-	-	-	-	-	-
D25Control	G/a	-	-	-	-	-	-
D25clone	G/a	-	-	-	-	-	-
D25clone	G/a	-	-	-	-	-	-
D25clone	G/a	-	-	-	-	-	-
D25 clone	G/a	-	-	-	-	-	-
D45Control	G/a	-	-	-	-	-	-
D45 control	G/a	-	-	-	-	-	-
D45Control	G/a	-	-	-	-	-	-
D45Control	G/a	-	-	-	-	-	-
D45clone	G/a	-	-	-	-	-	-
D45clone	G/a	-	-	-	-	-	-
D45clone	G/a	-	-	-	-	-	-
D45clone	GG	-	-	-	-	-	-
D75Control	-	G/a	G/a	G/a	G/a	G/a	G/a
D75 control	-	G/a	G/a	G/a	G/a	G/a	G/a
D75Control	-	G/a	G/a	G/a	G/a	G/a	G/a
D75Control	-	G/a	G/a	G/a	G/a	G/a	G/a
D75clone	-	G/a	G/a	G/a	G/a	G/a	G/a
D75clone	-	G/a	G/a	G/a	AA	G/a	G/a
D75clone	-	G/a	G/a	G/a	g/A	G/a	G/a
D75clone	-	G/a	G/a	G/a	G/A	G/a	G/a

Table 5.2 Allelic expression pattern of *IGF2r* gene in living and decease clones, aborted cloned fetuses and control derived from natural mating.

Organ	**Brain**	**Cotyle-don**	**Heart**	**Liver**	**Spleen**	**Intercoty-ledon**
Living 1	-	G/A	-	-	-	g/A
Living 2	-	g/A	-	-	-	g/A
Living 3	-	G/A	-	-	-	g/A
Living 4	-	g/A	-	-	-	G/A
Living 5	-	G/A	-	-	-	g/A
Living 6	-	G/A	-	-	-	G/A
Aborted 1	GG	G/A	GG	G/A	GG	GG
Aborted 2	GG	GG	AA	GG	AA	GG
Aborted 3	G/a	GG	G/a	G/a	G/a	GG
Decease 1	G/A	g/A	g/A	g/A	g/A	g/A
Decease 2	G/A	g/A	g/A	G/a	g/A	g/A
Decease 3	G/A	g/A	G/A	g/A	g/A	g/A
Decease 4	G/A	G/A	G/A	g/A	G/A	g/A
Control 1	G/A	g/A	g/A	g/A	g/A	g/A
Control 2	G/A	g/A	g/A	g/A	g/A	g/A
Control 3	G/A	G/a	G/a	G/a	G/a	G/a
Control 4	G/A	g/A	g/A	g/A	g/A	g/A

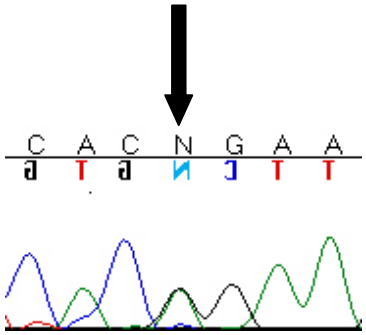

Figure 5.2. Sequence chromatogram of PCR product shows the SNP, a G to A transition (arrow) on 3'UTR of the cattle *IGF2r* gene.

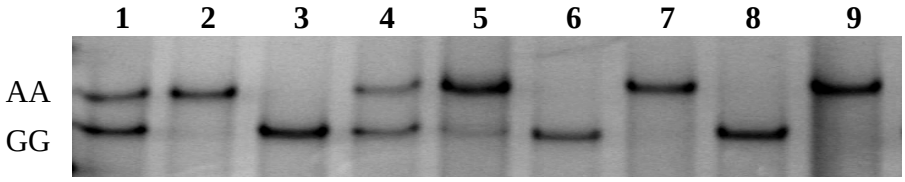

Figure 5.3. SSCP genotyping of the SNP in the 3'UTR of the bovine *IGF2r* gene. Lanes 1-3 represent a heterozygous (G/A) sample and homozygous (AA) and (GG) samples, respectively. Lanes 4-9 demonstrates different patterns derived from all samples; 4-heterozygous (G/A) pattern; 5-homozygous AA leakage G (g/A); 6,8-homozygous GG and 7,9 –homozygous AA.

5.4.3 Real time reverse transcription (RT)-PCR

The expression of the *IGF2r* gene in the spleen of cloned fetuses was significantly lower than the expression in the same tissue from control fetuses. The expression of the *IGF2r* gene in all of the other tissues was similar for cloned fetuses and control fetuses. Despite no significant differences found between controls and clones in *IGF2r* gene expression in most tissues, individual clones showed extremely high or low expression in some tissues, as evidenced by the large standard error bars for the clones (Fig. 5.) For instance, A day 45 cloned fetus had 36 fold less expression in the cotyledon, 12 fold less expression in the liver and 2 fold less expression in the spleen while also having 102 fold more expression in the brain when compared to the average expression levels of the corresponding tissues in the controls. A day 25 Clone had 4 fold less expression in the spleen and 8 fold less expression in the heart while showing 5 fold more expression in the liver.

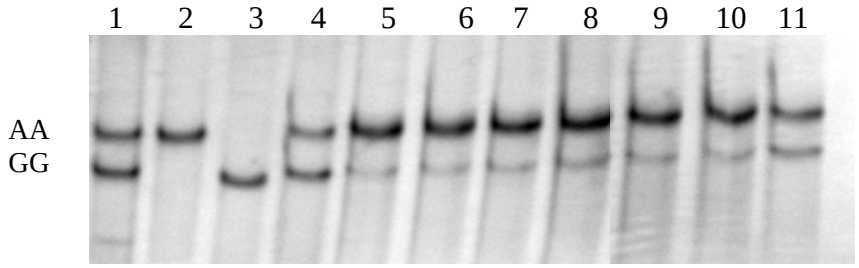

Figure 5.4. SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2r* gene derived from control calve.

Lanes 1-3 represents a heterozygous G/A, homozygous AA and GG genotype, respectively.

Lane 4 represents the genotype from the donor cell DNA.

Lane 5 represents the allelic expression pattern derived from donor cell fibroblasts.

Lanes 6-11 show the allelic expression patterns in the cotyledon, heart, kidney, liver, placenta and brain, respectively, which were found by studying control animal.

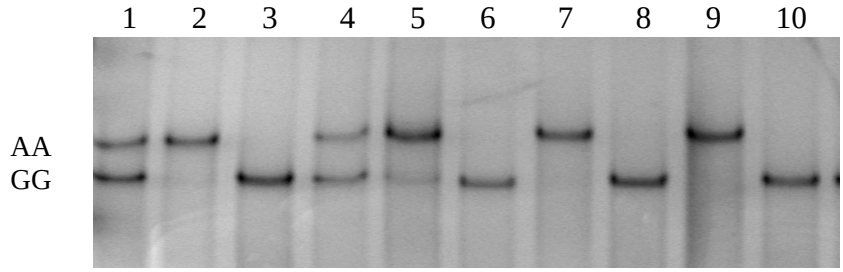

Figure 5.5. SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2r* gene derived from aborted cloned fetuses.

Lanes 1-3 represents a heterozygous G/A, homozygous AA and GG genotype, respectively.

Lane 4 represents the genotype from the donor cell DNA.

Lanes 5-10 show the aberrant monoallelic expression patterns and gene switching in the cotyledon, heart, kidney, liver, placenta and brain, respectively, which were found by studying aborted cloned fetuses.

5.4.4 Expression of the bovine *IGF2r* antisense transcript, AIR

All control and cloned organ/tissue samples expressed *AIR* (Fig. 5.).

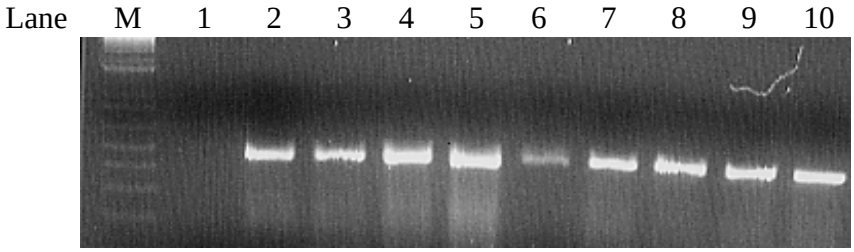

Figure 5.6. Expression of the bovine *IGF2r* antisense transcript, *AIR*, in control and clone tissues. Lane M: 100 bp ladder maker, lane 1: negative control and lane 2-10: PCR product of *AIR* from control and interest samples.

5.4.5 Discussion

In this study, the *IGF2r* allelic expression pattern and expression levels throughout the development of the placenta has been established by examining day 25 trophectoderm (before implantation), day 45 trophectoderm (after implantation) and day 75 placenta and cotyledons (after the differentiation of placental structures) from control and age matched cloned fetuses by SSCP and real time RT-PCR. Allelic expression was found to be somewhat monoallelic with some leakage of the imprinted allele in day 25, day 45 and day 75 placental structures. No significant differences were found in the expression of *IGF2r* between control day 25 and day 45 trophectoderm using the Kruskal-Wallis subroutine of the SAS. No significant differences were found between cloned and control trophectoderm within the day 25 and day 45 age groups or when comparing the rate of change between the day 25 to

day 45 trophectoderm to that of the control group. While no overall significant differences were found in the cotyledon and intercotyledon of day 75 fetuses between clones and controls, individual clones showed high variations such as a cloned fetus showed 36 fold less *IGF2r* expression in the cotyledon when compared to the control group. These data are consistent with the results of Lonergan et al. (2005) whose data suggest that large calf syndrome is manifested after day 45 of gestation. The allelic expression pattern of *IGF2r* in the cotyledon and intercotyledon of healthy, live cloned cattle was nearly consistent with the data from the control cotyledon and intercotyledon with most allelic expression patterns showing somewhat monoallelic expression with leaky expression of the imprinted allele.

IGF2r allelic expression patterns and expression levels in day 75 cloned and control fetuses were examined and the expression pattern and levels was found to be Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F., Ogura, A. (2002). Faithful expression of highly variable in some individual clones as compared to the controls. For example, most of the organs were found not to be significant as a group, and the allelic expression data correlates well with this observation with most samples showing somewhat monoallelic expression which, also compares well with the data from Yang et al. (2005). However, the expression of *IGF2r* in the spleen was found to be significantly different when compared to the control group, which correlates well with the allelic expression data that shows two instances of nearly monoallelic, switched expression as well as most samples appearing biallelic instead of displaying the typical somewhat leaky expression.

Major organs such as the brain, heart, spleen and liver of prenatal fetal tissues

from controls also showed a typical somewhat leaky expression pattern and the expression patterns of *IGF2r* from spontaneously aborted cloned calves and calves that died shortly after birth were compared to these. The group of spontaneously aborted fetuses showed the highest level of variability in the expression pattern of *IGF2r* with many instances of monoallelic expression and allele switching. Cloned calves that died shortly after birth show relatively normal allelic expression patterns.

The noncoding *AIR* RNA is believed to specifically target silencing to the *IGF2r* promoter in mice therefore, the expression of *AIR* was checked for in our samples. We found that air was expressed in all of the organs of clones and controls.

Taking these data together it can be shown that proper allelic expression of *IGF2r* probably plays a greater role in terms of carrying a fetus completely through gestation, than it does in early embryonic lethality or in determining a successful outcome after birth.

5.4 Conclusion

Our observations conclude that the fidelity of allelic expression patterns and expression levels of imprinted genes also relates to normal fetal development. We suggest that proper *IGF2r* expression is necessary for survival to term, but is most likely not a cause of early fetal lethality or an indicator of postnatal fitness.

5.5 References

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Yang, L., Chavatte-Palmer, P., Kubota, C., O'Neill, M., Hoagland, T., Renard, J. P., Taneja, M., Yang, X., Tian, X. C. (2005). Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. **Mol. Reprod. Dev.** 71(4): 431-438.

CHAPTER VI

OVERALL CONCLUSIONS

A complete epigenome is important to normal development in mammals. Abnormalities that are found in clones have been thought to be caused by aberrant epigenetic mechanisms during the developmental period. Here, we have first explored several epigenetic characteristics during the early developmental stages of swamp buffalo embryos and during the developmental period of cloned cattle.

Cloned embryos display heterogeneous global DNA methylation. A global demethylation wave occurs at the earliest embryonic stages after fertilization, as is found in other species. Cloned swamp buffalo embryos display abnormal demethylation patterns associated with higher mRNA levels of *DNMT1, 3A* and *3B*. Re-methylation occurs at the 8-cell to morula stage and a higher level of *de novo* DNA methylation, as represented by *DNMT3A* and *3B,* was identified in clones.

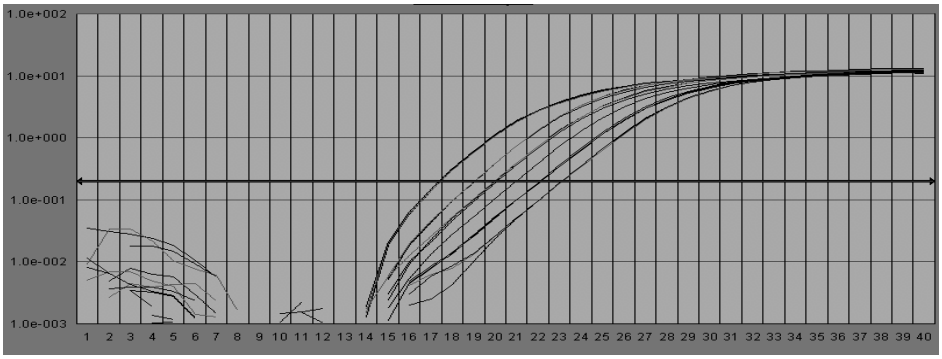
An abnormal correlation pattern between the global DNA methylation and histone acetylation exhibits in cloned swamp buffalo embryonic development and may be the cause of abnormal chromatin remodeling and developmental failure during embryonic stages.

In the gene imprinting study, abnormal allelic patterns were expressed in every cloned group but the severest abnormalities occurred in spontaneously aborted clone's samples. *IGF2R* expression is necessary for survival to term, but is most likely not a cause of early fetal lethality or an indicator of postnatal fitness.

APPENDIX

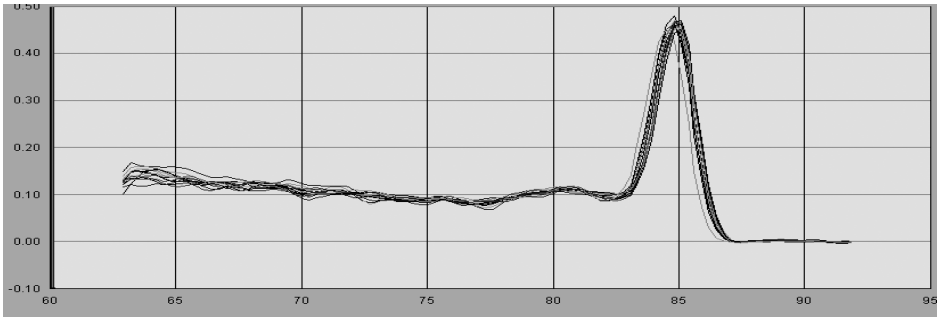
Figure 1. A representative validation curve of real time RT-PCR for *DNMT1* gene.

(A) The real time analysis of 6 triplicate concentrations of cDNA using *DNMT1* primer.



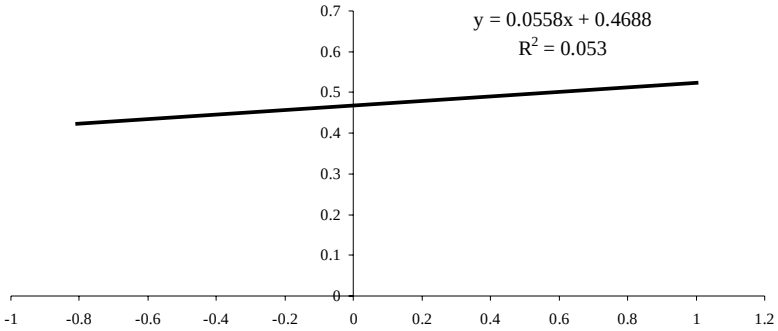
Cycle number

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



Temperature

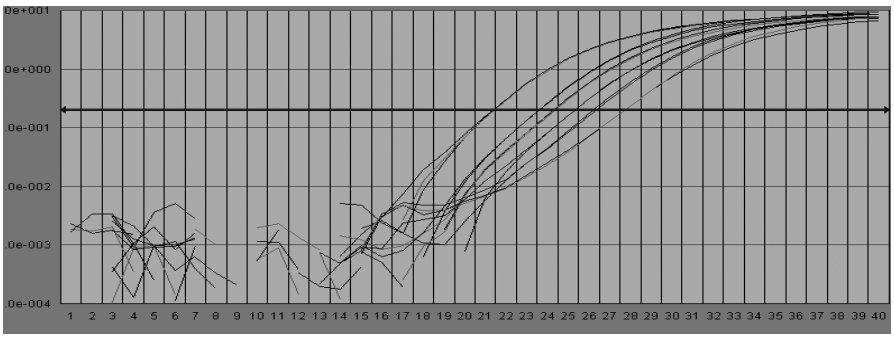
(C) The linear regression curve and the equation for *DNMT1* primer validation



Log10 of cDNA concentration

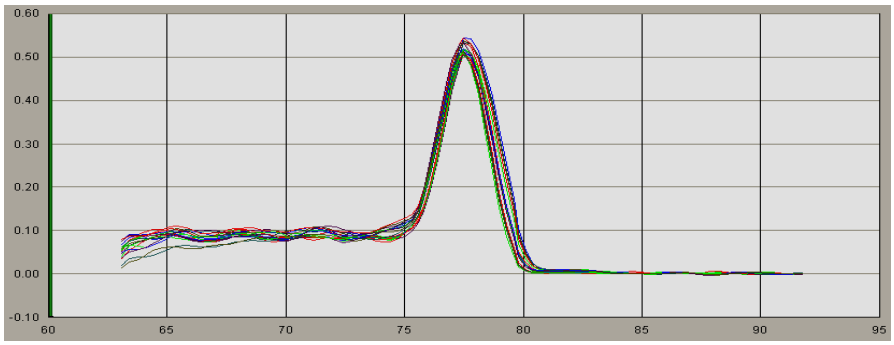
Figure 2. A representative validation curve of real time RT-PCR for *DNMT3A* gene.

(A) The real time analysis of 6 triplicate concentrations of cDNA using *DNMT3A* primer.



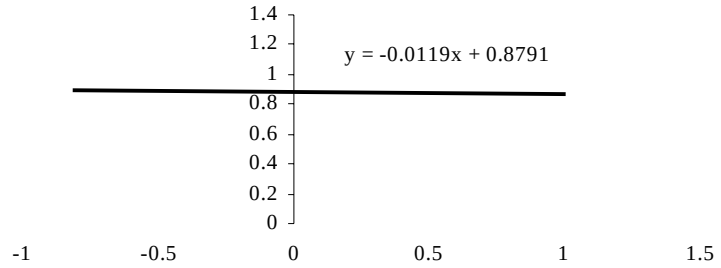
Cycle number

(B) The dissociation curve of PCR products of *DNMT3A*



Temperature

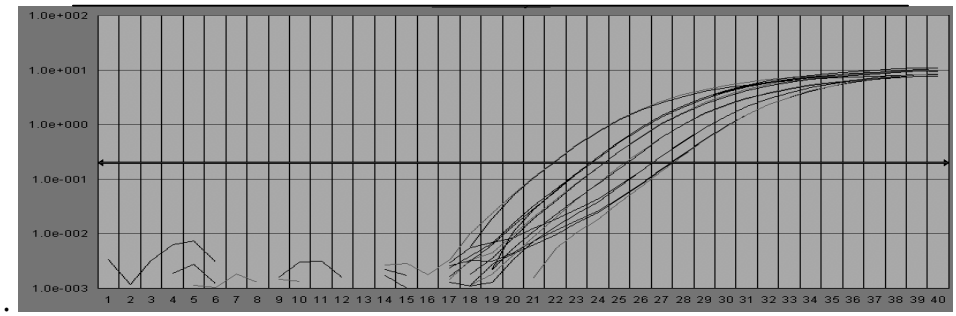
(C) The linear regression curve and the equation for *DNMT3A* primer validation



Log10 of cDNA concentration

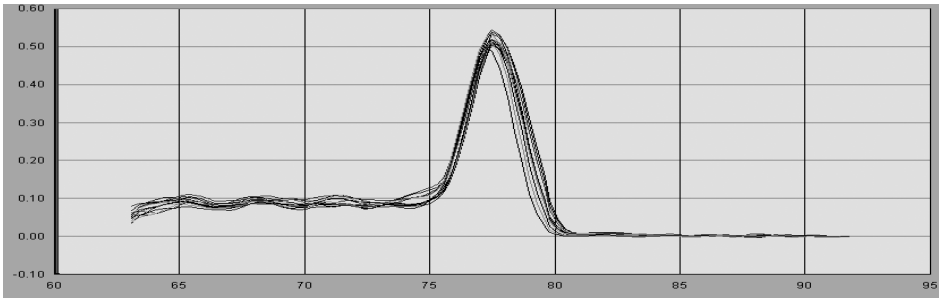
Figure 3. A representative validation curve of real time RT-PCR for *DNMT3B* gene.

(A) The real time analysis of 6 triplicate concentrations of cDNA using *DNMT3B* primer



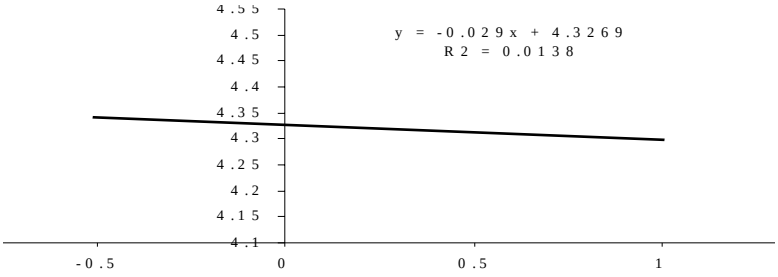
Cycle number

(B) The dissociation curve of PCR products of *DNMT3B* gene.



Temperature

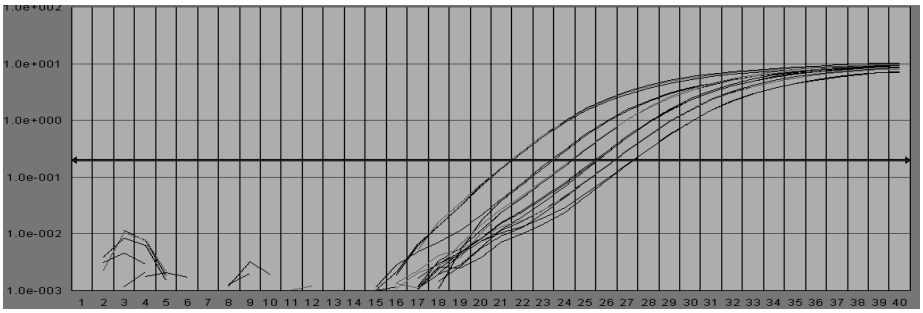
(C) The linear regression curve and the equation for *DNMT3B* primer validation



Log10 of cDNA concentration

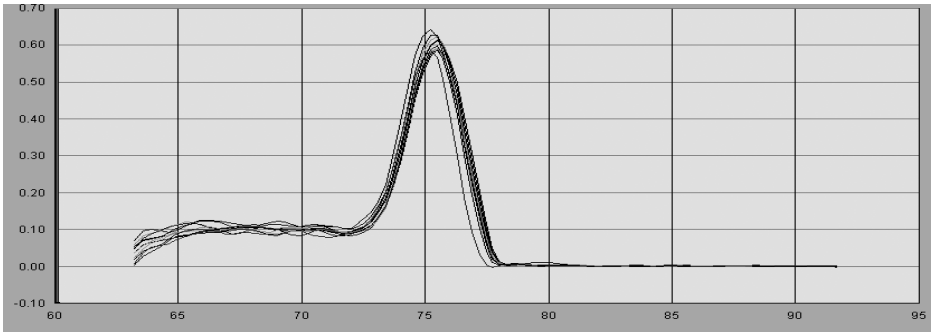
Figure 4. A representative validation curve of real time RT-PCR for *HAT1*gene.

(A) The real time analysis of 6 triplicate concentrations of cDNA using *HAT1*primer.



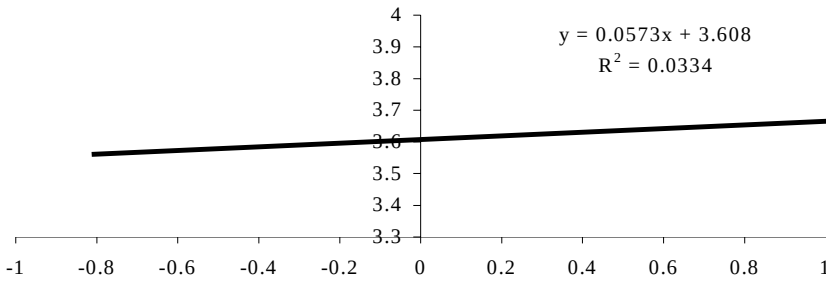
Cycle number

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



Temperature

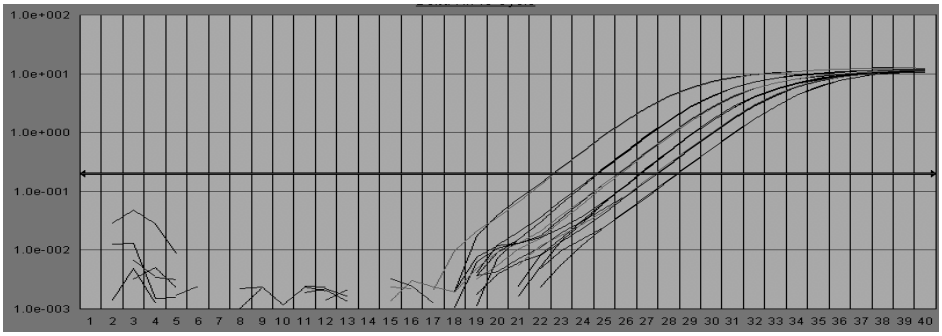
(C) The linear regression curve and the equation for *HAT1* primer validation



Log10 of cDNA concentration

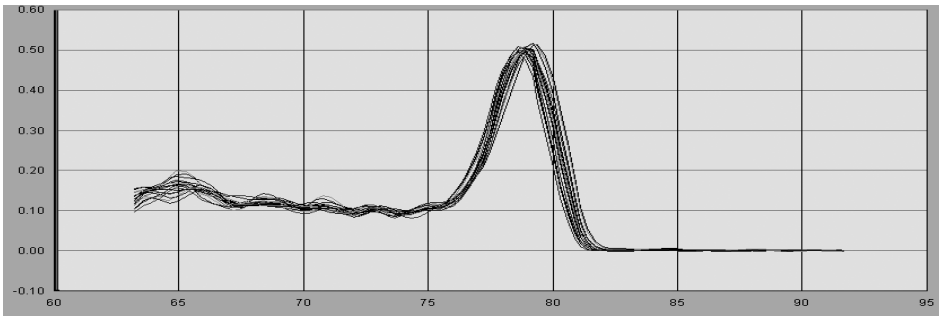
Figure 5. A representative validation curve of real time RT-PCR for *HDAC1* gene.

(A) The real time analysis of 6 triplicate concentrations of cDNA using *HDAC1* primer.



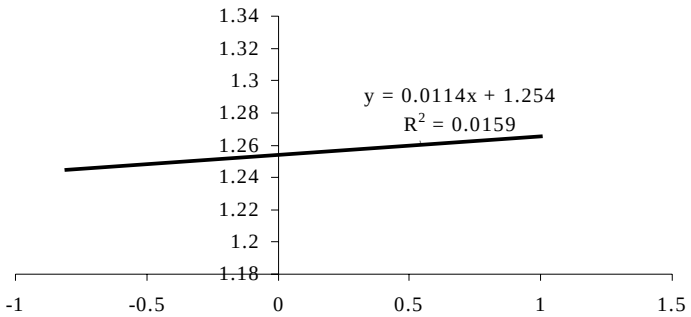
Cycle number

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



Temperature

(C) The linear regression curve and the equation for *HDAC1* primer validation



Log10 of cDNA concentration

Figure 6. The relation of chromatin remodeling gene expression patterns of cloned and IVF swamp buffalo oocytes and developmental stage embryos.

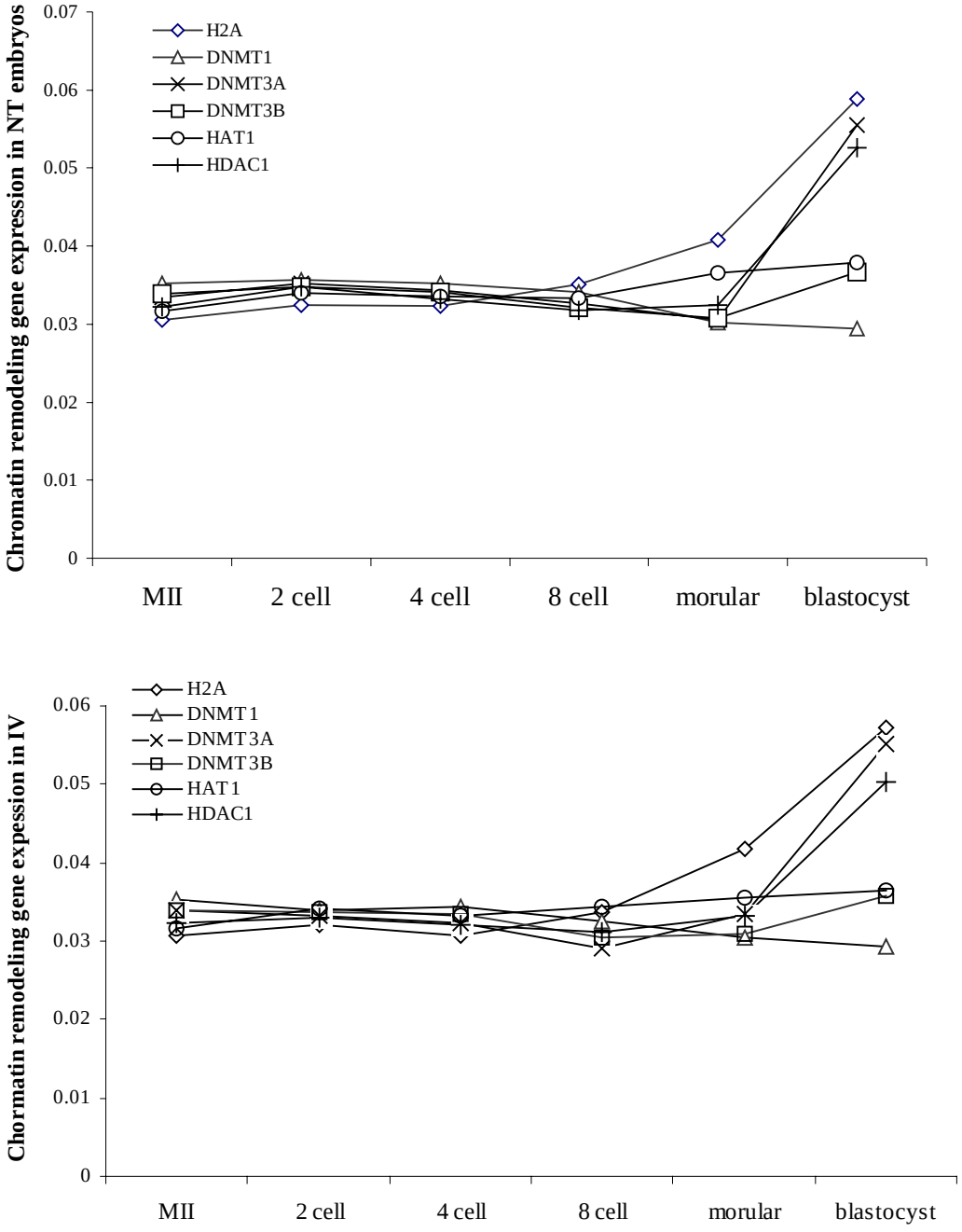
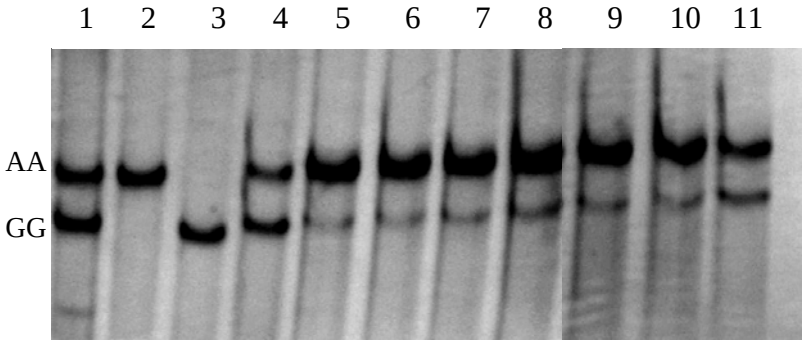


Figure 7. SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2R* gene.



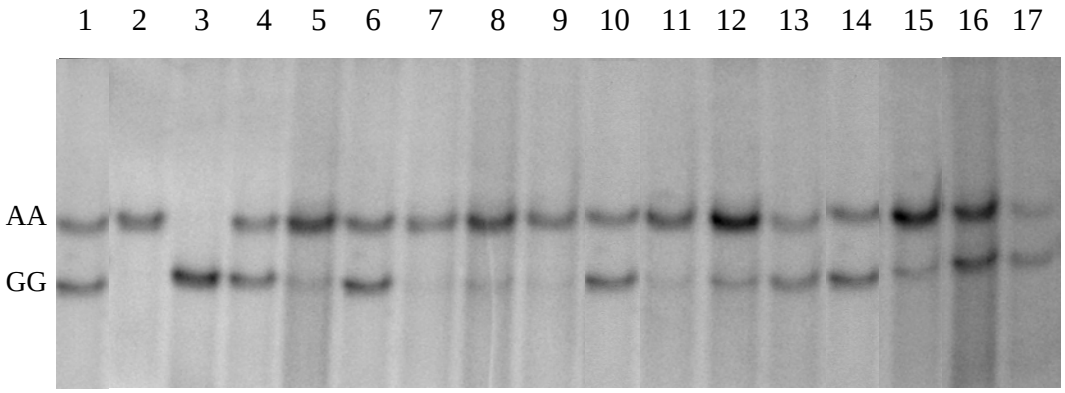
Lanes 1-3 represents a heterozygous G/A, homozygous AA and GG genotype, respectively.

Lane 4 represents the genotype from the donor cell DNA.

Lane 5 represents the allelic expression pattern derived from donor cell fibroblasts.

Lanes 6-11 show the allelic expression patterns in the cotyledon, heart, kidney, liver, placenta and brain, respectively, which were found by studying control animal.

Figure 8. Results of SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2r* gene in living cloned animals.



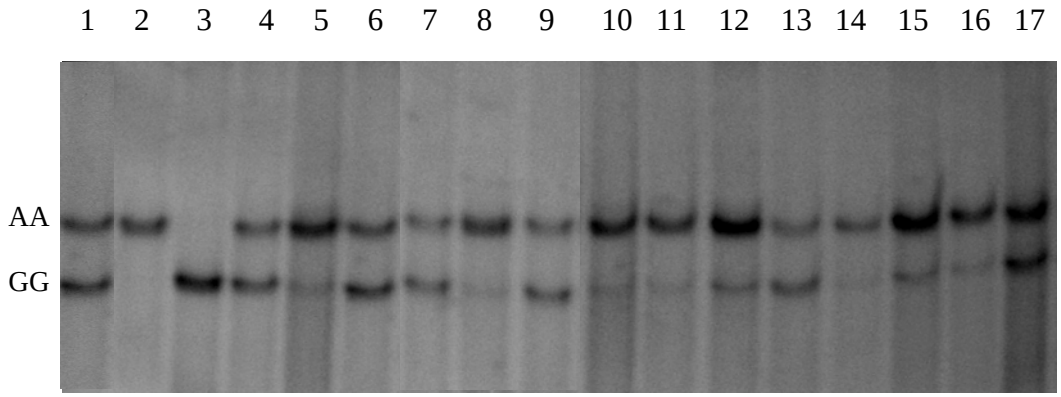
Lanes 1-3 represents a heterozygous G/A, homozygous AA and GG genotype, respectively.

Lane 4 represents the genotype from the donor cell DNA.

Lane 5 represents the allelic expression pattern derived from donor cell fibroblasts.

Lanes 6-7, 8-9, 10-11, 12-13, 14-15 and 16-17 show the different aberrant allelic expression patterns in the cotyledon and placenta, which were found by studying living clones number 1-6, respectively.

Figure 9. Results of SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2r* gene in dead after birth cloned animal



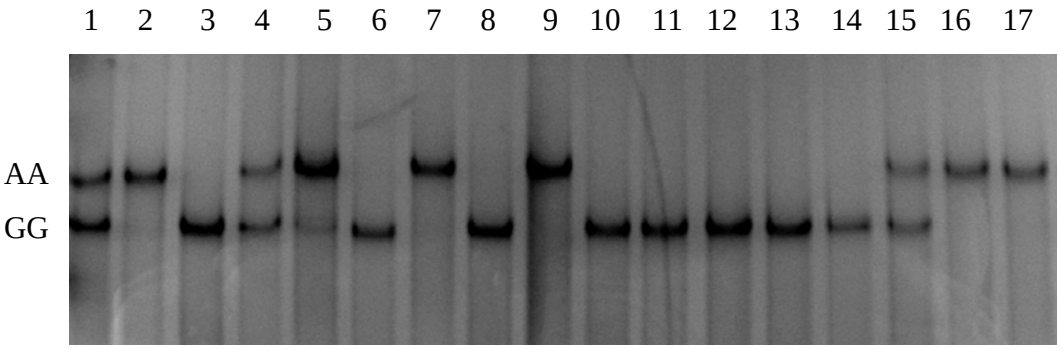
Lanes 1-3 represent the heterozygous G/A, homozygous AA and GG genotype, respectively.

Lane 4 represents the genotype from the donor cell DNA.

Lane 5 represents the allelic expression pattern derived from donor cell fibroblasts.

Lanes 6-11 and 12-17 show the different aberrant allelic expression patterns in the cotyledon, heart, kidney, liver, placenta and brain, respectively, which were found by studying dead after birth cloned animals.

Figure 10. Results of SSCP genotyping and allelic expression of a SNP in the 3'UTR of *IGF2r* gene in aborted cloned fetuses



Lanes 1-3 represents a heterozygous G/A, homozygous AA and GG genotype, respectively.

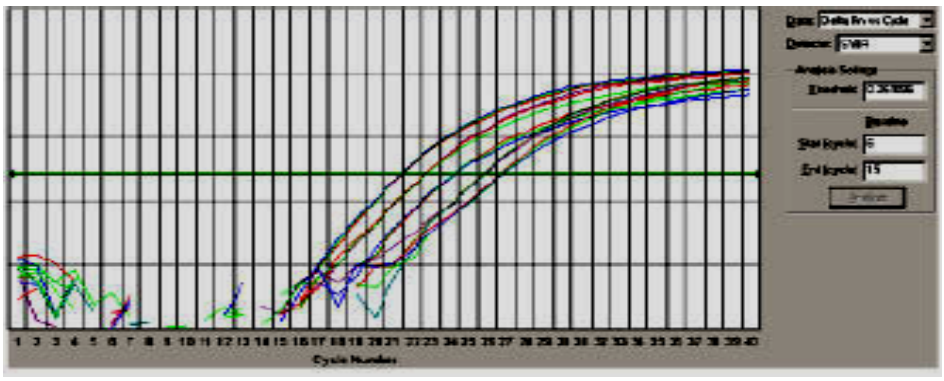
Lane 4 represents the genotype from the donor cell DNA.

Lane 5 represents the allelic expression pattern derived from donor cell fibroblast.

Lanes 6-11 and 12-17 show the different aberrant allelic expression patterns in the cotyledon, heart, kidney, liver, placenta and brain, respectively, which were found by studying aborted cloned fetuses.

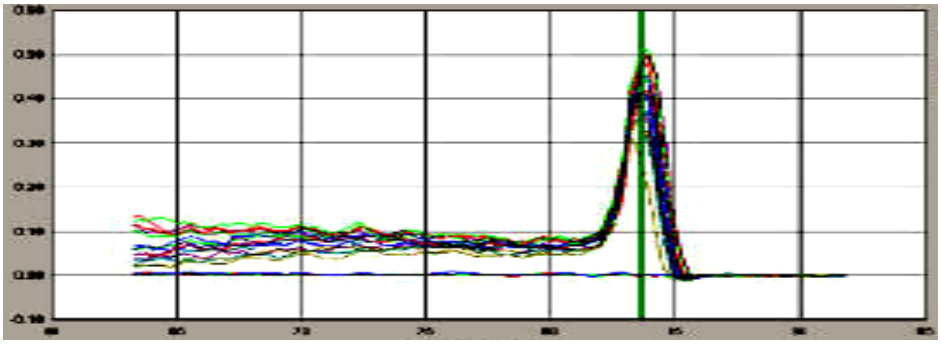
Figure 11. A representative validation curve of real time RT-PCR for *IGR2r* gene (Yang et al., 2005).

(A) The real time analysis of 6 triplicate concentrations of cDNA using *IGR2r* primer.



Cycle number

(B) The dissociation curve of PCR products of *IGR2r* gene.



Temperature

(C) The linear regression curve and the equation for *IGR2r* primer validation

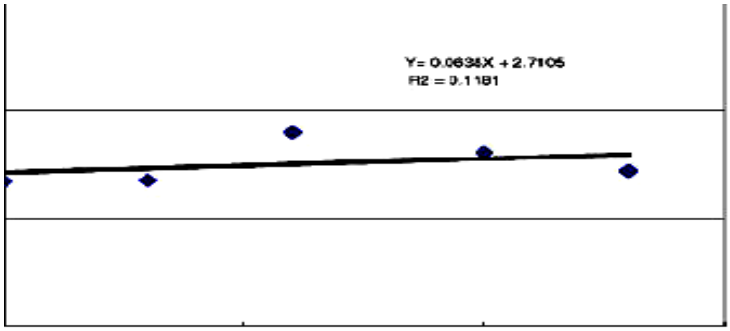


Table 1. The fold dilution of single cloned and IVF swamp buffalo oocytes and embryos used in each stage, for real time PCR analysis

Gene / Stages	H2A	DNMT1	DNMT3A	DNMT3B	HAT1	HDAC1
Metaphase II	2	6	6	6	6	6
2 cell	2	6	6	6	6	6
4 cell	2.5	5	5	5	5	5
8 cell	8	8	2	4	8	4
Morula	10	2.5	5	2.5	5	10
Blastocyst	32	-	32	8	8	32

Reference :

Yang, L., Chavatte-Palmer, P., Kubota, C., O'Neill, M., Hoagland, T., Renard, J., Taneja, M., Yang, X., Tian, X. (2005). Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. **Mol Reprod Dev.** 71: 431-438.

BIBLIOGRAPHY

Miss Tatsanee Suteevun was born on May 21, 1972 in Bangkok, Thailand. She graduated with the Bachelor's degree in Medical Technology, Mahidol University, Bangkok, Thailand in 1993. She was Medical Technologist at Siriraj Hospital, Bangkok in 1993-1995. In 1996, she started work at the Medical Scientist (embryologist) position in Infertility unit, Department of Obstetrics and Gynecology, Siriraj Hospital and she received the Royal Siriraj scholarship from faculty of Medicine, Siriraj Hospital to study the Master in Medical Biochemistry at the Department of Biochemistry, Faculty of Medicine, Mahidol University during 1997-2000. I studied at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand and received a scholarship from the Royal Golden Jubilee (RGJ) grant of the Thailand Research Fund in 2002. She had oral presentation in the Thailand Medical Congress in topic of "C677T mutation in Thai CAD patients" in July, 2002 and the 6th Siriraj Medical Congress in topic the "DNA methylation: a cause of abnormal development in cloned embryos" two years later.