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Functional analysis of bovine DNMT1 during bovine embryo development and its association with Bull fertility traits

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Parinya Wilaiphan

aus

Chachoengsao, Thailand

Referent:	Prof. Dr. K. Schellander
Korreferent:	Prof. Dr. B. Petersen
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Dedicated to my parents

Funktionelle Analyse des bovinen DNMT1 während der embryonalen Entwicklung und seine Assoziation mit der Fruchtbarkeit von Bullen

Diese Studie wurde durchgeführt, um den repressiven und hemmenden Einfluss von DNMT1 (DNA methyltransferase 1) auf Merkmale der Bullenfruchtbarkeit und der embryonalen Entwicklung zu untersuchen. Im ersten Untersuchungsschritt wurden *invitro* erzeugte Zygoten zufällig in vier Gruppen aufgeteilt. Diese wurden mit drei unterschiedlichen Injektionen behandelt: der Injektion (a) mit Smartpool siRNA (SpsiRNA), (b) mit 5 aza-2'-deoxycytidine (5-AZA) und (c) mit Nuklease freiem Wasser. Gruppe 4 verblieb als unbehandelte Kontrolle bestehen. Das Verhältnis der unterschiedlichen Entwicklungsstadien der Embryonen wurde 48 und 72 hr post Mikroinjektion (pmi) erfasst, wohingegen die Rate der Blastocysten 8 Tage pmi aufgezeichnet wurde. Im zweiten Abschnitt dieser Forschungsarbeit wurde der Einfluss der SNP von DNMT1, DNMT3a und DNMT3b in zwei unterschiedlichen Merkmals-komplexen geprüft. Zum einen wurde die Fruchtbarkeit von Bullen an Hand der Parameter Non-Return-Rate (NNR), Spermienqualität, sowie Plasma Membran Integrität (PMI), Akrosomen Integrität (PAS) und DNA Integrität (DFI) untersucht. Des Weiteren standen Merkmale der Embryonalentwicklung im Mittelpunkt. Zu diesem Zweck wurden die DNA von 310 Spermienproben von Bullen und 350 Embryonen an den entsprechenden Genorten genotypisiert. Die Anzahl der sich um 8-Zell-Stadium befindlichen Embryonen 72 hr nach pmi war geringer in den Gruppen die mit SpsiRNA und 5-AZA injiziert wurden. Die geringste Blastocystenrate wurde in der mit 5-AZA behandelten Gruppe beobachtet. Mikroinjektion von SpsiRNA bewirkte eine Reduktion der Target mRNA in Blastocysten und 8-Zell-Embryonen. Die Mikroinjektion von SpsiRNA und 5-AZA steigerten die Expression von IGF2. Die Varianzanalyse wies eine Assoziation des SNP in DNMT1 mit NRR und PAS vor, während DNMT3a und DNMT3b einen signifikanten Einfluss auf NNR und Spermienmotilität hatten. Zusätzlich zeigte eine kombinierte Genort Varianzanalyse von DNMT1 x DNMT3a x DNMT3b einen signifikanten Effekt auf NNR, Spermienmotilität und Überlebenfähigkeit nach dem Auftauen. Das Gen DNMT1 spielt eine entscheidende Rolle in der bovinen Preimplantation und es lässt sich mit Merkmalen der Bullenfruchtbarkeit und embryonalen Entwicklung assoziieren. Dies könnte ein Hinweis auf einen nützlichen, genetischen Marker zur Verbesserung der Merkmale sein, der durch weitere unabhängige Studien bewiesen werden kann.

Functional analysis of bovine DNMT1 during bovine embryo development and its association with bull fertility traits

This study was conducted to investigate the effects of suppressing and inhibiting DNMT1 on the embryonic development and bull fertility traits. In the first approach, *in vitro* produced zygotes were assigned randomly into four groups namely: those injected with Smartpool siRNA (SpsiRNA), 5aza-2'-deoxycytidine (5-AZA), nuclease free water and non-injected control. The proportions of different stages of embryos were assessed 48 and 72 hr post microinjection (pmi) while blastocyst rate was assessed at day 8 pmi. A second objective was to identify the effects of SNPs in DNMT1, DNMT3a and DNMT3b on bull fertility traits namely: non-return rate (NRR), sperm quality traits namely: sperm volume per ejaculate, sperm concentration, sperm motility, survivability after thawing, and sperm flow cytometric parameter namely: positive acrosome status (PAS), plasma membrane integrity (PMI) and DNA fragmentation index (DFI): and embryonic development in terms of time at first cleavage, late cleavage and blastocyst. For this, 310 breeding bull sperms obtained station and 350 embryos were genotyped at those loci using DNA samples. The proportions of the 8-cell embryos were lower in SpsiRNA and 5-AZA injected groups. The lowest total blastocyst rate was observed in 5-AZA treatment group. Microinjection of SpsiRNA has reduced the target mRNA by 80 and 50% in 8-cell and blastocyst stage embryos. Lower protein expression was also observed at 8-cell stage in embryos that were injected with SpsiRNA. The highest apoptotic index was found in SpsiRNA and 5-AZA injected groups. The microinjection of SpsiRNA and 5-AZA has increased the expression of IGF2 by 1.67 and 1.55 times. Analysis of variance revealed association of SNP of DNMT1 with NRR and PAS, while DNMT3a and DNMT3b were found to be associated with NRR as well as sperm motility. In addition, combined loci analysis of variance among DNMT1 x DNMT3a x DNMT3b showed significant association with NRR, sperm motility and survivability after thawing. SNP of DNMT1 gene was significant correlated with embryonic development. In conclusion, this gene evidently plays a critical role in bovine preimplantation and associates with bull fertility traits and embryonic development. Following validation of this result in an independent population, there is a great potential to use these loci as markers of fertility to enhance embryonic development.

List of abbreviations

5-AZA	5-aza-2'-deoxycytidine
AI	Artificial insemination
AP	Alkaline phosphatase
API	Apoptotic index
APS	Ammonium peroxydisulfate
ATP	Adenosine triphosphate
BMP-15	Bone morphogenetic protein 15
BSA	Bovine serum albumin
cDNA	Complementary DNA
CG	Cytosine-Guanine
CpG	Cytosine-phosphate-guanine
C. elegans	Caenorhabditis elegans
CO_2	Carbondioxide
COC	Cumulus oocyte complex
CONC	Sperm concentration
cont.	Continue
C_T	Threshold cycle
CTCF	CCCTC-binding factor
DEPC	Diethyl pyrocarbonate
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DNMT2	DNA methyltransferase 2
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
DNMT3L	DNA methyltransferase 3L
dNTP	Deoxyribonucleotide
DMRS	Different methylation regions
dsRNA	Double strand RNA
DTCS	Dye Terminator Cycle Sequencing
DTT	Dithiothreitol

eCG	Equine chronic gonadotropin
E. coli	Escherichia coli
EDTA	Ethylenediaminetraacetic acid
EGA	Embryonic genome activation
ES	Embryonic stem
ExoSAP	Exonuclease I and Shrimp Alkaline Phosphatase
EST	Expressed sequence tag
FITC	Fluorescein isothyocyanate
FSH	Follicle stimulating hormone
GAPDH	Glycerealdehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
GLM	General Linear Model
GVBD	Germinal vesicle breakdown
hr	Hour
hCG	Human chorionic gonadotropin
hpi	Hours post insemination
HRP	Horseradish peroxidise
ICE	Imprinted control element
ICM	Inner cell mass
ICR	Imprinted control region
IGFs	Insulin-like growth factors
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBP-4	Insulin-like growth factor binding protein 4
IPTG	Isopropyl β-D-thiogalectosidase
Itpr1	Inositol 1,4,5-triphosphate receptor 1
IVC	In vitro culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
IU	International unit
kDa	Kilo dalton
LH	Luteinizing hormone

LOS	Large offspring syndrome
LSM	least square means
M	Molar
MII	Second meiosis
mA	Milliampere
min	Minute
miRNA	Micro RNA
mm	Millimeter
mM	Mili mole
M phase	Meta phase
MOT	Sperm motility
MPM	Modified parker medium
mRNA	Messenger RNA
MW	Molecular weight
MZT	Maternal-zygotic transition
n	Number
nM	Nanomolar
NRR	Non-return rate
nt	Nucleotide
OAS	Oligoadenylates synthetase
OCS	Oestrus cow serum
OPN	Osteopontin
PAS	Positive acrosome status
PBS	Phosphate buffered saline
PMI	Plasma membrane integrity
PMP	Modified paker medium
PSA	Pisum sativum agglutinin
pmi	Post microinjection
PVP	Polyvinyl pyrolidone
qPCR	Quantitative real-time PCR
RdRP	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RISC	RNA induced silencing complex

RNA	Ribonucleic acid
RNAi	RNA interference
RNasin	Ribonuclease inhibitor
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
SAS	Statistical Analysis System
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SLS	Sample loading solution
SNP	Single nucleotide polymorphism
SNT	Somatic nuclear transfer
S phase	Synthesis phase
SpsiRNA	Smartpool siRNA
SUVR	Survivability after thawing
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TBST	Tris-buffered saline with Tween-20
TCM	Tissue culture medium
TE	Trophectoderm
TEMED	N, N, N', N'-Tetramethylendiamine
TPGS	Transcriptional gene silencing
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U	Enzyme unit
UTR	Untranslated region
VOL	Sperm volume per ejaculate
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3indolyl-β-D-galactopyranoside
ZGA	Zygotic gene activation

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1 Introduction

Fertility is a very complex trait and strongly influenced by environmental effects and partly by paternal and maternal genetics. The success outcome of insemination is due to the quality of semen and both the quality of egg and appropriate environment. In mammalian preimplantation, the embryonic development depends on DNA methylation which is crucially involved in controlling gene expression, cell differentiation, X-chromosome inactivation and imprinting (Bird 2002). DNA methyltransferase 1 (DNMT1) is a maintenance enzyme adding a methyl group at CpG dinucleotides of the newly synthesized strand in hemimethylated DNA after replication (Bestor 1992). DNMT3a and DNMT3b are a *de novo* methylation establishing DNA methylation during development (Hsieh 1999; Okano et al. 1999). Mutation analysis has shown that they are all essential genes regulating several imprinted gene expression in mice (Lei et al. 1996; Li et al. 1992; Okano et al. 1999). Depletion of DNMT1 also cause embryonic lethality and inappropriate gene expression in *Xenopus laevis* embryos (Stancheva and Meehan 2000).

Gene expression pattern have demonstrated altered expression of imprinted genes using *in vitro* culture and somatic cell nuclear transfer (sNT) procedures observed in embryos, fetuses, and placentas in mouse, sheep, and bovine (Bertolini et al. 2002; Blondin et al. 2000; Doherty et al. 2000; Wrenzycki et al. 2004; Young et al. 2001). The epigenetic specific changing in the pattern of methylation has been hypothesized (Wrenzycki et al. 2001). Methylation has long been known to be involved in printing the process by which certain alleles are expressed or silenced depending on the parental sex from which they are inherited (Sapienza 1990). Imprinted genes such as IGF2, IGF2R, H19 are preferentially involved in the control of embryonic, placental, fetal and neonatal growth in sheep and bovine (Young et al. 2001; Zhang et al. 2004). Aberrant expression patterns of imprinted genes have been implicated in embryonic and fetal abnormalities (Moore and Reik 1996). In human, imprinting deviations are responsible for conditions known as Beckwith-Weidmann syndrome (Robertson 2005), in bovine the large offspring syndrome (Young et al. 1998).

So far, the functional study of DNMT1, DNMT3a, and DNMT3b genes are limited in bovine. Suppression or inhibition of those genes reflecting phenotypic development of

embryo subsequently influencing on other imprinted gene expressions would be a baseline to understand loss of embryonic development. In addition, the silent mutation due to its exonic/intronic splicing of those genes which may effect the fertility traits recorded, such as non-return rate, sperm quality traits, sperm flow cytometric parameters, would be accountable to develop a potential marker for future selection.

The DNMT1 alone is sufficient to maintain the methylation of imprinted genes during preimplantation in mouse (Hirasawa et al. 2008). Suppression of DNMT1 has been documented to decrease cell survival and proliferation and increase apoptosis in human and mouse (Jackson-Grusby et al. 2001; Rhee et al. 2000). Inhibition of DNMT1 with 5-AZA results in lower cellular maintenance and methyltransferase activity, demethylation of global and gene-specific expression in human cell line (Robert et al. 2003) and inhibition of cell differentiation in mouse sperm (Mizukami et al. 2008; Raman and Narayan 1995). In other species, it halts embryonic development at early gastrula stage and subsequently induces apoptosis in *Xenopus* laevis embryos (Kaito et al. 2001).

The candidate genes developed as a marker for boar fertility and semen quality has been reported in swine (Huang et al. 2002; Lin et al. 2006c; Wimmers et al. 2005).

In spite of its crucial role in preimplantation embryonic development and fertility traits in other species, the biological function study during preimplantation embryonic development and its association with fertility traits of this gene have not been done in bovine.

Therefore, the objectives of this study were elucidated:-

1. Suppression and inhibition of DNMT1 and its effect on embryonic development, apoptosis and the expression of imprinted genes during bovine preimplantation stage embryos
2. To identify polymorphisms in the candidate genes DNMT1, DNMT3a, and DNMTT3b and their association with bull fertility traits and embryonic development of embryos

2 Literature review

Fertility is a very complex trait and strongly influenced by environmental effects and a part by genetics of males and females. The successful outcome of insemination is attributed to the service bull, the inseminated cow, and the embryos. The service bull provides the quality of semen and genes that are directly transmitted to the embryo. On the other hand, the cow provides the quality of egg and appropriate environment. Furthermore, the cow develops a direct genetic effect transmitting its genes to the embryo. With respect to the embryos, physiological studies reveal a high fertilization rate, that is, each insemination yields 90% of successful fertilization occurs, suggesting that the embryo plays an important role in the reproductive process through its own development (Miglior 1998). In addition, during preimplantation and postimplantation development, there are many genes analyzed involved in numerous biological processes including compaction/cavitation, metabolism, transcription/ translation, DNA methylation, stress (e.g., oxidative), growth factor/cytokine signalling, cell cycle regulation and apoptosis (Wrenzycki et al. 2004).

2.1 Mammalian preimplantation development

The preimplantation development in mammals is characterized by a highly dynamic process including phenomena such as oogenesis, oocyte maturation, fertilization and implantation of the embryo. The development of the fertilized zygote through several morphologic changes results in the storage of maternal mRNA and protein that support the early embryonic development (Nothias et al. 1995). Despite a number of zygotic and embryonic genes which are expressed in a stage-specific manner, this leads to genome activation of the embryo (Rodriguez-Zas et al. 2008). The start of zygote genome activation in mammals varies between 1- and 8-cell stage embryos, depending on the species (Telford et al. 1990). In porcine, the first synthesis of nucleolar RNA was observed at 4-cell stage (Anderson et al. 2001; Viuff et al. 2002). In mice, the first major wave of gene activation starts at the 2- to 4-cell stage, and the peak is reached at the 8-cell stage (Hamatani et al. 2004).

In bovine, the major genomic activation of zygotic gene occurs at 8-16 cell stage (Dean et al. 2001; Frei et al. 1989; Kopecny et al. 1989). However, a few studies have also reported

the minor genomic starts at an earlier stage of development before the 8-cell stage (Memili et al. 1998; Sirard et al. 2005). During the 8-16 cell stage, a set of genes is transited by turning off the maternal and turning on the zygotic translation called maternal-zygotic transition (MZT) (Sirard et al. 2005). This is absolutely essential for the processes of embryonic development and differentiation.

Preimplantation development is critical for the generation of the genomic methylation pattern. Reprogramming in early embryos occurs by active and passive mechanisms (Reik et al. 2001). During early cleavage, a genome is widely dropped in methylation to reset the genome where it can be reprogrammed to direct embryonic development. A *de novo* wave of methylation is then remethylating a new pattern during preimplantation or postimplantation depending on the species. These processes appear to be conserved across mammalian species and are essential for normal development (Dean et al. 2001; Okano et al. 1999; Reik et al. 2001). During the preimplantation stages, the reprogramming in the genome is controlled by epigenetics.

2.2 Epigenetics

Epigenetic refers to the different patterns of gene expression based on the biochemical properties without a change of the DNA sequence. Two known major mechanisms responsible for these specific properties are DNA methylation and post-translational histone modification (Bird and Wolffe 1999; Jenuwein and Allis 2001).

2.2.1 DNA methylation

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring occurring at CG dinucleotides (Larsen et al. 1992; Li et al. 1993). It is mainly within particular regions termed CpG islands which occupy up to 70% in the genome and represent one of the major epigenetic modifications in mammals (Li 2002; Robertson and Wolffe 2000). The active and inactive alleles are due to the differential DNA methylation in a critical regulatory region. These differentially methylated regions (DMRs) are essential for expression or repression. It is noted that a high number of imprinted genes are found to be methylated on the maternal allele (Reik and Walter 2001).

DNA methylation also plays an important role in imprinting, both in silencing genes as well as activating other genes (Reik and Walter 2001; Sleutels and Barlow 2002). X-chromosome inactivation also depends on methylation (Avner and Heard 2001). In cancer cell lines, gene repression is mediated by DNA methylation, where tumors are often globally hypomethylated but locally hypermethylated, especially in tumor-suppressor genes (Bird and Wolffe 1999; Jones and Takai 2001; Rountree et al. 2001).

2.2.2 Histone modifications

Histone modification refers to the addition or removal of phosphate, acetyl and/or methyl groups to the histone proteins that form the nucleosome composing of histone H2A, H2B, H3 and H4. These modifications are dynamic during development, vary among different tissues, and interact with other epigenetic control systems such as DNA methylation (Jenuwein and Allis 2001; Richards and Elgin 2002; Turner 2000). Acetylation of various amino acid residues of histones H3 and H4 is generally associated with an active chromatin configuration and expressed genes mainly found in euchromatin area. In contrast, histone methylation is generally associated with condensed or heterochromatic chromatin and results in gene repression (Richards and Elgin 2002).

Post-translation histone modification refers to the addition or removal of phosphate, acetyl and/or methyl groups to the histone protein in DNA package. The modification results in transcription or repression of a specific gene based on whether it lies in an open (acetylated) or closed (phosphorylated, methylated or unmethylated) conformation (Jenuwein and Allis 2001).

Thus, modifications of either DNA methylation or histone status which result in a differential gene expression refer to genetic reprogramming. In addition, pattern of DNA methylation and affect of histone status have been implicated in transcriptional regulation both in a global and gene specific manner, X-chromosome inactivation, genomic imprinting, as a mechanism for controlling cell differentiation (Reik and Walter 2001).

2.3 Genes involved in DNA methylation and genomic imprinting

In mammals, methylation patterns are established and maintained by several DNA methyltransferases subsequent to DNA replication within DMRs of imprinted genes.

2.3.1 DNA methyltransferases (DNMTs) enzyme family

The DNMT enzyme family is involved in the production and modulation dynamics of the global genomic methylation pattern in mammalian embryo development (Bestor 2000). It has been identified and grouped into three families: DNA methyltransferase 1 (DNMT1), DNA methyltransferase 2 (DNMT2) and DNA methyltransferase (DNMT3) (Bestor 2000; Li 2002). All DNMTs have a similar molecular structure and most of them contain ten specific sequence motifs within the C-terminal catalytic domain, six of which are highly conserved (Lauster et al. 1989).

DNMT1 is the maintenance enzyme responsible for methylation of hemimethylated CG dinucleotides after DNA replication (Bestor 1992). DNMT3a and DNMT3b are required for *de novo* methylation to establish new DNA methylation patterns during embryonic development (Hsieh 1999; Okano et al. 1999). In addition, DNMT3L by itself has no methylation activity, colonizes with DNMT3a and DNMT3b and is thought to be essential for establishing methylation (Bourc'his et al. 2001; Hata et al. 2002)

DNMT1 is the first member of the DNMTs family identified and shows its most abundant activities in mammalian cells (Robertson et al. 1999). DNMT1 maintains the methylation status during or after replication by copying the methylation patterns from the parental to the newly synthesized strand (Li et al. 1992; Pradhan et al. 1999). Additionally, DNMT1 is also involved in certain types of *de novo* methylation activity as seen in embryo lysate (Yoder et al. 1997b). This enzyme is about 1620 amino acids long. The first 1100 amino acids constitute the regulatory domain or N-terminus region of the enzyme. The remaining residues contain the catalytic domain or C-terminus. The domains are joined by Glycine-Lysine repeats (Pradhan et al. 1999). The DNMT1 protein interaction with several cellular proteins plays an important role in methylation mechanism such as *De novo* DNA methyltransferase (Kim et al. 2002), histone deacetylase (HDAC1/2) (Fuks et al. 2000; Rountree et al. 2000), methyl CpG binding

(MeCP2) (Kimura and Shiota 2003), and transcription/RNA processing (Carty and Greenleaf 2002).

DNMT1 has several isoforms, including an alternative splice variant known as DNMT1 oocyte specific isoforms (DNMT1o), DNMT1 somatic isoforms (DNMT1s), and DNMT1 sperm isoforms (DNMT1p) (Mertineit et al. 1998). The DNMT1o introduces an oocyte-specific 5'-exon (exon 1o) start position at the ATG codon in exon 4, which makes an N-terminal part 118 amino acids shorter than the somatic isoforms (Bestor 2000). DNMT1o expression is restricted during the oocyte and early preimplantation development (Howell et al. 2001; Mertineit et al. 1998; Ratnam et al. 2002). The DNMT1o is highly accumulated in the nucleus only during at earliest stages of oocyte growth, and it becomes localized in the cytoplasma within the oocyte cortex (Carlson et al. 1992). Subsequently, the DNMT1o protein is cytoplasmic at the beginning of oocyte growth and during 1-cell to 4-cell stage, but it specifically enters and then exits the nuclei at the 8-cell stages (Carlson et al. 1992; Mertineit et al. 1998). The DNMT1o protein does not become fully nuclear until the implantation is done, when it is replaced by the full-length somatic isoforms (Bestor 2000).

The promoter and the exon (exon 1s) of DNMT1s are active in all somatic cells and they are working as the housekeeping promoter (Yoder et al. 1997a). The protein contains 1620 amino acids (Mertineit et al. 1998). The promoter of DNMT1s is activated shortly after implantation and at the post-insemination Day 7 in mice (Mertineit et al. 1998). In bovine, the DNMT1s mRNA isoforms were found to be expressed throughout the preimplantation stages (Golding and Westhusin 2003).

The promoter and the exon (exon 1p) is only active with high amount of mRNA transcription in the pachytene spermatocyte but the translation of this gene does not happen (Mertineit et al. 1998).

Recently, a splice variant of DNMT1s, called DNMT1b was identified. It is truncated at the 5' end lacking 887 nucleotides (DNMT1, position 1 to 887 Genebank accession number AY173048). The truncation extends until exon 12, which lacking 15 nt at its 5'

end. The function of DNMT1b has not yet been studied but it might be necessary for maintenance and *de novo* methyltransferase activity (Russell and Betts 2008).

The architecture of the DNMT3 enzyme family is similar to DNMT1 with a large amino terminal regulatory region attached to a catalytic domain. The DNMT3a protein binds to RP58, a DNA-binding transcriptional repressor, leading to methylation independent repression of the gene (Fuks et al. 2001). It further binds to HDAC1, leading to a histone deacetylase mediated gene silencing (Pradhan and Esteve 2003). DNMT3a and DNMT3b consist of a large regulatory N-terminal domain and a smaller catalytic C-terminal domain, but no intra-molecular interaction was found between the two domains (Margot et al. 2003). The isolated C-terminal domain remains capable of methylating DNA (Gowher and Jeltsch 2002). DNMT3a and DNMT3b are predominantly active in *de novo* methylation processes during embryonic development and they are involved in the establishment of maternal and paternal imprinting (Hata et al. 2002; Kaneda et al. 2004; Santos et al. 2002).

DNMT3a is predominantly localized in retroviral sequences, major satellite repeats, IAP repeats, non-imprinted genes as well as paternally imprinted genes, and the *X*ist gene on the X-chromosome (Chen et al. 2003). Knock out of DNMT3a maternal or paternal alleles leads to embryonic motility and impaired spermatogenesis (Kaneda et al. 2004).

Mutant DNMT3b has no effect on phenotype after crossing with wild-type female mice (Kaneda et al. 2004). In human, deletion of DNMT3b causes immunodeficiency and chromosome instability disease (ICF syndrome) (Okano et al. 1999; Xu et al. 1999).

2.3.2 Genomic reprogramming

The change in the paternal and maternal genome after fertilization is a critical stage for the methylation pattern in mammals. The DMRs within imprinted genes and methylation pattern of the entire genome are parts of the reprogramming process (Reik et al. 2001).

2.3.2.1 Demethylation

The methylation patterns of the paternal genome are active demethylation in the embryo after fertilization (Dean et al. 2003; Mayer et al. 2000). The timing of the onset of active demethylation is conserved among species. In mouse, rat, pig and human, the male pronucleus is demethylated shortly after fertilization, while demethylation was observed only at the blastocyst stage in sheep (Beaujean et al. 2004). In bovine, the genomic methylation is further reduced from the beginning of the early cleavage until the 8-cell stage (Dean et al. 2001) (Figure: 1). On the other hand, the maternal genome is passively demethylated due to the absence of maintenance DNMT1.

It was proposed that passive demethylation after fertilization occurs because the oocyte specific DNMT1o is excluded from the nucleus (Cardoso and Leonhardt 1999; Carlson et al. 1992; Howell et al. 2001). The DNMT1o is supposed to play an important role to maintain the imprinting at 8-cell stage (Howell et al. 2001; Ratnam et al. 2002).

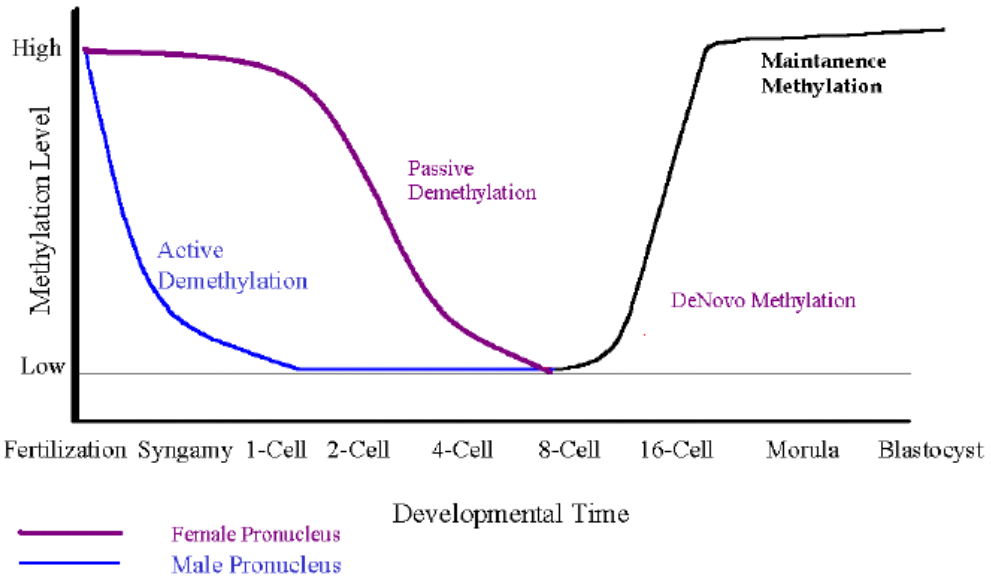


Figure 1: The diagram shows the timing of the epigenetic alternations in the male and female genome during the bovine preimplantation development

2.3.2.2 Remethylation

The genome starts remethylation by *de novo* methylation from 8-16-cell stage or blastocyst stage in bovine or mouse onward, respectively (Dean et al. 2001; Santos et al. 2002), which is presumably catalyzed by methyltransferase of the DNMT3 family (Reik et al. 2001). Thus, the highest methylation level takes place at the blastocyst stage when the first two cell lineages are differentiated: the inner cell mass (ICM) and the trophectoderm (TE). The ICM cells are hypermethylated while TE cells are hypomethylated. These differences are reflected later during the development (Reik et al. 2001; Reik et al. 2003; Santos et al. 2002).

There is little known about the function of bovine DNMT family and its isoforms on methylation pattern during in preimplantation development. Up to now, Hirasawa and colleagues demonstrated that DNMT1 alone is sufficient to maintain the methylation of imprinted genes during cleavage. The DNMT3a and DNMT3b are not required for the maintenance of the imprints in mouse preimplantation embryos (Hirasawa et al. 2008).

2.3.3 DNMTs expression

During mammalian preimplantation development, specific DNA methyltransferase mRNAs have been identified as shown in table 1. In bovine, DNMT1s and DNMT1b are expressed throughout all stages of preimplantation, whereas DNMT3a and DNMT3b are expressed from the at 8-16 cell stages onwards. The DNMT1o is not expressed during in preimplantation. In mouse, DNMT1o (except at 4-cell stage and 8-16 cell stage), DNMT1s and DNMT3a are expressed throughout the preimplantation stages whereas DNMT3b is only expressed at the blastocyst stage.

2.4 Regulation of gene expression

There are many mechanisms and chemicals regulating gene expression as well as embryo production protocol.

2.4.1 RNA interference and micro RNA

RNA is more profound and plays a complex role during regulating of the gene expression. The regulation is through RNA-associated silencing that can be transcriptional in nature,

Table 1: DNA methyltransferases (DNMTs) mRNA expression during preimplantation

Expression		Zygote	2-cell	4-cell	8-16 cell	Morula	Blastocyst	Ref.
Bovine	DNMT1o	-	-	-	-	-	-	1
	DNMT1s	+	+	+	+	+	+	1
	DNMT3a	-	-	-	+	+	+	1
	DNMT3b	-	-	-	+	+	+	1
	DNMT1b	+	+	+	+	+	+	2
Mouse	DNMT1o	+	+	-	-	+	+	3,4
	DNMT1s	+	+	+	+	+	+	3,4
	DNMT3a	+	+	+	+	+	+	3,4
	DNMT3b	-	-	-	-	-	+	3,4

Remark: 1/Golding and Westhusin 2003; 2/Russell and Betts 2008; 3/ Ratnam et al., 2002; 4/Ko et al., 2005

and is operable through an RNA interference (RNAi). This mechanism is mediated by small-interfering (siRNA) RNA (Kawasaki et al. 2005). Double-strand RNA (dsRNA)-induced post-transcriptional gene silencing (PTGS) is known as RNAi in animals (Fire et al. 1998). In this system, dsRNA is processed to 21-25 bp nucleotides long by RNase III Dicer (Elbashir et al. 2001). These siRNA are incorporated to the RNAi-induced silencing complex (RISC) where they then promote degradation of sequence-specific mRNAs mediated by Argonaute 2 (Ago 2) in the cytoplasm of cells (Hammond et al. 2000; Liu et al. 2004; Tuschl et al. 1999).

Silencing mechanisms by microRNA (miRNA) at the level of translation are operated by targeting of partially complementary sequences which are located within the 3' untranslated region (UTR) (Ambros 2004; Bartel 2004). miRNAs are first transcribed as a long RNA and then processed to a premiRNA of approximately 70 nucleotides (Lee et al. 2003). This premiRNA is transported to the cytoplasm (Lund et al. 2004) and processed by RNase III dicer to produce the mature miRNA. The mature miRNA is incorporated into a ribonucleoprotein complex including eIF2C2 and FMRP, which play a role during RNAi-mediated gene silencing (Mourelatos et al. 2002).

Both siRNA and miRNA have their main function to guide the cleavage of sequence-complementary mRNA (Mansfield et al. 2004; Yekta et al. 2004). Additionally, it was found that siRNAs targeted to promoters can induce transcriptional silencing via DNA methylation in human cells (Kawasaki and Taira 2004; Morris et al. 2004).

The mechanism by which the promoter-directed siRNAs are guided to and gain access to genomic DNA, however, remains unknown. siRNAs might gain access to genomic DNA during cell division when the nuclear membrane disappears, which involves both DNMT1 and DNMT3b RNA (Kawasaki and Taira 2004). The siRNAs can induce not only DNA methylation but also histone methylation (Kawasaki and Taira 2004). Thus, the methylation of histones by RNAi also has the potential to induce DNA methylation. This mechanism of siRNA-induced DNA methylation may be complicated and might actually involve a chromatin remodelling complex at least at some stage of the silencing (Morris et al. 2004).

2.4.2 DNA methylation inhibitors

DNA methylation in promoter regions can be targeted by inhibiting DNA methyltransferases resulting in re-expression of genes (Strathdee and Brown 2002). The first DNA methyltransferase inhibitors synthesized are 5-Azacytidine and 5-aza-2'-deoxycytidine (5-AZA) (Figure: 2). They are chemical analogues and similar to the natural substrate of DNA methyltransferase. The inhibitor mechanism appears to be through the incorporation into DNA strands (after phosphorylation) and subsequent trapping of DNMT1 onto the DNA. Concurrently with the trapping, free DNMT1 disappears from the whole cell causing methylated gene re-expression (Liu et al. 2003).

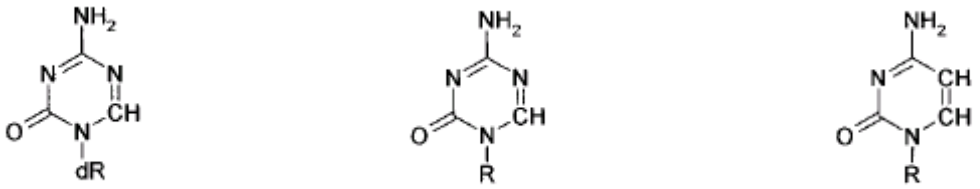


Figure 2: Structure of cytidine and its 5-aza-analoges. (R= robes, dR = doeoxyribose)

Embryos treated with 5-AZA showed delayed gastrulation and developmentally arrested at the neurula stage. They subsequently loose cellular adhesion and finally die. Gene expression profiles of 12-hr old embryos treated with 5-AZA revealed 91 unregulated genes and 168 down regulated genes in comparison with wild-type embryos. In addition, genes associated with the stress response and cell defence were up regulated, whereas genes involved in cell adhesion were down regulated (Sasaki and Satoh 2007).

2.4.3 Embryo production protocols

Gene expression varies according to the respective IVP and sNT protocol (Lonergan et al. 2003; Niemann and Wrenzycki 2000; Niemann et al. 2002). Imprinted genes appear to be more susceptible to alterations in epigenetic modifications (Moore 2001), especially after *in vitro* culture (IVC) of ovine (Young et al. 2001), bovine embryos (Blondin et al. 2000; Tveden-Nyborg et al. 2008), and mice (Dean et al. 1998; Doherty et al. 2000; Humpherys et al. 2001; Khosla et al. 2001).

It has been shown that *in vitro* culture of embryos and embryonic stem cells affected the methylation pattern of the DNA upstream of H19 in mouse (Mann et al. 2003) and disrupted the DMR2 within the imprinted IGF2R gene which subsequent affected on phenotype and growth in sheep (Young et al. 2001). The hypothesis to account for these abnormality phenotype is epigenetic deregulation of genes. It would affect in particular expression of genes that are subject to imprinting (Khosla et al. 2001; Young and Fairburn 2000).

2.5 Functional study of genes

To study the function of genes in mammalian embryogenesis, the posttranscriptional gene silencing (PTGS) by double-stranded RNA (dsRNA), or RNA interference (RNAi), has emerged as a new tool for studying gene function in an increasing number of organism (Review see Schellander et al. 2007). The first study has been successively reported that injection of E-cadherin, Mos and Plat dsRNA interfered the expression of targeted mRNA in mouse oocytes (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). In bovine, injection of C-mos, cyclin B1, and Oct-4 dsRNA in immature oocytes resulted in a decrease in mRNA and protein in mature oocyte stage cultured *in vitro* (Nganvongpanit et al. 2006; Paradis et al. 2005) as well as the injection of Connexin 43 and E-Cadherin dsRNA at zygote decreased in mRNA and protein in blastocyst stage either cultured *in vivo* or *in vitro* (Tesfaye et al. 2007). For the RNAi, since Tuschl's group showed that 21-nucleotide siRNA suppressed expression of endogenous and heterologous genes in mammalian cell lines (Elbashir et al. 2001), then siRNA has been widely used to knock down the targeted mRNA in several genes (Review see McManus and Sharp 2002). In bovine, Adams et al. (2007) showed that transfection of DNMT1s siRNA leads to a moderate reduction in mRNA level in bovine fibroblast cells.

2.6 Effect of DNMT1 suppression

Suppression of DNMT1 affects embryonic development, apoptosis, and imprinted gene expression

2.6.1 Embryonic development

Embryos enter several divisions after fertilization. Cell division or cleavage occurs without increasing cell mass. In bovine, cleavage of embryos of the first, second, third and fourth cell cycle *in vitro* has been estimated to be 32-34, 9-14, 10-11 and 48-52 hrs, respectively (Grisart et al. 1994; Holm et al. 1998). The fourth cell cycle is usually prolonged in embryos developed *in vitro* (Gordon 1994). To maintain genomic stability, the genetic code must be copied faithfully from cell to cell and from generation to generation.

Mechanistic insights into the role of DNA methylation and the establishment of methylation patterns during the development came from phenotypic analyses of mice mutations. Deletion of DNMT1 in mice (Lei et al. 1996; Li et al. 1992) and antisense RNA-mediated inhibition of xDNMT1 expression in frogs (Stancheva and Meehan 2000) results in global demethylation and embryonic lethality. This variation in DNMT1 expression alters the phenotype of the embryo. DNMT1 activity is also required for progression through mitosis (Milutinovic et al. 2003). Deficient DNMT1o leads to significant loss of post-implantation embryos between Day 14 to 21 of gestation (Howell et al. 2001). It suggests that developmental abnormalities in DNMT1o-deficient embryos are largely due to imprinting defects (Toppings et al. 2008). So far, the knowledge about suppression of DNMT1 and its effect on bovine embryonic development is limited in *in vitro* bovine preimplantation embryos.

2.6.2 Apotosis

Apoptosis is a programmed cell death that can be considered as normal process to eliminate damaged cells. It is required at normal mid-to-late stage blastocyst in preimplantation embryos (Jacobson et al. 1997). The dead cells were phagocytized by ICM and polar trophectodermal cells and digested in phagocytic vacuoles. The dead cells are believed to be ones that had failed to be differentiated normally (Pierce et al. 1989).

In bovine, apoptosis has been observed in embryos after the 8-cell stage using the TUNEL assay (Fahrudin et al. 2002; Matwee et al. 2000). In *in vitro* produced blastocysts, the percentage of apoptotic cells is significantly higher than their *in vivo*-development counterpart in mouse (Brison and Schultz 1997; Hardy 1997) and bovine (Gjorret et al. 2001). Furthermore, the presence of glucose (Moley et al. 1998), fetal bovine serum (Byrne et al. 1999) and synthetic oviduct fluid medium (Watson et al. 2000) can affect the incidence of apoptosis in mouse and bovine, respectively.

Stress has been also reported to increase apoptosis. Heat stress inducing TUNEL labeling at the late 8- to 16-cell stage has been reported in bovine (Paula-Lopes and Hansen 2002). Thus, increased apoptosis is probably related to embryo losses and to lower developmental competence of *in vitro* fertilized and cultured embryos (Betts and King 2001). However, the relationship between the rate of individual cell death and the level of whole embryo loss is unclear.

It has been hypothesized that epigenetic modification may be responsible for the programmed cell death. Transient depletion of DNMT1 in frog embryos induces DNA hypomethylation producing an altered phenotype and causes apoptosis (Stancheva et al. 2001). Conditional inactivation of DNMT1 causes genomic demethylation which then leads to apoptosis in embryos (Panning and Jaenisch 1996; Stancheva et al. 2001) and in primary fibroblasts (Jackson-Grusby et al. 2001). TUNEL assays showed that the cell undergoes apoptosis (Jackson-Grusby et al. 2001) as well as DNMT1 mutants also showed apoptotic cell death in ES cells (Panning and Jaenisch 1996). The widespread apoptotic phenotype of these DNMT1-deficient cell suggested that DNA demethylation might represent an endogenous signal of DNA damage. The *Trp53* tumor-suppressor gene was considered to rescue this cell lethal phenotype, as it is activated in response of DNA

damage (Jackson-Grusby et al. 2001; Levine 1997). Additionally, other embryonic lethal mutations genes, such as *Rad51, Brca1* and *Brca2*, have been rescued (Hakem et al. 1997; Lim and Hasty 1996; Ludwig et al. 1997).

2.6.3 Imprinted gene expression

Epigenetic modulations of DNA and histone determine the pattern of gene expression and silencing (Jaenisch and Bird 2003). The epigenetic regulation of gene expression seems inevitable for multicellular organisms as it underlies the development of cell lineage-specific gene expression (Jablonka and Lamb 1998). In general, known effects of DNA methylation on gene expression evident in frog showed that demethylation induces of premature gene activation (Stancheva et al. 2002; Stancheva and Meehan 2000). Similarly, widespread activation of tissue-specific genes has been seen in fibroblasts (Jackson-Grusby et al. 2001). The 5-AZA interferes with the activity of DNMT1, leading to genomic hypomethylation. It also reactivates silenced tumor suppressor genes (Esteller and Herman 2002; Santini et al. 2001). DNMT1 knockout led to biallelic IGF2 expression. In contrast, the imprinted gene IGF2R, was completely resistant to *de novo* methylation even when DNMT1 was overexpressed (Biniszkiewicz et al. 2002). The table below (Table 2) summarizes the imprinted genes in bovine.

2.6.3.1 Insulin-like growth factor 2 (IGF2)

The IGF2 gene is a polypeptide growth factor hormone, which plays an important role for the regulation of cellular growth and division during embryonic development (Ferguson-Smith et al. 1991) and the development of the placenta (Reynolds et al. 1997). IGF2 is maternally imprinted. With the exception of the central nervous system where the gene is biallelically expressed, only the paternal allele is normally transcribed in all tissues (Hu et al. 1997; Hu et al. 1995).

Table 2: Imprinted genes indentified in bovine

Imprinted loci	Chromosome	Expression parental	Name	References
H19	-	Maternal		Zhang et al. 2004
IGF2	29	Paternal	Insulin-like growth factor 2	Dindot et al.2004
IGF2R	9	Maternal	Insulin-like grow factor 2 receptor	Killian et al. 2001
XIST	X	Maternal	X-inactive specific transcript	Dindot et al. 2004
NAPIL5		Paternal	Nucleosome assembly protein 1-like 5	Zaitoun and Khatib 2006
NESP55	2	Maternal	Neuroendocrine cecretory protein	Khatib 2004
NNAT	13	Paternal	Neuronatin	Zaitoun and Khatib 2006
PEG1 (Mest-1)	4	Paternal	Paternally expressed gene 1	Tveden-Nyborg et al. 2008
tPEG3	18	Paternal	Paternally expressed gene 3	Kim et al. 2004
GTL2	21	Paternal	Gene trap locus 2	Dindot et al. 2004

The imprinted status of the IGF2 gene is conserved among rodents, humans, and ruminants (Dindot et al. 2004; Young et al. 2003). The function of IGF2 is mediated through the insulin-like growth factor1 (IGF1), the insulin-like growth factor 2 receptor (IGF2R) and several serum IGF binding protein. Disruption of only one of the IGF family members can affect the embryonic development (Sara and Hall 1990). However, overexpression of IGF2 or IGF2R, which is paternally and maternally expressed, respectively, results in overgrowth of mouse embryos (Sun et al. 1997).

Genomic imprinting is associated with allele-specific DNA methylation. In mouse embryos that are deficient in the DNA methyltransferases gene, the expression of IGF2 (Li et al. 1993). Mono-allelic expression of the IGF2 gene is regulated by a methylation-

sensitive insulator element (Eden et al. 2001) which is located between enhances and promoters, and prevent gene expression. The function of insulator elements is related to chromatin boundary, which is "closed" in its unmethylated status but "open" when methylated (West et al. 2002). Silencing of the maternal IGF2 allele which is only possible when the imprinting control element (ICE) remained constantly unmethylated on the maternal allele (Srivastava et al. 2000) is performed through binding of the repressor factor *CTCF* (CCCTC-binding factor) to a repressor element of the maternal allele. *CTCF* cannot interfere with the paternal allele protected by methylation (Fedoriw et al. 2004; Schoenherr et al. 2003; Szabo et al. 2000). CTCF-binding seems to be initiated after fertilization when primary imprints have already been established to protect the maternal imprinting from methylation (Verona et al. 2003).

DNA demethylation induced by the demethylating agent 5-AZA leads to loss of IGF2 imprinting in tissues (Hu et al. 1997), dramatically increased the expression of IGF2 which was primarily derived from the activation of the normally imprinted maternal alleles. The normal expression from paternal allele also remains active (Hu et al. 1996). In cells treated with 5-AZA, the IGF2 expression increased 2- to 4-fold compared to the imprinted allele (Eversole-Cire et al. 1993).

2.6.3.2 Insulin-like growth factor 2 receptor (IGF2R)

IGF2R gene is generally imprinted on the paternally inherited allele and expressed from maternal allele depending on the imprinting control region (ICR) differentially methylated (Sleutels et al. 2002; Zwart et al. 2001). IGF2R is imprinted in bovine, sheep, pig and mice (Killian et al. 2001). There are two imprinting control regions (ICR) in the mouse IGF2R gene which are differentially methylated on the two parental alleles. The first ICR includes the sense IGF2R RNA promoter and is only methylated on the suppressed paternal allele. The second ICR2 encompasses the promoter of the IGF2R antisense RNA (Wutz et al. 1997) which is preferentially methylated on the maternal allele (Hu et al. 1998). The IGF2R gene encode including the promoter for the sense IGF2R transcript, which is located within the second intron of the gene, includes the promoter for *Air* gene (Sleutels et al. 2002).

The silencing activity of ICR2 on the paternal allele correlates with the absence of methylation and the presence of *Air* RNA. In contrast, expression of the IGF2R on the

maternal allele correlates with region 2 methylation and *Air* repression (Wutz et al. 1997). The presence of a methylation imprint on the active maternal allele indicates that this allele is epigenetically activated by DNA methylation (Zwart et al. 2001). This interpretation contrasts with results obtained *in vitro* based on demethylating agents (Hu et al. 1999), but is consistent with results obtained *in vivo* based on mouse mutants lacking the maintenance methyltransferase gene (Jackson-Grusby et al. 2001).

Aberrant methylation patterns at IGF2R significantly regulates the expression of IGF2R in bovine cells which is correlated with developmental abnormalities such as heavy liver, heart and abnormal brain in cloned bovine (Long and Cai 2007).

2.6.3.3 Insulin-like growth factor binding protein 4 (IGFBP-4)

The IGFBP-4 gene contains 15.3 kb bp which the transcription initiation site located 28 bp downstream of a TATA box and 286 bp 5' of the translation initiation codon. The IGFBP-4 gene is composed of four exons separated by three introns (Zazzi 2000). The function of IGFBP-4 appears to protect cells from overstimulation by IGF genes, since it acts as an inhibitor of cell growth by binding to IGF genes (Culouscou and Shoyab 1991). However, IGFBP-4 is suppressed by a specific protease that cleavages IGFPB-4 into two 18- and 14-kDa protein fragments (Conover et al. 1993). Expression of IGFPB-4 was related to growth and differentiation of colon cancer cells due to up-regulation of IGFBP-4 expression (Dai et al. 1997). High expression of IGFBP-1 and IGFBP-4 favoured early embryonic development *in vitro* fertilized oocytes aspirated from human follicles (Wang et al. 2006). Demethylation by culturing cell lines with 10 µmol/L 5-azaC for 48 hr and then adding IGF-1 induced IGPB-4 and IGFBP-2 expression (Sato et al. 2006). In cloned bovine claves dying within 48 hrs of birth, IGFBP-4 was found to be aberrant in five tissues, with lower levels in livers, lungs, and kidneys of adult and fetal fibroblast cell-derived clones, but higher levels in brains and hearts of fetal fibroblast cell-derived clones (Li et al. 2007).

2.7 Genetic of male fertility

Male fertility is the result of a combination of genetic potential and environment. It seems likely that substantial genetic effects are contributing to the fertility, despite the low heritability of most fertility traits (Veerkamp and Beerda 2007).

2.7.1 Factors affecting male fertility

Factors that influence semen and sperm quality include genetic factors (genes) and environmental factors

2.7.1.1 Genetic factors

Molecular evidence suggests that the regulation of normal spermatogenesis is mediated by several genes. Mutants in ubiquitin specific protease (USP) 26 gene and mitochondrial ND4 gene causes hypogonadism (Paduch et al. 2005) and low sperm motility in humans (Selvi Rani et al. 2006) respectively. Mutants in the haemochromatosis gene (HFE)H63 D shows an association with abnormal sperm motility in human (Gunel-Ozcan et al. 2008). Premature translation of transition protein 2 (Tnp2) mRNA causes abnormal head morphogenesis, reduced sperm motility and male infertility in mouse (Tseden et al. 2007). Mutation in Nsun7 depresses motility of sperm in mouse (Harris et al. 2007). Petrunkina et al (2007) showed that inhibition of protein kinase increased the number of sperm cells and ejaculate volume in the boar. Two SNPs in the porcine testis-specific phosphoglycerate kinase 2 (PGK2) gene resulting in amino acid substitutions decreased semen volume in boar (Chen et al. 2004). In bovine, the defect of complex vertebral malformation (CVM) gene reduced reproductive performance as measured in terms of non return rate (NRR) (Berglund et al. 2004). The polymorphisms of GFG2 and STAT5 gene is associated with fertilization success and survival rate of embryos during in bovine preimplantation (Khatib et al. 2008a; Knatib et al 2008b)

2.7.1.2 Environmental factors

Season alters endocrine profiles and influences fertility of males. Spermatogenesis is impaired, and testosterone is lower during early exposure to hyperthermia. However, season of semen collection did not affect 56-day NRR, but calving rate was significantly higher for semen collected in the period when the photoperiod was increasing over time across of the year (Haugan et al. 2005). Cows with higher milk production had lower probability of pregnancy and higher probability of embryonic loss (Vasconcelos et al. 2006). In mice, scrotal heat stress, 42°C for 30 min, resulted into a lower concentration of spermatozoa which reduced viability, low motility, and higher degree of DNA damage in mice (Perez-Crespo et al. 2008). In addition, there is a significant increase in the number of immotile sperm (Rizvi et al. 2008).

2.8 Male fertility traits of bull

Reproductive efficiency of bull is evaluated by several traits; NRR, semen quality (sperm concentration, motility and morphology), and sperm flow cytometric parameters.

2.8.1 NRR

NRR is defined as the percentage of cows that were inseminated and not reinseminated within a specified interval, typically 56 days (Grossman et al. 1995). It is also the result of conception (Koops et al. 1995) and, therefore, is regarded as a field fertility measure. Bulls different in their reproductive performance can be evaluated on the basis of NRR. NRR is under the influence of several factors, such as the reliability of the estrus control systems, season (Stalhammar et al. 1994), herd, technician, age of the cow (Guaita et al. 1996), heat stress, and humidity (Ravagnolo and Misztal 2002; Ravagnolo et al. 2000). Other factors not related to fertility may affect NRR, among these, misidentification of the cow at subsequent services, inaccurate heat detection and recording (Rycroft and Bean 1991).

The NRR has been found to be correlated with semen quality such as sperm morphology (Barth, 1992), and sperm motility (Hallap et al. 2006; Kjaestad et al. 1993).

2.8.2 Sperm quality traits

The sperm quality is routinely evaluated providing descriptive information on morphology, motility, and numbers of spermatozoa in the ejaculation. These parameters also determine primarily the fertility of bull. A minimum of 80% motility and 70% normal sperm of freshly ejaculated semen represents an acceptable standard for breeding soundness evaluation of bulls (Hopkins and Spitzer 1997). The major defects of sperms including abnormalities of the head, mid piece, and proximal cytoplasmic droplets are evidently correlated with low bull fertility bulls; however, it can be compensated by increasing sperm dose per insemination (Saacke et al. 2000). Sperm quality differs with age of the sires. Young bulls have high percentages of proximal droplets (Arteaga et al. 2001), whereas older bulls display a lower number of total sperm per ejaculation (Al-Makhzoomi et al. 2008).

The percentage of morphologically normal and abnormal spermatozoa is related positively to NRR (Fitzpatrick et al. 2002). Reduction of total sperm per insemination from $16x10^6$ to $10x10^6$ has no affect on NRR (Foote and Kaproth 1997). Sperm motility and sperm motility index (SMI) showed significant relation with NRR (Hoflack et al. 2005; Kjaestad et al. 1993; Stalhammar et al. 1994). However, based on using sexed semen (91.6% females), an overall numerical decline of 13.6 in NRR was observed (Frijters et al. 2009).

2.8.3 Sperm flow cytometric parameters

2.8.3.1 Membrane integrity

The sperm outer membrane is essential for sperm metabolism, capacitation, sperm attachment and acrosome reaction. The plasma membrane is responsible for the mechanism of maintaining the cell osmotic equilibrium and acts as a barrier between intra- and extra cellular medium. Damages in this structure can lead to homeostasis loss, leading to cellular death (Flesch and Gadella 2000). The most common adverse effect of freeze-thawing is the dramatic and sharp decrease in plasma membrane integrity (PMI) of sperm in human (Lin et al. 1998), mouse (Nishizono et al. 2004; Sztein et al. 2001) and bovine (Bollwein et al. 2008). Sperm require an active membrane during fertilization and they will fail to fertilize if the plasma membrane is physically not intact. Vital stains measure whether the membrane is functionally active. Propidium iodide (PI) is a

fluorescent probe that binds to DNA of cells possessing a damaged plasma membrane (Graham et al. 1990). Other fluorescent probes with DNA specificity have also been used such as Hoechst 33258 (H258), Hoechst 33342 (H342) (Casey et al. 1993) and SYBR-14 (Thomas et al. 1998). PMI values are not correlated with fertility as measured by NRR 56 days due to the fact that the PMI was required only for fertilization, but not for embryonic development (Watson 1995). Thus, it was compensated by increasing sperm concentration or volume (Evenson, 1999). This result was not consistent with Bollwein and colleagues who found that the PMI was correlated with NRR (56 days) (Bollwein et al. 2008).

2.8.3.2 Acrosome integrity

The acrosome is a large lysosome-like vesicle overlying the sperm nucleus containing a large array of powerful hydrolyzing enzymes including hyaluronidase and acrosin (Zaneveld et al. 1991). A spermatozoa must maintain an intact acrosome up to the time it binds to zona pellucida of the mature oocyte and undergoes the acrosome reaction to release acrosomal enzymes (Graham and Moce 2005). It is an important feature to evaluate the sperm fertilizing potential (Silva and Gadella 2006). The acrosome can be examined by several methods. The most commonly used is fluorescein isothiocyanate (FITC) labelled plant lectins such as pisum sativum agglutinin (PSA) and peanut agglutinin (PNA) (Gillan et al. 2005). The lectin can penetrate an acrosomal membrane when it has been damaged.

2.8.3.3 DNA integrity

The integrity of sperm DNA is of prime importance for the paternal genetic contribution to normal offsprings. Spermatozoa with DNA defects affects on spermatogenesis, delays in initiation of the zygotic S-phase (Eid et al. 1994) and blocking embryonic development (Fatehi et al. 2006), and subsequently reduces number of fetus developed to term (Ahmadi and Ng 1999). Several techniques have been developed to detect DNA abnormalities (Fraser 2004). These assays include the single cell gel electrophoresis assay (COMET) (Irvine et al. 2000), the terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) assay (Host et al. 2000a; Host et al. 2000b), in situ nick translation (NT) (Sakkas et al. 1996), sperm chromatin structure assay (SCSA) (Evenson et al. 1999; Evenson et al. 2002), and acridine orange test (AOT) (Duran et al. 1998).

The degree of DNA-stability determined by SCSA within the sperm chromatin structure is related to 56-day NRR (Bollwein et al. 2008; Januskauskas et al. 2001; Januskauskas et al. 2003), 60-day NRR (Waterhouse et al. 2006), and 90-day NRR (Madrid-Bury et al. 2005).

2.9 Genotype dependent embryonic development

In *in vitro* bovine embryo production, approximately one-thirds of oocytes develop to the blastocyst stage (Gordon 1994). There is a relationship between the time of the first cleavage of a bovine oocyte in vitro and its development ability. The earliest-cleaving oocytes are more likely to develop the blastocyst stage than those that cleave late (Lonergan et al. 1999). With regard to the mechanisms, the sperm influences the time of first cleavage and thereby development without involvement of the maternal genotype (Comizzoli et al. 2000; Ward et al. 2001). Therefore, early embryonic development can be used to measure embryo quality and bull field fertility. Ward et al (2001) reported that the proportions of early-cleaving oocytes developing to blastocysts stage are higher than the late-cleaving oocytes. In addition, the 33-hpi-cleavage rate was the best predictor of field fertility (NRR 150-days). There was also a high correlation between Day 7 blastocyst yield and NRR (Ward et al. 2001).

In addition, concentration of sperm in IVF also reflected blastocyst yields and bull field fertility. By this regard, a minimum concentration of 0.125×10^6 sperm/ml resulted in higher Day 8 blastocyst yield regardless of sire. A concentration of 0.5×10^6 sperm/ml showed significant correlation between cleavage rate (48 phi) and NRR (Ward et al. 2003). Bovine IVF and embryo culture techniques showed the relationship between *in vivo* bull fertility and IVF outcomes (Lonergan et al. 1994; Zhang et al. 1997). Individual bulls differ in their ability to fertilize oocytes following IVF procedures (Ward et al. 2001) and even after intracytoplasmic sperm injection (Wei and Fukui 1999).

The polymorphisms of GFG2 gene is reported to be associated with fertilization success and survival rate of embryo during in bovine preimplantation (Khatib et al. 2008a). Knatib et al (2008b) showed that STAT5 affected the survival rate of embryo by 2 mechanisms: prefertilization involving sperm factors and causing lower fertilization rate and postfertilization causing incompatibility between the male pronucleus and the oocyte, which in turn leads to death of the embryo before the blastocyst stage (Khatib et al. 2008b).

2.10 Association of candidate genes with bull fertility traits and embryonic development

Association analyses of genes that play a major role during bovine preimplantation development have been done with bull fertility traits namely NRR, sperm quality, and sperm flow cytometric parameters. Polymorphisms of the leptin gene were found to be associated with shorter calving intervals and the time of day open in Jersey cows (Komisarek and Antkowiak 2007). A growth hormone gene polymorphisms was observed to have an effect on volume of ejaculation and NRR (Lechniak et al. 1999). Kia (2007) reported that association analysis revealed significant association of SNP of desmocollin 2 (DSC2) and tight junction protein 1 (TJP1) with volume of ejaculated, aldo-keto reductase family1 member1 (AKR1B1) and CDH1 with sperm motility, CD9, AKR1B1, COX-2, DSC2 and TJP1 with sperm motility, CD9, N-PAC, CDH1 and Plakophilin 1 (PKP1) with sperm concentration. However, there was no association of those SNPs with NRR and sperm flow cytometric parameters.

In porcine, the candidate genes associated with boar fertility traits namely NRR and sperm quality traits have also been investigated. Lin (2005) reported the significant associations of the following candidate genes with traits: actinin alpha 1 (ACTN1) and acrosin (ACR) locus with NRR; prolactin (PRL), inhibin beta B (INHBB), ACR, and follicular stimulating hormone beta (FSHB) locus with sperm concentration; actin gamma (ACTG2), relaxin (RLN) and follistatin (FST) with semen volume per ejaculate; ACTG2, retinol-binding protein 4 (RBP4), OPNin6, ACR and gonadotropin releasing hormone receptor (GnRHR) with motility; GnRHR, inhibin alpha (INHA), inhibin beta A (INHBA), OPNpro and androgen receptor (AR) with abnormal sperm rate.

For the development of embryos in IVP, there was a difference of genotype frequency of the IJP1 gene observed at blastocyst stage. The IJP1 gene might play an important role during early bovine preimplantation development (Kia 2007).

3 Materials and methods

3.1 Experimental design

The aim of this study was to study the function of DNMT1 gene in bovine preimplantation and the association analysis of DNMT1, DNMT3a, and DNMT3b sequence variants with bull fertility traits and embryonic development.

3.1.1 Suppression of DNMT1

Firstly, alternative transcripts of DNMT1 isoforms namely; DNMT1o and DNMT1s, and their expression profiles were analysed in preimplantation stage embryos. To identify the isoforms, two sets pairs of primer were designed as shown in Figure 3. The cDNA from immature oocytes, mature oocytes, 2-cell, 4-cell, 8-cell, 16-cell and blastocyst was obtained for DNMT1 mRNA expression by using semi-quantitative RT-PCR. The transcript profile of DNMT1 isoforms was done by quantitative RT-PCR.

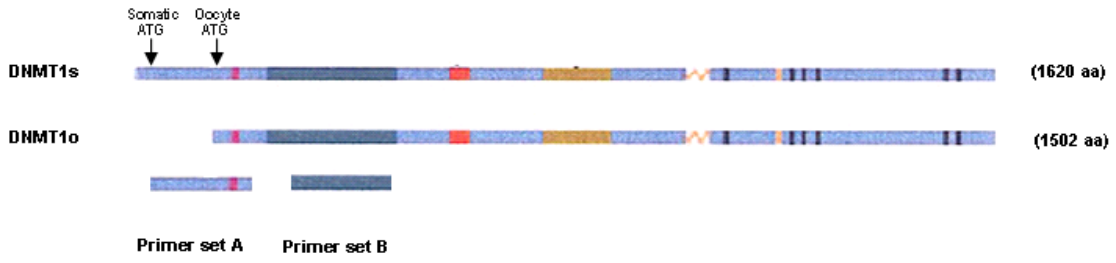


Figure 3: The primer set designer to identify the different isoforms of DNMT1 gene.

It is due to the fact that DNMT1o initiates at oocyte ATG in exon 4, resulting in the 118 amino acids shorter than the somatic isoforms (Mertineit et al. 1998). The pair of primer A was designed to cover exon 4 which is specific to amplify only the somatic-specific DNMT1 isoforms and the pair of primer B is amplified both isoforms. Therefore the specific DNMT1 isoforms could be identified due to the intensity of the PCR product.

Secondly, it was to investigate the effects of suppression of DNMT1 on the embryonic development, the levels of apoptosis, and the expression of imprinted genes during bovine preimplantation. *In vitro* produced zygotes were categorized into four groups, namely those injected with Smartpool siRNA (SpsiRNA), 5-aza-2'-deoxycytidine (5-AZA), nuclease free water, and uninjected control. The mRNA and protein expression data were

generated using Real Time polymerase chain reaction (RT-PCR) based on the relative standard curve method employing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene for normalization and western blotting analysis, respectively. As shown in the overview of the first experiment (Figure 4), the microinjection was performed at the zygote stage. The survival rate was recorded 3-4 hr post microinjection (pmi). Phenotype assessments of the proportion of 2-, 4-, 8- cell and 2-, 4-, 8 and 16-cell embryos were assessed 48 and 72 hr pmi, respectively. The mRNA and protein expression was performed at 8-cell stage to check whether the cognate mRNA and protein were degraded. Moreover, an independent transcript of DNMT3a and DNMT3b was quantified to access the specificity of the mRNA suppression. In a second experiment (Figure 5), the zygotes were cultured until Day 8 blastocyst. For phenotype assessment development to Day 7 and Day 8 blastocyst was observed. RT-PCR and western blotting analysis was performed to check whether the cognate mRNA and protein had degraded at Day 8 blastocyst. The TUNEL staining was performed to calculate the apoptotic index (API) by dividing the number of apoptotic cells by the total cell number. Moreover, the expression of imprinted genes, namely IGF2, IGF2R, and IGFBP-4 was measured.

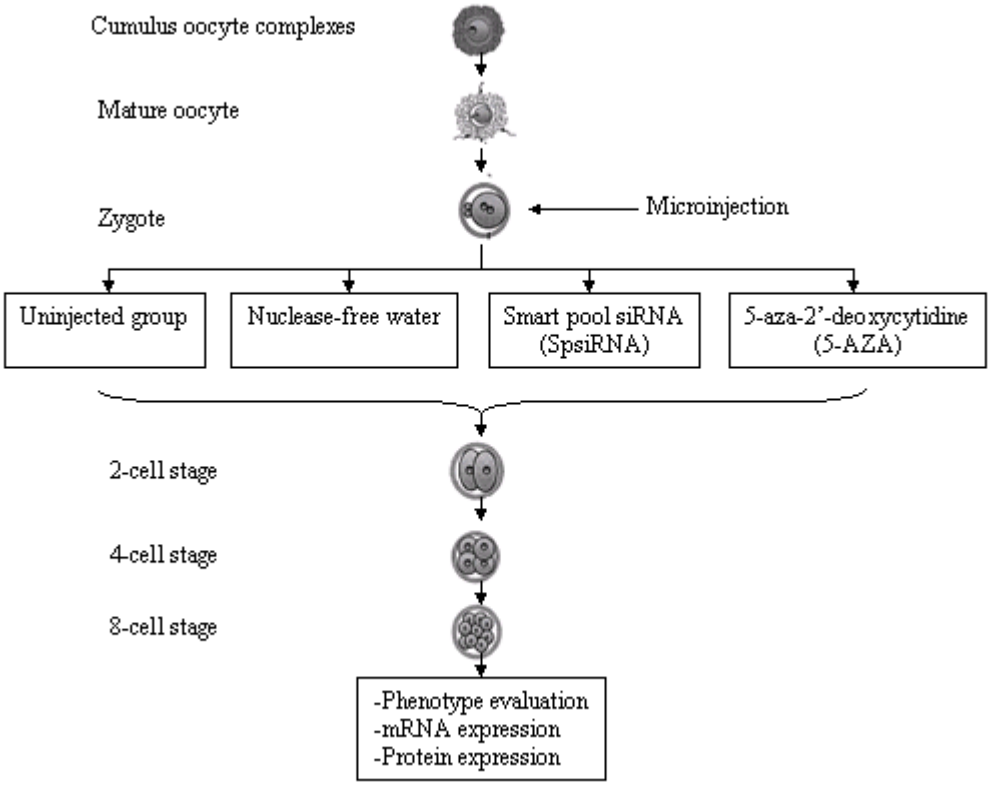


Figure 4: An outline of the experiment designed to investigate the effect of suppressing and inhibiting DNMT1 on the embryonic development, mRNA and protein expression at 8-cell stage bovine preimplantation.

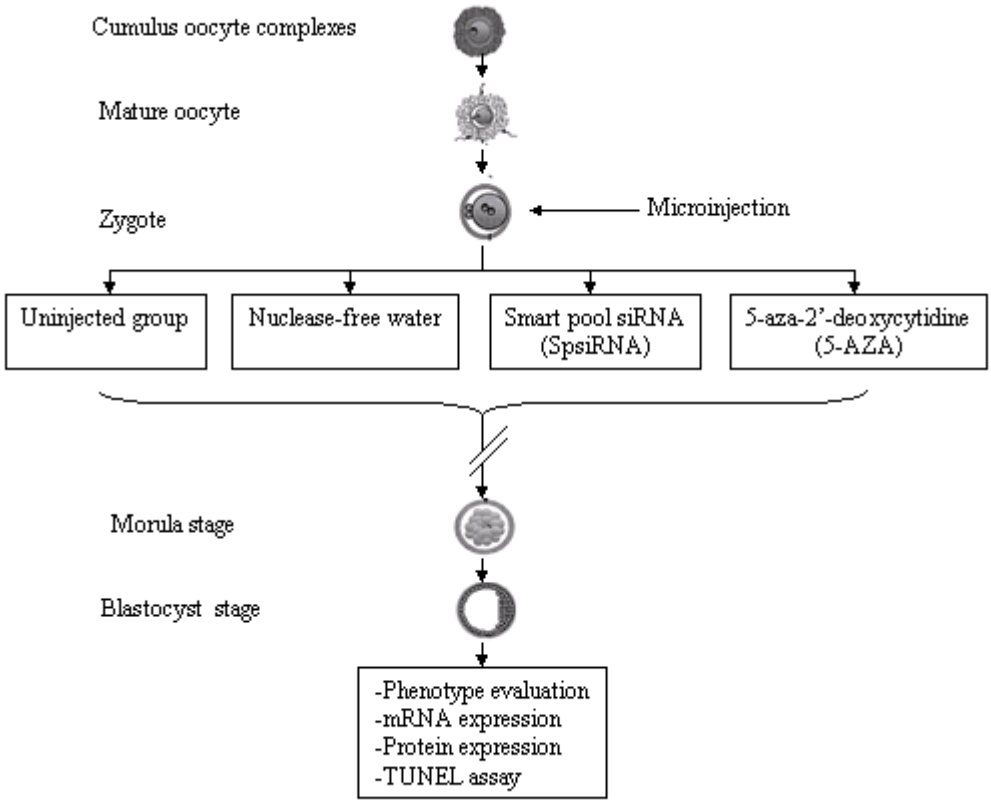


Figure 5: An outline of the experiment designed to investigate the effects of suppressing and inhibiting DNMT1 on blastocyst rate, apoptotic index, mRNA and protein expression at blastocyst stage bovine preimplantation.

3.1.2 Association analysis of DNMT1, DNMT3a, and DNMTT3b sequence variant

This study was to elucidate effects of gene DNMT1, DNMT3a, and DNMT3b gene on bull fertility traits namely; non-return rate (NRR; 56 days, %), sperm quality traits which are sperm volume per ejaculate (VOL), sperm concentration (CONC), sperm motility (MOT), and survivability after thawing (SUVR), sperm flow cytometric parameters such as positive acrosome status (PAS), plasma membrane integrity (PMI), and DNA fragmentation index (DFI), and embryonic development such as early cleavage, late cleavage, and blastocyst embryos (Figure 6). For this, The single nucleotide polymorphisms studied namely; DNMT1 (rs41256891; C/T), DNMT3a (rs41569254; C/T), and DNMT3b (rs41700758; A/G) were retrieved from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). The SNPs of interest were confirmed with 11 different cattle breeds namely; Limousin, Gelbvieh, Blond d'Aquitaine, Salers,

Vorderwälder, Hinterwälder, Charolais, Red Angus, Piemontese, Pinzgauer and Galloway by using comparative sequencing analysis and the PCR-RFLP method. A total of 310 bull sperms and 210 embryo samples were used for genotyping.

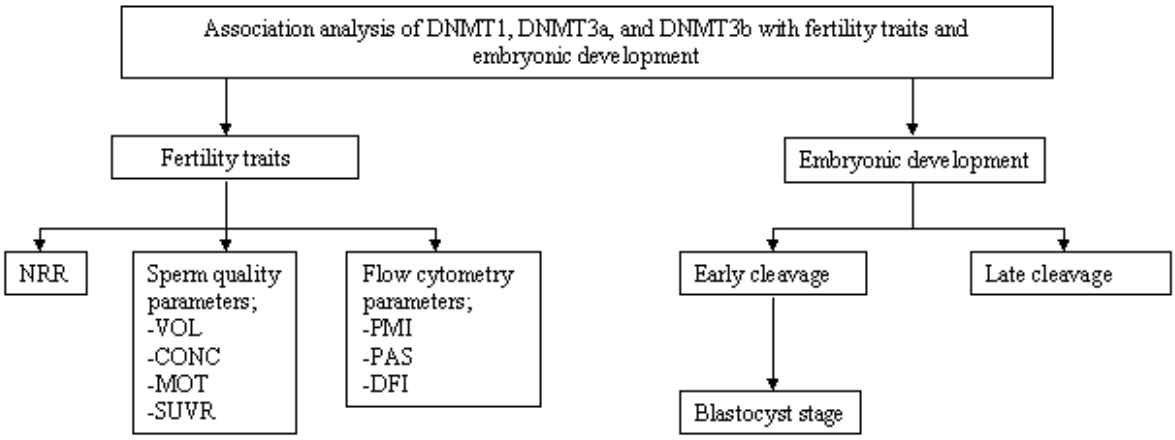


Figure 6: Flowchart of the experiment design to study the association analysis of DNMT1, DNMT3a and DNMT3b sequence variants with bull fertility traits and embryonic development

3.2 Material

3.2.1 Samples

3.2.1.1 Suppression of DNMT1

Bovine ovaries were collected from two different local slaughterhouses. A total of 1,470 embryos were obtained by *in vitro* production (IVP) after *in vitro* maturation, fertilization and culture at the experimental farm Frankenforst of the University Bonn.

3.2.1.2 Association of DNMT1, DNMT3a, DNMT3b

3.2.1.2.1 Bull

Sperms from 310 black and red Holstein-Friesian AI bulls with a range of age 1 to 8 years obtained from the Rinder-Union West eG (RUW) station were used for genotyping and association analysis. The phenotypes in this study were included the bull fertility traits namely NRR (56 days), semen quality traits namely; VOL (ml), CONC ($x10^6$/ml), MOT (%), and SUVR (%) and sperm flow cytometric parameters namely PMI, PNA, and DFI. Semen qualities of each bull were evaluated with a standard method based on the guidelines of the World Health Organization (WHO). The sperm flow cytometric parameters were tested and recorded by the University Hanover (Prof. Dr. Bollwein lab). The additional information on bull status (testing bulls and approved bulls), inseminator (veterinarian, technician, and farm owner), and bull race (black and brown bulls) were obtained from the RUW.

3.2.1.2.2 Embryos

A total 350 embryos obtained from two different local slaughterhouses and produced by IVP at the experimental farm Frankenforst of the University Bonn. The mature oocytes were fertilized with the heterozygote genotype DNMT1 (C/T), DNMT3a (C/T), and DNMT3b (A/G). The zygotes which had developed to 2-cell stage at 30 hr post insemination (hpi) were placed in new droplets and cultured in separated groups. Half of them were individually frozen in liquid nitrogen. The rest were cultured until blastocyst stage and frozen individually in liquid nitrogen. The zygotes which did not cleavage at 30 hpi (late cleavage) were individually frozen in liquid nitrogen. These three groups of samples, early cleavage, late cleavage, and blastocyst were used for the genotyping.

3.2.2 Chemicals

Abcam plc (UK):	DNMT1 antibodies
Affymetrix, Inc (USA):	ExoSAP-IT®
Applied Biosystems (Foster City):	SYBR® Green Universal PCR Master Mix,
Beckman Coulter (Krefeld):	CEQ™ 8000 Genetic Analysis System Sequencing (DTCS) kit, Sample loading solution (SLS), Glycogen
Biomol (Hamburg):	Phenol, Phenol:Chlorophorm:Isoamyl alcohol (25:24:1)
Dharmacon RNA technologies:	Smartpool RNA DNMT1
DYNAL Biotech (Hamburg):	Dynabeads oligo (dT)25
Fermentas (Germany):	Restriction emyzmes (BcnI, BspLI, and NdeI)
GeneCraft (Germany):	BioTherm® Taq DNA polymerase
Invitrogen Life Technologies (Karlsruhe):	DTT, SuperScript™ II RNase H⁻ Reverse transcriptase, 5x first strand buffer, random primers
Kodak (Japan):	Autoradiography film (Kodak® Biomax XAR film)
MWG biotech:	Oligonucleotide primers
Promega (Mannheim):	BSA, pGEM®-T vector, RQ1 RNase-free DNase,

	RNasin ribonuclease inhibitor, 2x rapid ligation buffer, T4 DNA ligase
Qiagen (Hilden):	RNeasy® Mini kit, QIAquick PCR Purification Kit, Mini Elute™ Reaction Cleanup Kit
Roth (Karlsruhe):	2- Propanol, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyra-noside (X-gal), Acetic acid, Agar-Agar, Ampicillin, Ammonium peroxydisulfate (APS), Bromophenol blue, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl -D-thiogalactoside (IPTG), Ponceau-S, Proteinase K, Sodium dodecyl sulfate (SDS), Sodium acetate, Sodium carbonate, Sodium chioride, Sodium hydroxide, TEMED, Tris, Tris-HCl, T-octylphenosypolyethosyethanol (Triton X-100), Trichloromethane/chiorophorm, Tyrode, Yeast extract
Sigma (Steinheim, Germany):	5-aza-2'-deoxycytidine, 10× Buffer for PCR, Albumin bovine, Bisbenzemide, Dulbecco´s Phosphate Buffer Saline (D-PBS), Heparin, Hepes, Hyaluronidase, Hypotaurin, Igepal, Isopropanol, L-Glutamin, Magnesium chloride, Medium 199, Mineral oil, Penicillin, Polyvinyl pyrolidone (PVP), Propidium iodide, Protease inhibitor cocktail, Sodium hydrogen carbonate, Sodium hydrogen sulphate, Sodium lactate solution (60%), Sodium pyruvate, Streptomycin sulfate,
	SYBR® Green JumpStartTM *Taq* ReadyMix, Tween-20, TRIReagent

StarLab (Germany): Agarose (StarPure Agarose)

Stratagene (Amsterdam): 5 c-DH *Escherichia coli* competent cells

3.2.3 Reagents and media

All solutions used in these investigations were prepared with deionized Millipore water (ddH_2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylenczanol	0.0625 g
	Glycerol	7.5 ml
	Water added to	25 ml
Ampicillin (10 mg/ml)	Ampicillin powder	2 g
	Water added to	40 ml
Anode buffer I	Tris-HCl (pH 10.4)	300 mM
	Methanol	10 ml
	Water added to	100 ml
Anode buffer II	Tris-HCl (pH 10.4)	25 mM
	Methanol	10 ml
	Water added to	100 ml
Binding buffer	Tris-HCl (1 M pH 7.5)	1,000 µl
	Lithium chloride (5M)	10 ml
	EDTA (0.005 M pH 8)	20 ml
	Water added to	50 ml

Blocking buffer	Polyvinyl pyrolidone	1 g
	TBST added to	100 ml
BSA (3 %)	Bovine serum albumin (BSA)	0.15 g
	PBS+PVA added to	5 ml
Capacitation medium	Sodium chloride	0.2900 g
	Potasium chloride	0.0115 g
	Sodium hydrogen carbonat	0.1050 g
	Sodium dehydrogen sulphate	0.0017 g
	Hepes	0.1190 g
	Magnisium chloride $6H_2O$	0.0155 g
	Calcium chloride	0.0145 g
	Sodiumlactate solution (60%)	184 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water add to	50 ml
Cathode buffer	Tris-HCl (pH 9.4)	25 mM
	Methanol	10 ml
	6-aminohexanoic acid	60 mM
	Water added to	100 ml
Culture medium	Hemicalcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potasium chloride	0.0112 g
	Sodium hydrogencarbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamin	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl

DEPC-treated water	DEPC	1 ml
	Water added to	1,000 ml
Digestion buffer	NaCl	100 mM
	Tris-HCl	50 mM
	EDTA pH 8.0	1 mM
dNTP solution	dATP (100 mM)	10 µl
	dCTP (100 mM)	10 µl
	dGTP (100 mM)	10 µl
	dTTP (100 mM)	10 µl
	Water added to	400
Epinephrin solution	Sodiumdisulphate	0.0400 g
	Epinephrin	0.0018 g
	Water added to	40 ml
Fertilization medium	Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin	0.0032 g
	Magnesium chloride hexahydrate	0.0050 g
	Calcium chloride dehydrate	0.0150 g
	Sodium lactate solution (60%)	93 µl
	Phenol red solution	100 µl
	Water added to	50 ml
Glycine+PBS	Glycine	0.02252 g
(30 mmol/ml)	PBS+PVA solution added to	10 ml

IPTG solution	IPTG	1.2 g
	Water added to	10 ml
LB-agar	Sodium chloride	8 g
	Pepton	8 g
	Yeast extract	4 g
	Agar	12 g
	Sodium hydroxide (40 mg/ml)	480 µl
	Water added to	800 ml
LB-broth	Sodium chloride	8 g
	Pepton	8 g
	Yeast extract	4 g
	Sodium hydroxide (40 mg/ml)	480 µl
	Water added to	800 ml
Lysis buffer	Igepal (0.8%)	0.8 µl
	RNasin	5 µl
	Dithiothreitol (DTT)	5 µl
	Water added to	100 µl
Modified parker medium	Sodium hydrogencarbonat	0.080 g
	Hepes	0.140 g
	Sodium pyruvat	0.025 g
	L-Glutamin	0.010 g
	Gentamycin	500 µl
	Medium 199	99 ml
	Hemicalcium lactate	0.06 g
	Water added to	110 ml
Paraformaldehyde 16%,	Paraformaldehyde	1.6 g
	Water added to	10 ml

PBS + PVA	Polyvinyl alcohol (PVA)	300 mg
	PBS added to	50 ml
Permeabilization solution	Tritonx-100	5 µl
	Glycine + PBS added to	10 ml
PHE medium	Physiological saline (0.9%)	16 ml
	Hypotaurin solution	10 ml
	Epinephrin solution	4 ml
Physiological saline	Sodium chloride	9 g
	Water added to	1,000 ml
Running buffer, 10×	Tris-HCl (0.25 M)	30.3 g
	Glycin (1.92 M)	144 g
	SDS	10.0 g
	Water added to	1,000 ml
Sample loading buffer, 4×	Tris-Hcl (1M pH 6.8)	13 ml
	SDS	6 g
	2-Mercaptoethanol	10 ml
	Glycerine	20 ml
	Bromophenol blue	10 mg
	Water added to	50 ml
Separating gel	Acrylamide (30%), bis-acrylamide (0.8%)	5 ml
	Tris (1 M pH 8.8)	5.60 ml
	SDS (10%)	0.15 ml
	APS (20%)	30 µl
	TEMED	10 µl
	Water	4.25 ml

Stacking gel	Acrylamide (30%), bis-acrylamide (0.8%)	1.50 ml
	Tris-HCl (1M pH 6.8)	1.30 ml
	SDS (10%)	0.15 ml
	APS (20%)	30 µl
	TEMED	10 µl
	Water	7.05 ml
TAE buffer, pH 8, 50X	Tris-HCl	242 mg
	Acetic acid	57.1 ml
	EDTA (186.1 mg/ml)	100 ml
	Water added to	1,000 ml
TBE buffer, 10X	Tris-HCl	108 g
	Boric acid	55 g
	EDTA	40 ml
	Water added to	1,000 ml
TBS	Tris-HCl	121.14 g
	Water added to	1,000 ml
TBST	Tween-20	1 ml
	TBS added to	1,000 ml
TE buffer, 1X	Tris-HCl (1M)	10 ml
	EDTA (186.1 mg/ml)	2 ml
	Water added to	1,000 ml
Washing buffer	Tris-HCl (1 M pH 7.5)	500 µl
	Lithium chloride (LiCl)	1,500 µl
	EDTA (0.005 M pH 8.0)	1,000 µl
	Water added to	50 ml

X-gal solution	X-gal	50 mg
	N,N´-dimethylformamide	1 ml

3.2.4 Kits

CEQ DTCS-Quick Start Kit:	Beckman Coulter (CA, USA)
ECL Plus Western Blotting Detection:	Amersham Biosciences, (Buckinghamshire, UK)
GenEluteTM Plasmid Miniprep Kit	Sigma (Steinheim, Germany)
pGEM®-T vector:	Promega (Medison, USA)
In Situ Cell Death Detection Kit :	Roche Diagnostics GmbH, Germany

3.2.5 Software

ABI PRISM® 7000 Sequence	Applied Biosystems, Foster city, USA
BLAST program	http://www.ncbi.nlm.nih.gov/BLAST/
Image analysis	BIo-Rad Laser Sharp MRC-1024 CLS Software
Multi sequence alignment	http://prodes.toulouse.inra.fr/multalin/multalin.html
Primer Express® Software	Applied Biosystems, Foster city, CA, USA
Restriction enzyme analysis	http://tools.neb.com/NEBcutter2/index.php
Weight to Molar Quantity	http://www.molbiol.ru/eng/scripts/01_07.html
SAS (version 8.02)	SAS Institute Inc, NC, USA

3.2.6 Equipments

ABI Prism® 7000 Sequence	Applied Biosystems (Foster Detectio System City, CA, USA)
Binocular microscope (TS-100)	Nikon TS-100 inverted microscope, (Japan)
Carbon dioxide incubator (BB16)	Heraeus (Hanau, Germany)
Carbon dioxide incubator (MCO-17AI)	Sanyo (Japan)
Centrifuge	Hermle (Wehingen, Germany)
CEQ™ 8000 Series Genetic Analysis System	Beckman Coulter GmbH (Krefeld, Germany)
CLSM LSM 510	Carl Zeiss (Germany)
Cryotube	Nunc (Roskilde, Germany)
Electrophoresis chamber	BioRad (Munich, Germany)
Epifluorescence microscope	Leica (Bensheim, Germany)
Four-well dish	Nunc (Roskilde, Germany)
HERA safe Bioflow safety hood	Heraeus Instruments, Meckenheim
Incubator (BB16)	Heraeus (Hanau, Germany)
Injection capillary (K-MPIP-3335-5)	Cook (Ireland)
Microinjector pipettor (Femto Jet 5247)	Eppendorf (USA)
Millipore apparatus	Millipore Corporation (USA)

Nitrocellulose transfer membrane (Protran®)	Schleicher & Schuell BioScience, (Germany)
PCR thermal cycle (PTC 100)	MJ Research (USA)
pH meter	Kohermann
Power Supply PAC 3000	BioRad (München, Germany)
Power Supply Mini-Protan®	BioRad (Italy)
Spectrophotometer, Ultrospec™ 210(*pro* UV/Visible	Amersham Bioscience (Munich, Germany)
Trans/Blot®Semi/Dry transfer Cell	BioRad (CA, USA)
Tuttnauer autoclave	Connections unlimited (Wettenberg Germany)
Ultra low freezer (-80^{o}C)	Labotect GmbH (Göttingen, Germany)
Ultraspec 2100 pro spectrophotometer	Amersham Biosciences (Buckinghamshire UK)

3.3 Methods

3.3.1 Experiment 1

3.3.1.1 *In vitro* embryo production

Bovine ovaries were collected from two different local slaughterhouses and transported to the laboratory in a thermoflask containing 0.9% physiological saline solution at 39°C. Cumulus oocyte complexes (COCs) were aspirated from follicles (2-8 mm in diameter) with 18-gauge needle and COCs with multiple layers of cumulus cells were selected for in vitro maturation. The selected oocytes were washed in maturation medium (modified Paker medium, PMP) supplemented with 15% oestrus cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 µg/ml gentamycin sulphate and 10 µl/ml FSH (Folltropin, Vetrepharm, Canada) before set into culture. The COCs were cultured in groups of 40 in 50 µl of maturation medium under mineral oil in four-well dishes (Nunc, Roskilde, Denmark). Maturation was performed at 39°C for 24 hours under humidified atmosphere containing 5% CO_2 in air. A group of 50 matured oocytes were transferred into a four-well dishes containing fertilization medium.

Matured oocytes were washed two times in the fertilization medium and transferred into a four-well dish containing 400 µl of fertilization medium supplement with 6 mg/ml bovine serum albumin (BSA), 2.2 mg/ml sodium pyruvate and 1 mg/ml heparin. Ten microliter of PHE medium was added to each well and covered with mineral oil (Sigma).

The sperms of selected bull were thawed and motile spermatozoa were obtained by swim-up procedure (Parrish et al. 1988). Sperm cell were incubated in a tube containing 5 ml capacitating medium supplement with heparin for 50 min at 39°C in an incubator with humidified atmosphere of 5% CO_2. The motile sperm cells found in the upper layer of the solution were transferred into new falcon tube. The sperm cells collected by centrifugation at 10,000 rpm for 10 min. The sperm cell pellets were washed two times and then resuspended in 3-5 ml capacitating medium and further used for the *in vitro* fertilization.

The motile spermatozoa were added to the fertilization medium with a final concentration of $1x10^6$ spermatozoa/ml and added to a group of 50 oocytes in each well. Sperms and

oocytes were co-cultured for 18 hr at standard incubation conditions, 39ºC and humidified atmosphere containing 5% CO_2.

After IVF, the presumptive zygotes were transferred into 15 ml falcon tube containing 1 ml of culture medium (CR1aa) supplemented with 10% OCS, 10 µl/ml BME (essential amino acids) and 10 µl/ml MEM (non essential amino acids). The fertilized oocytes were gently vortexed to separate them from dead spermatozoa and the cumulus cells. The cumulus free zygotes were selected and washed two times with culture medium before being transferred in group of 50-60 cells into four-well dish, each well containing a 400 µl culture medium cover with mineral oil.

3.3.1.2 Microinjection of zygotes

Each groups of 50-70 zygotes were placed in 10 µl droplet injection medium (H-TCM) under mineral for microinjection. Dharmacon synthesized smartpool siRNA (SpsiRNA), 5-aza-2'-deoxycytidine (5-AZA), and nuclease-free water were used. The microinjection was performed with a 0.5 µm diameter injection capillary (Femtojet II, Eppendorf, USA) under an inverted microscope (Nikon TS-100). The injection volume of ~7 pl was estimated from the displacement of the minisque of mineral oil in the capillary. All groups of injected zygotes were cultured for 3-4 hr after that the survival rates were recorded. For this experiment, a total of 1470 zygotes were produced and categorized into four groups: those injected with SpsiRNA (n= 374), 5-AZA (n=382), nuclease-free water (n=380), and uninjected control zygotes (n=340) (control).

3.3.1.3 Embryo collection

Eight-cell stage and Day 8 blastocyst embryos were collected at specific time points to access the effects of suppression and inhibition on mRNA transcript abundance and protein expression using RT-PCR and western blotting analysis. During experiment 1 (Figure 4), each group of 50-70 injected zygotes were cultured for 48 and 72 pmi to allow phenotypic development, those zygotes used for transcription and protein expression studies were collected at 8-cell stage. In experiment 2 (Figure 5), each group of 50-70 injected zygotes were cultured *in vitro* until Day 8 blastocyst to assess the developed and resulting blastocysts. For each treatment group of blastocyst was used for transcription, protein expression and apoptosis studies. All samples from 8-cell and blastocyst were

washed two times with PBS (Sigma) and treated with acidic Tyrode pH 2.5-3.0 (Sigma) to dissolve the zona pellucida. The zona free embryos were further washed two times in drops of PBS and frozen in cryo-tubes containing lysis buffer. The samples for western blot study were additionally treated with protease inhibitor (Sigma). Finally, all embryos were stored in liquid nitrogen.

3.3.1.4 RNA isolation and cDNA synthesis

Three pools biological replicates of 20-25 embryos per each pool from each treatment group of 8-cell stage and 10-15 embryos per each pool from each treatment of Day 8 blastocysts were used for mRNA isolation using T7-oligo $(dT)_{23}$ attached magnetic beads (Dynal, Oslo, Norway) according to the manufacture's instruction. The isolated mRNA samples were eluted in 11 µl DEPC treated water and reverse transcribed in 20 µl of total reaction volume containing 1 µl oligo $(dT)_{23}$ primer, 4 µl 5x first stand buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris –HCl pH (8.3), 2 µl DTT, 1 µl dNTP, 0.3 µl RNase inhibitor (Promega) and 0.7 µl superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). Messenger RNA and oligo $(dT)_{23}$ primer were mixed and incubated at 70ºC for 3 min and placed on ice until the remaining reaction components were added. The reaction was incubated at 42ºC for 90 min and terminated by heat inactivation at 70ºC for 15 min.

3.3.1.5 Preparation of RNA template for RT-PCR quantification

Primer pairs were designed according to the bovine cDNA sequences as assigned in GenBank (see Table 3 for details) using Primer Express® Software v2.0 (Applied Biosystems). The primers were designed to amplify fragments of the genes covering the coding sequence. The identity of the product was confirmed by sequencing. PCR amplification was performed in a 20 µl reaction volume containing 1.0 U *Taq* DNA polymerase, 50 ng of each primer, 200 µM of each dNTP, 2.0 µl 10x PCR buffer, and 2 µl RNA template. PCR started with denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing step (temperatures as indicated in table 3) for 30 s ,and 72°C for 1 min. The final extension was at 72°C 10 min. The PCR product was visualized on 0.8% agarose gel stained with ethidium bromide.

The amplified fragments were cut from the 0.8% agarose gel and displaced in 1.5 ml tube and kept at -20°C overnight. The product fragments with the gel were homogenized in 500 ml 1x TE buffer. Five hundred microliters of phenol: chloroform: isoamyl (1:1:1 v/v) were added to the homogenized solution and mixed well by vortexing. The mixture was centrifuged at 12,000 rpm for 10 min at 4°C, then the aqueous phase of the solution was transferred to new 2.0 ml tube followed by an equal volume of chloroform. The mixture was centrifuged by 12,000 rpm for 10 min at 4°C and the aqueous phase of the solution was transferred to a new 2.0 ml tube. 1:10 volume of sodium acetate (3 M, pH 5.2) and an equal volume of 100% ethanol was added. The DNA was allowed to precipitate at -20°C overnight. Thereafter, the pellet was recovered by centrifugation for 30 min at 4°C. The pellet was washed two times with 75% ethanol and resuspended in 7 µl ddH_2O and stored at -20°C until further use.

3.3.1.6 Cloning and transformation

The fragments isolated from the agarose gel were then ligated using pGEM®-T vector (Promega). The ligation reaction was performed in 5 µl reaction volumes containing 2.5 µl ligation buffer, 0.5 µl vectors, 0.5 µl T4 DNA ligase (3 U/µl) and 1.5 µl RNA template. The reaction was incubated at 4 °C overnight.

Table 3: Details of the primers for gene expression study by RT-PCR

Gene	Primer sequences	Annealing temperature (°C)	Product size (bp)
DNMT1 (AY244709)	5'-AGGGAGACGTGGAGATGCTG-3'	57	194
	5'-CATGGAGCGCTTGAAGGAG-3'		
DNMT1 set A[1] (AY244709)	5'-GCCTTCTCACTGCCTGACGAT-3'	57	193
	5'-TAGCCAGGTAGCCCTCCTCA-3'		
DNMT1 set B[1] (AY244709)	5'-AGAACGGGAGCCAGACAAGTG-3'	57	221
	5-CCCCGTGGGAAATGAGATG-3'		
DNMT3a (AY271298)	5'-AGACATGTGGGTTGAACCCG-3'	58	188
	5'-GGCTCCCACAAGAGATGCAG-3'		
Dnmt3b (AY244710)	5'-CAGGATGGGAAGGAGTTTGGA-3'	56	151
	5'-CACCAAACCACTGGACCCAC-3'		
IGF2[2] (X53553)	5'-GGGATCAGAACAACATCTCT-3'	58	176
	5'-GCTAGTTTGCTTTTCTGGTG-3'		
IGF2R[2] (AF342811)	5'-GCCTACAGCGAGAAGGGGTTAGT-3'	62	293
	5'-GAAAAGCGTGCACGTGCGCTTGT-3'		
IGFBP-4[2] (NM174557)	5'-TGTGAGGAGCTGGTGCGAGA-3'	60	281
	5'-TTCGTGGGTGCGGCTCTGT-3'		
GAPDH	5'-AATGGAAAGGCCATCACCATC-3'	60	203
	5'-GTGGTTCACGCCCATCACA-3'		

(1)Primer designed for semi-quantitative RT-PCR

(2)Primers from Li et al. (2007)

Three microliters of the ligation product was co-incubated with 60 µl DH5α E. *coli* competent cells (Stratagen) for 30 min on ice. The mixture was heat shocked by putting it into a 42°C water bath for 90 s and immediately transferred on ice for 2 min. LB-broth (750 ml) was added to the bacteria solution and shaked at 150 rpm at 37°C for 90 min. Each bacterial suspension was plated on two ampicillin containing LB-agar plates. The medium contained 20 µl X-gal and IPTG solutions, incubated at 37°C was done overnight. Colonies were differentiated by the activity of ß-galactosidase as white and

blue for the presence of inserted DNA fragment. Due to the activation of LacZ gene on the vector, colonies containing the insert target DNA appear as white colonies and those with active LacZ gene without insert DNA formed blue colonies.

To identify the inserted fragments, two white colonies were picked up from each plate and suspended in 30 µl 1X PCR buffer. One blue colony was picked up as a control to differentiate the presence of the target insert by comparing the length of amplified DNA fragments from white and blue colonies with M13 primers.

3.3.1.7 M13 amplification

M13 PCR was performed to confirm the insertion of the fragment into the plasmid. Bacterial suspensions were boiled at 95°C for 10 min and then the solution was used as template. The M13 PCR was carried out in a 20 µl reaction including 1 µl 10x PCR buffer, 10 µl lysed bacterial solution, 0.5 µl dNTP (10 mM), 0.5 µl (10 µM) of each M13 primer (forward: 5'-TTG-TAA-AAC-GAC-GGC-CAG-T-3'; reverse: 5'- CAG-GAA-ACA-GCT-ATG-ACC-3') and 0.1 U Taq polymerase. The PCR reaction was performed with a thermal cycling program of 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 70°C for 1 min and an additional extension step for 10 min at 72°C. An aliquot of 5 µl PCR product was then electrophoresed in 2% (w/v) agarose gel with 0.8 µg/ml ethidium bromide in 1xTAE buffer. Under UV-transilluminator, length differentiation of PCR fragments was identified. The successful white colony ligation was kept and then cultured in 5 ml LB-broth at 37°C overnight in a shaking incubator for plasmid isolation.

3.3.1.8 Plasmid isolation

Plasmid was isolated using the GenElute™ Plasmid Miniprep Kit followed the manufacturer's instructions. Briefly, 5 ml of bacterial culture were centrifuged at 14,000g for 1 min for harvesting cells, the supernatant was discarded. These cells were resuspended and vortexed in 200 µl of resuspension solution before adding 200 µl of lysis solution. The mixture was subsequently mixed by inversion of tubes until it became clear and viscous. After incubating at room temperature for 4 min, cell precipitation was done by adding 350 µl of neutralization/binding buffer, mixed gently and centrifuged at 14,000 g for 10 min. The GeneElute Miniprep column was prepared by adding 500 µl of preparation solution, centrifuging shortly and discarding the flow-through. After that, the clear supernatant was transferred to this binding column and centrifuged at 14,000 g for 1 min. The flow-through was discarded and the column was washed by adding 750 µl of wash solution followed by centrifugation at 14,000 g for 1 min. To elute plasmid, the column was transferred to a fresh collection tube; 50 µl of ddH_2O was added and centrifuged at 14000 g for 1 min. The column was discarded and the plasmid was then collected. For determination of plasmid size and quality, 5 µl of plasmid together with 2 µl loading buffer was checked by agarose gel electrophoresis. In addition, the quantity of the plasmid was measured by reading the absorbance at 260 nm in a spectrophotometer UV/visible light (Beckman Du® 62). The plasmid was kept at -20°C for further used to set up the standard curve for RT-PCR.

3.3.1.9 Sequencing

The fragments inserted in the plasmid were sequenced using CEQ™ 8000 Series Genetic Analysis System (Beckman Coulter). A mixture of 1 µl of ExoSAP-IT with 5 µl of PCR product was incubated at 37°C for 30 min followed by ExoSAP-IT inactivation at 80°C for 15 min to purify the PCR product. Five microliters of purified sample were used for sequencing, with specific primers (Table 3), and Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter). Twenty microliter of sequencing PCR were performed for each primer. 3 M NaOAc, 100 mM EDTA, and glycogen were added to the PCR product. After that 60 µl of 100% ethanol were added and mixed well by vortexing, then centrifuged at 18,000 rpm at 4°C for 15 min. The liquid was removed and the pellets were washed twice times with 200 µl 70% ethanol. Finally, the ethanol was removed and the sample was air dried. The sample was then resuspended in 40 µl of sample loading

solution (SLS) (Beckman Coulter). Samples were transferred to a CEQ sample plate and overlaid with mineral oil and then sequenced using CEQ™ 8000 Genetic Analysis System. The completed sequencing result of the inserted fragment was compared by using BLAST program (http://www.ncbi.nlm.hih.gov/BLAST/). The fragments from cloning were considered to be right gene if they were identified with the fragment from GenBank with a percentage of ≥90.

3.3.1.10 Gene expression analysis by semi-quantitative RT-PCR

The cDNA from immature oocyte, mature oocyte, 2-cell, 4-cell, 8-cell, 16-cell, blastocyst and muscle was performed to measure the DNMT1 expression isoforms using semi-quantitative RT-PCR. GAPDH was adopted to give the same plateau phase PCR signal strength. Primer sequence and optimal PCR annealing temperature are listed in Table 3. The PCR program initially started with a 95°C denaturation for 5 min, followed by 29 cycles of 94°C for 30 s, annealing step for 30 s and 72°C for 1 min. The final extension was at 72°C 10 min. The PCR product was visualized on 2.0% agarose gel stained with ethidium bromide.

3.3.1.11 Gene expression analysis by quantitative RT-PCR

The ABI Prism® 7000 apparatus (Applied Biosystems) was used to perform the quantitative real time PCR analysis using SYBR® Green Universal PCR Master Mix (Applied Biosystem) incorporation for dsDNA-specific fluorescent detection dye. The amount of cDNA present in a sample was measured as a function of how quickly a fluorescent signal is first observed above threshold (C_T value) during the process of sequence amplification. The threshold cycle (C_T) is the point at which the fluorescence values are recorded during every cycle and represents the amount of the product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach this point.

The plasmid concentration was converted into number of copies (molecules) using the program Weight to Molar Quantity (www.molbiol.ru/eng/scripts/01_07.html). The plasmid solution was diluted several folds to be at a concentration range similar to the target in the embryos. Serial dilutions were freshly prepared for RT-PCR from 10^1 to 10^8 copy numbers in 50 µl volume.

The SYBR® Green dye can bind to any DNA and generate fluorescence. Therefore an additional verification was achieved by plotting fluorescence as a function of temperature to generate a melting or dissociation curve of the amplicon, which is sequence specific. This curve has been used to distinguish between the amplicon and non-specific DNA. The dissociation curve was produced at the end of PCR by monitoring fluorescence continuously while slowly heating the sample from 60°C to 95°C at 0.2°C intervals to observe the loss of fluorescence at the denaturing temperature. As the products specificity is determined entirely by its primers, an optimization step has been done for each pair of primers before quantification.

Optimization of the primers concentration has been done to determine the minimum primer concentrations giving the lowest threshold cycle without primer dimer formation. For each primer pairs, nine reactions using different combinations of each primer with a total 20 µl were carried out. The dissociation curves generating the lowest primer dimer by observing the absence of non specific amplification, the combination was selected for target quantification.

GAPDH was quantified as endogenous control for RT-PCR. The DNMT1 transcript was quantified at 8-cell and blastocyst stage to assess the suppression and inhibition of DNMT1. Moreover, independent transcript of DNMT3a and DNMT3b had been quantified at 8-cell stage to assess the specificity of mRNA suppression by the SpsiRNA. In addition, the IGF2, IGF2R, and IGFBP-4 transcripts have been also quantified to investigate the subsequent suppression and inhibition of DNMT1 at blastocyst stage. The ABI Prism® 7000 apparatus was used to perform the quantitative analysis using SYBR® Green Universal PCR Master Mix (Applied Biosystem) incorporation for dsDNA-specific fluorescent detection dye. Quantitative analyses of all studied transcripts were performed in comparison with GAPDH as an endogenous control and were run in separate wells. The primer sequences were designed for RT-PCR amplification according to the bovine cDNA sequence (Table 3) using Primer Express® Software v2.0 (Applied Biosystems). Standard curves were generated for both target and endogenous control genes using serial dilutions of plasmid DNA (10^1–10^8 molecules). The RT-PCRs were performed in 20 µl reaction volume containing 10.2 µl SYBR® Green Universal PCR Master Mix (Applied Biosystem), optimized concentration of gene specific forward and reverse primer

combinations, and 2 µl embryonic cDNA. During each PCR reaction samples from the same cDNA source were run in duplicate to control the reproducibility of the results. A universal thermal cycling program with an initial denaturation step at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s and 60°C for 60 s was used to quantify each gene of interest. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7 s interval until the temperature reached 95°C.

3.3.1.12 Protein analysis by western blotting

A total of 80 8-cell stage and 40 of Day 8 blastocyst embryos from each treatment group and uninjected control were lysed with sample loading buffer to denature protein structure. The equal amount of protein normalized by adjusting the same numbers of embryos from each treatment groups were used for protein analysis. The embryos were homogenized in sample loading buffer by boilng at 95 °C for 5 min.

Twelve percentage acrylamide SDS-PAGE gel was used for protein segregation. Two solutions were prepared a separating gel and a stacking gel. A separating gel was used to separate proteins into their respective sizes allowing sharp bands to be seen, whereas the stacking gel organizes proteins before they enter the separating gel. Glass plates were cleaned thoroughly using soap and distilled water and finally cleaned using 70% ethanol. The sandwich glass plates were introduced into the support piece and placed on a flat surface. First, the separating gel (30% acrylamide, 0.8 % bis acrylamide, 1M Tris-HCl with pH 8.8, 10% SDS, 20% APS, TEMED) was prepared, quickly mixed and poured in 3/4 volume of sandwich glass plate and the remaining space (1/4 volume) was filled with isopropanol to avoid air bubbles and normalize the surface the gel. The isopropanol was poured off after polymerization took place. The stacking gel (30% acrylamide, 0.8% bis acrylamide, 1M Tris-HCl with pH 6.8, 10% SDS, 20% APS, TEMED) was added on the top of separating gel and then a 10 well comb was inserted.

The sandwich glass plates were fixed with the U-shape rubbers and then put into the chamber. The upper reservoir was filled with running buffer and then the samples were loaded in the prepared wells. The SDS-PAGE was run 1 hr with a standard vertical gel electrophoresis apparatus (BioRad) at 10 mA.

Proteins were then transferred into a nitrocellulose transfer membrane (Protran®, Schleicher&Schuell, BioScience) using the Trans-Blot Semi-Dry Transfer Cell (BioRad). A transfer membrane was prepared by soaking it in wetting solution for a few seconds. Three pieces of filter paper were wetted in anode buffer I and placed on the anode plate of the blotter. Three piece of filter paper were soaked in anode buffer II and placed on top of filter papers previously placed on electrode. The membrane was equilibrated in water for 5 min, then removed from water and placed on top of filter paper stack. The gel was placed on top of the transfer membrane and finally, three pieces of filter paper were soaked in cathode buffer and placed on top of the gel. Time of transfer was 1 hr using 100 mA per each gel (1.75mA/cm^2/hr). After transfer was completed, the blot membrane was washed two times with water and then stained with ponceau-S (Roth). The blot membrane was washed twice times in TBST (10 min/time).

The blot membrane was placed into blocking buffer (1% PVP). It was incubated on the shaker at room temperature for 1 hr to prevent a non-specific absorption of the immunological reagent. The blocking solution was poured off and 10 ml of primary antibody in 0.1% blocking buffer was added. The primary antibody, anti-mouse monoclonal to DNMT1 protein (Abcam, Cambridge, UK) was used at a dilution of 1:800 of 0.1% PVP in TBST. The primary antibody was poured off and then the blot membrane was washed for 10 min in 10 ml washing buffer. It was incubated overnight on shaker at 4°C. The washing step was done with 20 ml TBST six times 10 min each. The secondary antibody, horseradish-peroxidase (HRP) conjugated anti-mouse antibody (Abcam, Cambridge, UK) was used a dilution of 1:25,000 in 0.1% PVP in TBST. The blot membrane was then incubated with 10 ml of secondary antibody on shaker at room temperature for 1 hr. The blot membrane was washed with 20 ml TBST six times 10 min each.

The ECL Plus Western Blotting Detection (Amersham Biosciences) was employed using the manufacturer's protocol. The detection solution A and solution B were mixed in a ratio of 40:1 and the final volume of detection reagent required was 0.1 ml/cm^2. The mixed detection reagent was poured on the blot membrane followed by incubation for 5 min at room temperature. The blot membrane was placed on to a fresh piece of saran wrap. The wrapped membrane was placed side up in an X-ray film cassette. A sheet of

autoradiography film (Kodak®Biomax XAR film, Kodak) was placed to cover over membrane. The film was exposed for 5-30 min in the dark room. To visualize the film, the exposed film was then soaked in developer solution (Kodak®Kodak) for 5 min, water for 5 min, and finally fixed with fixation solution for 5 min (Kodak®Kodak).

3.3.1.13 DNA fragmentation detection by TUNEL staining

A total of 114 blastocysts, those injected with SpsiRNA (n= 40), 5-AZA (n=22), nuclease-free water (n=25), and uninjected control (n=27) were fixed in 4% paraformaldehyde (P-6148, Sigma, Germany) in PBS for 1 hr at room temperature, and then washed three times with PBS. Embryos were placed into 50 µl drops of TUNEL reagent (Roche Diagnostics GmbH, Mannheim, Germany) covered with paraffin oil and incubated at 37°C for 60 minutes under dark and moist conditions. Negative control embryos were apoptosis induced by using the protein kinase inhibitor staurosporine (STS). Embryos were incubated in culture media containing 10 µM STS for 26 hr and they were finally subjected to TUNEL reagent in the absence of deoxynucleotide transferase enzyme. Positive control embryos were treated with 50 IU/ml of DNase (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min at 37°C before incubation with TUNEL reagent. Counter staining was performed by incubating embryos in the PBS solution containing 6.5 µg/ml bisbenzemide (Hoechst H33528, Sigma) for 4 min followed washing three times in PBS. Embryos were mounted on glass slides and examined under an epifluorescence microscope (DM-IRB, Leica, Bensheim, Germany). Two standard filters, DAPI filter (emission wavelength: 425 nm) were employed to determine the number of inner cell mass (ICM) and trophectoderm (TE) cells, while FITC (emission wavelength: 512 nm) was used to detect TUNEL stained nuclei. The total number of cells and the number of cells with DNA fragmented nuclei were counted.

3.3.2 Experiment 2

3.3.2.1 DNA isolation

Sperm samples were thawed from -80°C and then mixed with 4 ml 0.9% sodium chloride solution. The mix was centrifuged at 5,000×g for 10 min and the supernatant was discarded. The pellet was resuspended in 4 ml digestion buffer. In order to digest protein in the pellet suspension, 4 ml lysis buffer containing proteinase K, SDS and mercaptoethanol were added and the samples were incubated at 56°C overnight. Two

times an equal volume of phenol-chloroform (1:1 v/v) was added and mixed thoroughly and centrifuged at 5,000×g for 10 min. Two layers of aqueous supernatant solution were formed and the upper layer was collected and transferred into fresh tubes. To the supernatant, one-tenths volume of 3 M sodium acetate (pH 5.2) was added followed by an equal volume of isopropanol and gently shaking until precipitation of DNA. The DNA was washed with 200 µl of 70 % ethanol twice and dried at room temperature. The DNA pellet was resuspended with 1 ml TE buffer and kept at 4°C. The DNA was diluted to a final concentration of 50 ng/µl.

Embryos were digested with 10 µl of lysis buffer, a solution containing 1.13 mg/ml proteinase K in 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 0.5% Tween 20. The digestion was carried out at 56°C overnight. Finally, the digestion was heated to inactivate the proteinase K at 98°C for 8 min. The lysed sample was stored at 4°C for further genotyping.

3.3.2.2 Genotyping

Bull sperms and embryos were genotyped for single nucleotide polymorphisms of DNMT1 (C/T), DNMT3a (C/T), and DNMT3b (A/G) with different primer pairs as shown in Table 4. PCR amplification was performed in a 20-µl reaction volume containing 1.0 U *Taq* DNA polymerase, 50 ng of each primer, 200 µM of each dNTP, 2.0 µl 10x PCR buffer, and 2 µl DNA template. PCR started with denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing (temperatures as indicated in table 4) for 30 s and 72°C for 1 min. The final extension was at 72°C 10 min. In addition to genotyping the embryos, the PCR products were amplified in a nested PCR reaction using primer pairs; DNMT1 (forward primer: 5'-CAG-TGC-CTC-CAG-GAC-TTC-TC-3', reverse primer: 5'-TTC-CGA-ACG-TTC-TCC-AAG-AG-3'), DNMT3a (forward primer: 5'-CTC-TCC-TCC-GTC-CTG-AGT-TG-3', reverse primer: 5'-CCG-AGA-GAG-GCT-CTA-CAT-GC-3'), and DNMT3b (forward primer: 5'-TAA-GAC-TGT-GTG-GCC-CTG-TG-3, reverse primer: 5'-ACT-GGC-ACA-ATG-GTT-CT-TCC-3'), respectively. The nested PRC reaction included 4 µl of PCR product, 50 ng of each primers, 200 µM of each dNTP, 2 µl of 10x PCR buffer, and 1 U *Taq* DNA polymerase. The temperature cycles were as described for the first PCR.

Table 4: Detail of primers used for genotyping

Gene	Primer sequences	Annealing temperature (°C)	Product size (bp)
DNMT1 (AY244709)	5'-TTGAAGATCCTCCGAACCAC-3'	60	602
	5'-GACCACCAGGGAGTTCTTGA-3'		
DNMT3a (NW001492965)	5'-CACACGCACACACACTGAAG-3'	60	611
	5'-TCCTTTTAGCCAGTGGAGTA-3'		
DNMT3b (NW_001493157)	5'-CTGAAAGGAATCCAGCTTGC-3'	60	641
	5'-TGAGTGGGTCCATTCCTCTC-3'		

DNA samples from bulls and embryos were genotyped using PCR-RFLP (restriction fragment length polymorphisms) method. The restriction enzymes were selected according to their recognition size (http://tools.neb.com/NEBcutter2/index.php) of the polymorphism. For genotyping gene at the locus DNMT1 (position: 3593), DNMT3a (position: 308446), and DNMT3b (position: 337339), PCR products were digested with the restriction enzymes BcnI, BspLI, and NdeI (Fermentas, Germany), respectively. The digestions were carried out in 10 µl reaction volume containing 1 U restriction enzyme, 1 µl 10x restriction buffer (Fermentas, Germany), 3.9 µl ddH_2O and 5 µl PCR product. Finally, digests were incubated at 37°C for 6 hr. Digested products were then visualized on 3% agarose gel stained with ethidium bromide. The different fragment lengths between non- and digested DNAs reflected the genotype of a specific DNA sample as shown in figure 7.

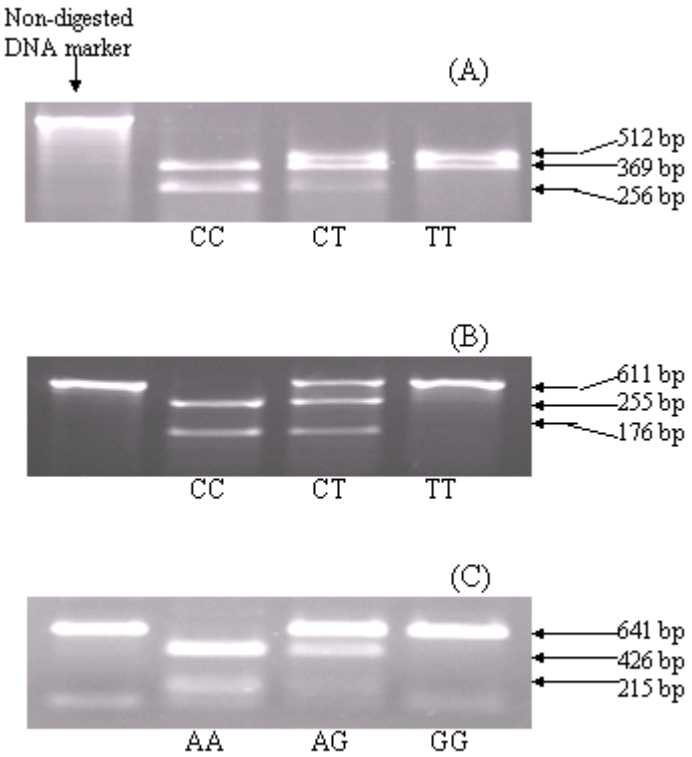


Figure 7: Picture of PCR-RFLP for genotyping of DNMT1 (A), DNMT3a (B), and DNMT3b (C) using restriction enzyme BcnI, BspLI, and NdeI, respectively.

3.4 Statistical analysis

3.4.1 Suppression of DNMT1

The mRNA expression of the gene silencing studies was analyzed based on the relative standard curve observed from the RT-PCR. The reference gene GADPH was used for normalization. The relative expression data were analyzed using the Statistical Analysis System (SAS) version 8.0 (SAS institute Inc.) software package. Differences in mean values between two or more treatment groups were tested using ANOVA variance analysis followed by a multiple pair wise comparisons using *t*-test. If the *P*-value is $\leq$ 0.05, it is considered to be significant.

3.4.2 Association analysis of DNMT1, DNMT3a, and DNMT3b sequence variant

Allele and genotype frequencies were calculated and tested for Hardy-Weinberg equilibrium by chi-square analysis.

For the association analysis of single nucleotide polymorphisms of DNMT1, DNMT3a and DNMT3b following traits were observed: NRR; semen quality traits namely VOL, CONC, MOT, and SUVR; and sperm flow cytometric parameter namely PMI, PAS, and DFI. Linear mixed model, using the procedure "Proc Mixed" in SAS were applied to the analysis. NRR was calculated by No. of non-return cows (56-days last service) dividing by No.of inseminations. The bull fertility traits were assumed to be normally distributed for each bull. Multiple pair wise comparisons were conducted using the Tukey-Kramer test. Differences of $P \leq 0.05$ were considered as significant association. All analyzes were conducted with the SAS 9.1 Package (SAS Inc., Cary, NC, USA).

3.4.2.1 NRR

The following linear mixed model was used (Model 1):

$$y_{ijkn} = \mu + TEC_i + BSTAT_j + BR_k + GEN_l + B_n + \varepsilon_{ijkln}$$

Where:

y_{ijkln}	NRR under investigation per cow i inseminated with bull n,
μ	Overall mean,
TEC_i	Fixed effect for the AI-technician, veterinarian, and farm owner who inseminated cow i,
$BSTAT_j$	Fixed effect for the status of bull, tested and approved bulls j ,
BR_k	Fixed effect for the colour of bull, black and brown bulls k
GEN_l	Fixed effect for the genotypes of the genes, DNMT1, DNMT3a, and DNMT3b under investigation for bull l,
B_n	Random effect of the bull n,
ε_{ijkln}	Error

3.4.2.2 Sperm quality traits and sperm flow cytometric parameters

The following linear mixed model was used (Model 2):

$$y_{kln} = \mu + BR_k + GEN_l + B_n + \varepsilon_{kln}$$

Where:

y_{ijkln}	Traits and parameters under investigation per cow i inseminated with bull n,
μ	Overall mean,
BR_k	Fixed effect for the colour of bull, black and brown bulls k
GEN_l	Fixed effect for the genotypes of the genes, DNMT1, DNMT3a, and DNMT3b under investigation for bull l,
B_n	Random effect of bull n,
ε_{kln}	Error

3.4.2.3 Embryo development

Analysis of variance using "GENMOD" was performed to investigate the effect of genotypes DNMT1, DNMT3a, and DNMT3b depending on embryonic development. Pair wise comparisons were done using the chi-square test. Differences of $P \leq 0.05$ were considered as significant. The following statistical model was used (Model 3):

$$y_i = \mu_i + C_i + \varepsilon_i$$

Where;

y_i	Embryonic development investigated of embryo i
μ	The overall mean of embryo i
C_i	Effect of genotype DNMT1, DNMT3a, and DNMT3b of embryo i
ε_i	Error

4 Results

4.1 Effect of DNMT1 suppression

4.1.1 DNMT1 expression isoforms and its profile

The results showed that both the primer set A and B amplified cDNA with the same PCR signal strength from muscle, immature oocyte, mature oocyte, 2-cell, 4-cell, and blastocyst except the 8-cell, 16-cell cells. (Figure 8). In addition, the cDNA product of pooled embryos was further confirmed by comparative sequence analysis with somatic cDNA from muscle (Figure 9). The results clearly indentifying the reading frame of embryonic ATG starting codon necessary to produce the DNMT1o mRNA; however, the DNMT1o was not able to amplify. Therefore, only the somatic DNMT1 isoform is expressed during bovine preimplantation stage embryos.

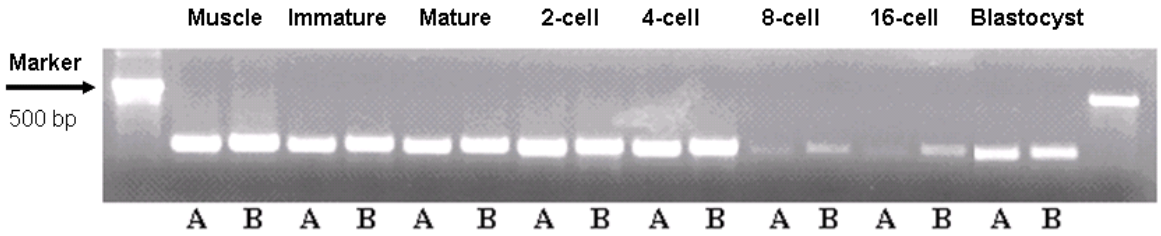


Figure 8: The semi quantitative PCR analysis demonstrating the PCR product amplified with primer set A and B using cDNA sample from different development stages of bovine preimplantation embryos. GADPH was adopted to give the same plateau phase (33 cycles) PCR signal strength (Data not shown)

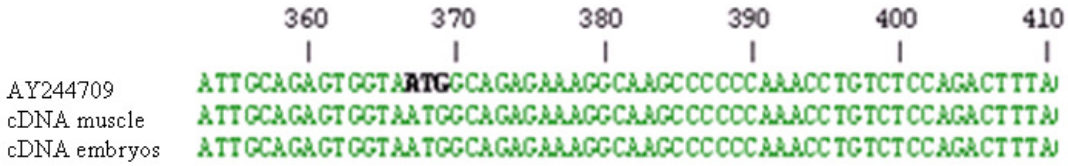


Figure 9: A sequence alignment of the oocyte-specific DNMT1 ATG initiation region of bovine DNMT1 gene from cDNA of muscle and embryos.

The DNMT1 mRNA expression profile was analysed throughout in vitro preimplantation embryo development RT-PCR (Figure 10). The relative gene expression of DNMT1 was higher during the mature oocyte stage and was gradually down-regulated at the blastocyst stage.

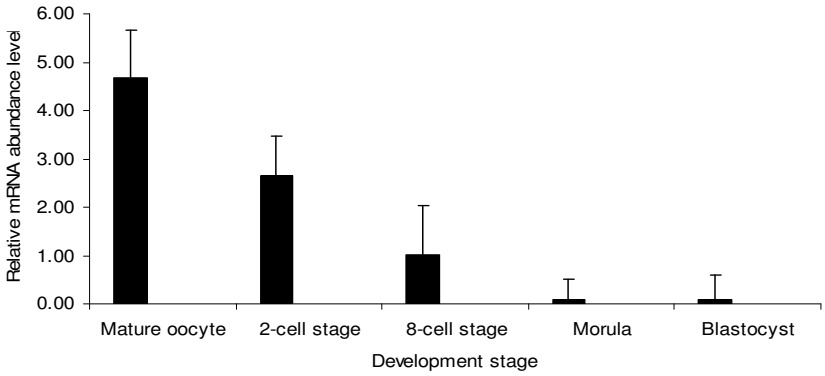


Figure 10: Relative abundance of DNMT1 mRNA (mean±SD) in *in vitro bovine* preimplantation stage embryos

4.1.2 Effect of suppression of DNMT1 on embryonic development at 8-cell and blastocyst stage

In order to avoid any effects of physical injury on further developmental competence, only those zygotes which survived the microinjection procedure were considered in the subsequent developmental data analysis. Developmental data were recorded 48 and 72 hr post microinjection (pmi). There was no significant difference among embryos of the different treatment groups on early cleavage rate at 48 hr pmi (data not shown). However, the proportions of 8-cell embryos was significantly lower *($P<0.05$)* in SpsiRNA and 5-AZA compared with embryos from water injected and uninjected control groups (Table 5).

Table 5: Proportions of phenotype development (mean±SD) of embryos in different treatment groups 72 hr pmi

Treatment	No.of zygote	Sur-vival rate	proportion of cleavage				Total cleavage
			2-cell	4-cell	8-cell	16-cell	
Uninjected	250	97.6	13.1±4.5	20.9±5.9	30.7±6.2[a]	2.0±1.0	66.8±8.8[a]
Water	350	83.7	15.3±4.4	17.9±1.9	26.8±2.9[a]	1.5±1.1	61.6±9.8[a]
SpsiRNA	352	81.3	13.9±5.4	20.5±4.5	16.3±4.5[b]	1.2±0.9	52.0±9.9[b]
5-AZA	355	80.2	16.3±3.0	20.3±3.5	17.7±4.9[b]	2.3±0.7	56.8±5.5[b]

a, b: *P<0.05*

Similarly, the proportions of embryos that developed to blastocyst stage were significantly lower *(P<0.05)* in 5-AZA compared with SpsiRNA, water injected and uninjected control (Table 6).

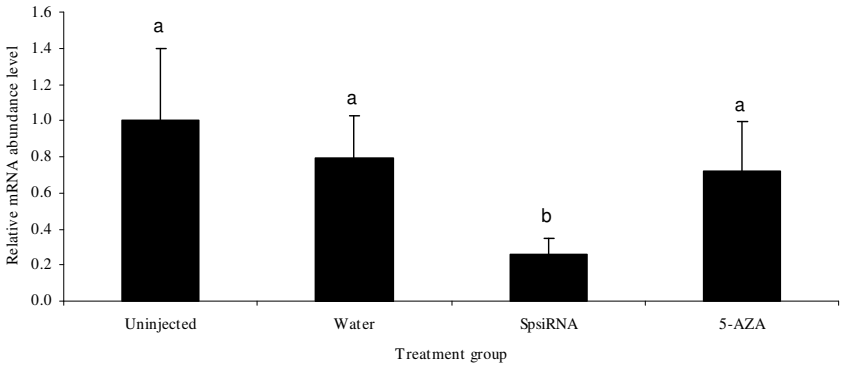
Table 6: *In vitro* development of bovine zygote at blastocyst stage after microinjection of water, SpsiRNA and 5-AZA

Treatment	No. zygotes	Survival rate (%)	Blastocyst rate (%)		Total blastocyst (%)
			Day 7	Day 8	
Uninjected	230	96.1	14.5±4.3[a]	14.9±2.7	29.4±2.1[a]
Water	350	82.7	10.4±3.2[a]	13.7±2.4	24.1±5.3 [a]
SpsiRNA	352	81.8	9.0±3.3[a]	14.2±6.1	23.4±2.1[a]
5-AZA	357	85.2	6.8±1.2[b]	10.1±4.9	16.9±4.9 [b]

a, b: *P<0.05*

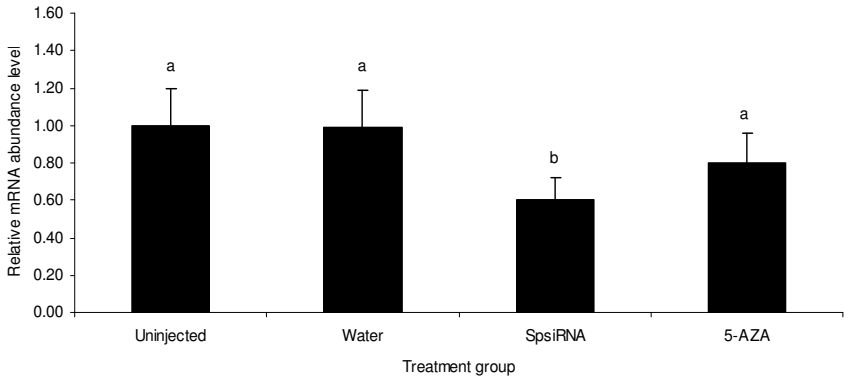
4.1.3 Effect of microinjection of SpsiRNA and 5-AZA on mRNA expression

As shown in table 5, the proportions of 8-cell embryos and blastocysts were lower in SpsiRNA and 5-AZA treated groups compared to embryos from water injected and uninjected control groups. Moreover, the selective suppression of DNMT1 mRNA was also confirmed by quantification of the DNMT3a and DNMT3b mRNA in the treatment groups. The result showed that the microinjection of SpsiRNA had reduced *(P<0.05)* the target mRNA by 80 and 50% in 8-cell and blastocyst stage embryos, respectively, compared to uninjected control groups (Figure 11 and Figure 12).



a, b: *P<0.05*

Figure 11: Relative abundance of DNMT1 mRNA levels (mean±SD) of 8-cell stage embryos from different treatment groups.



a, b: *P<0.05*

Figure 12: Relative abundance of DNMT1 mRNA abundance levels (mean±SD) at blastocyst stage from different treatment groups.

Microinjection of 5-AZA had no effect on DNMT1 mRNA levels at neither 8-cell stage embryos nor blastocyst stage. Microinjection of SpsiRNA and 5-AZA had no effect on the relative abundance of DNMT3a and DNMT3b observed at 8-cell stage *in vitro* bovine preimplantation stage embryos (Figure 13).

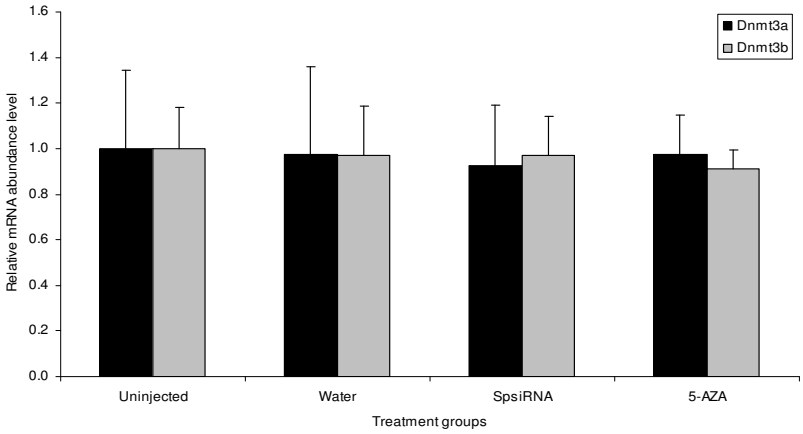


Figure 13: Relative abundance of DNMT3a and DNMT3b mRNA (mean±SD) at 8-cell stage embryos from different treatment groups.

4.1.4 Effect of DNMT1 mRNA suppression on protein product

Western blot analysis was performed to determine the subsequent suppression of DNMT1 mRNA using proteins extracted from each treatment group. As shown in figure 14 (A), reduced the intensity of DNMT1 protein (MW 198 kDa) at 8-cell stage embryos. However, microinjection of SpsiRNA had no affect on degradation of protein at blastocyst stage (Figure 14 B). In addition, microinjection of 5-AZA had no significant effect on protein found both at 8-cell and blastocyst stage embryos.

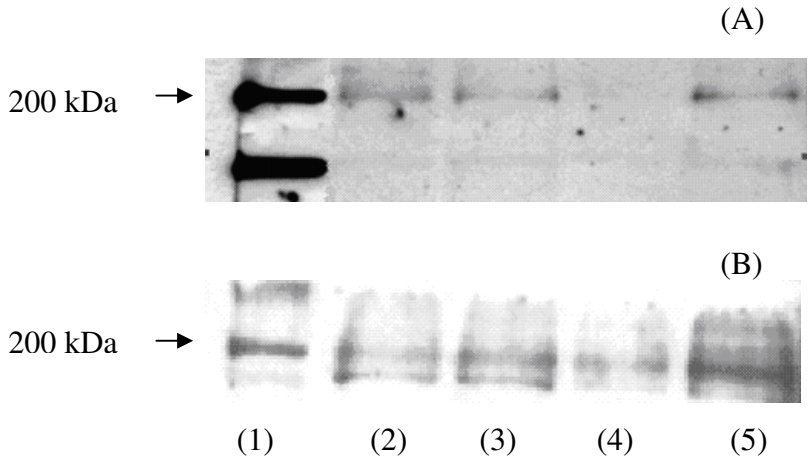


Figure 14: Western blot analysis for detection of DNMT1 protein at 8-cell stage (A) and blastocyst stage (B). (1) Marker, (2) Uninjected, (3) Water, (4) SpsiRNA, (5) 5-AZA

4.1.5 Effect of suppression on incidence of apoptosis

The TUNEL assay was performed to detect the fragments with DNA damage. The total cell number and the TUNEL stained nuclei were recorded to calculate the apoptotic index (API). As shown in figure 15, no difference was observed in the total cell number among treatment groups.

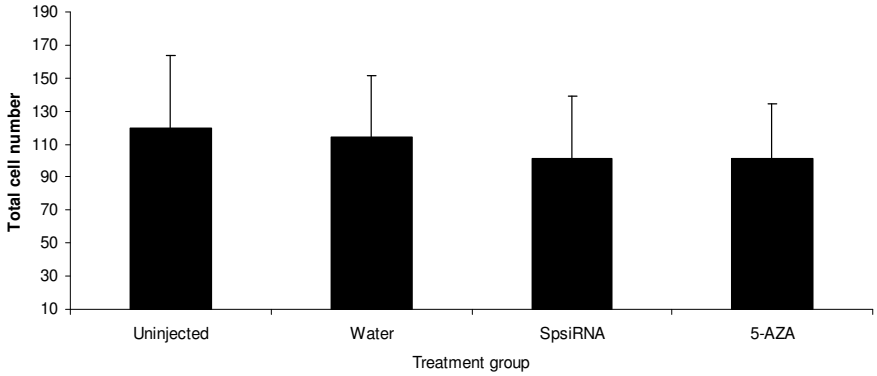
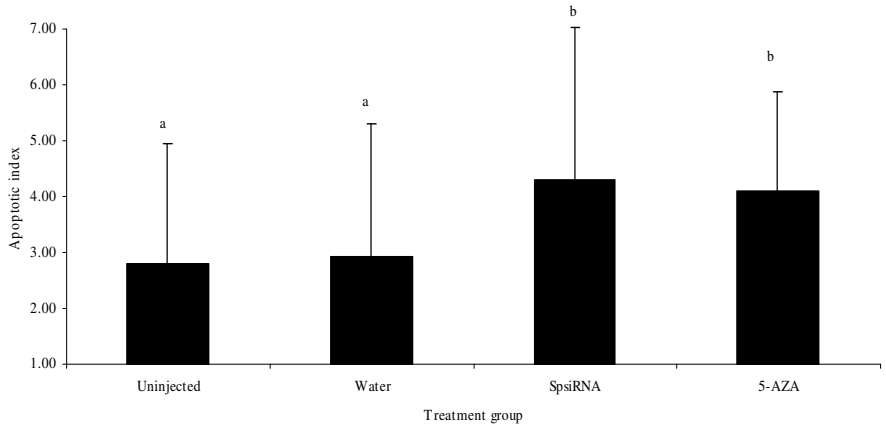


Figure 15: The total cell number of blastocysts stage between the different treatment groups

However, the highest API ($P<0.05$) was found in SpsiRNA (4.2±2.0) and 5-AZA (4.1±1.7) injected groups compared to water injected (2.8±2.1) and uninjected control (2.9±2.3) (Figure 16)

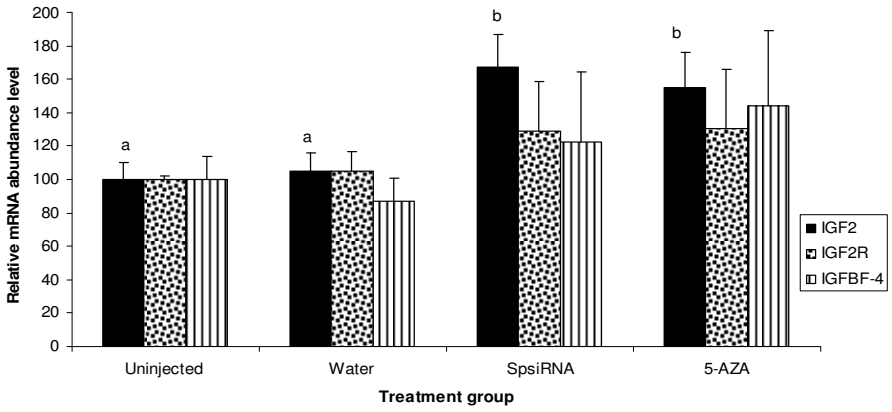


a, b: *P<0.05*

Figure 16: The apoptotic index of blastocysts stage between the different treatment groups.

4.1.6 Effect of gene expression of imprinted genes due to suppression of DNMT1

To evaluate the effect of suppression of DNMT1 on expression of some candidate imprinted genes, the relative expression of IGF2, IGF2R and IGFBP-4 was quantified. The results showed that microinjection of SpsiRNA had significantly increased the expression of IGF2 (1.67 and 1.55 times), respectively compared to uninjected control. No effects of the microinjection of SpsiRNA and 5-AZA on the expression of IGF2R and IGFPB-4 were found (Figure 17).



a, b: *P<0.05*

Figure 17: Relative gene expression of IGF2 (black bars), IGF2R (scattered bars) and IGFBP-4 (lined bars) at blastocyst stage.

4.2 Association analysis of DNMT1, DNMT3a and DNMTT3b sequence variants with bull fertility traits

4.2.1 Test of Hardy-Weinberg equilibrium

The Chi-square test revealed that the DNMT1 and DNMT3a loci were in Hardy-Weinberg equilibrium in the bull population while DNMT3b is not in Hardy-Weinberg. In the embryo population, the DNMT3a locus was in Hardy-Weinberg equilibrium (Table 7).

Table 7: Chi-square test revealing genotypes of the loci in Hardy-Wienberg equilibrium

Locus	N	Bull population		N	Embryos	
		X^2	*P*-value		X^2	*P*-value
DNMT1	300	0.45	0.96	249	13.99	0.001*
DNMT3a	306	0.63	0.53	286	1.72	0.12
DNMT3b	303	3.41	0.03*	310	0.0006	0.002*

* shows significant difference (P<0.05)

4.2.2 Description of bull fertility traits data

4.2.2.1 NRR

The NRR (%) was calculated by number of non-return cows (56 days last service) divided by number of inseminations. Number of bulls (N), number of inseminations (n), ranges (minimum and maximum), and mean, standard deviation (SD) in population are shown in table 8.

Table 8: Number of bulls (N), number of inseminations (n), mean, standard deviation (SD) and range of NRR

Number of bulls (N)	Number of inseminations (n)	Min	Max	Mean ± SD
310	368,694	58	92	74.08±6.87

For the NRR, the mean and standard deviations were 74.08 and 6.8, respectively. The NRR within all observation ranged widely from 58 to 92% within the bull population.

4.2.2.2 Sperm quality traits

The sperm quality traits including VOL (ml), CONC ($x10^6$/ml), MOT (%) and SUVR (%) were obtained from each ejaculation. The semen quality, number of bulls (N), number of inseminations (n), range (minimum and maximum), means, and standard deviation (SD) are shown in table 9:

Table 9: Number of bulls (N), number of inseminations (n), mean, standard deviations and range of sperm quality trait

Sperm quality traits	Number of bulls (N)	Number of inseminations (n)	Min	Max	mean± SD
VOL	145	2,465	3	6	4.50±2.49
CONC	145	2,465	0.51	2.15	1.33±3.31
MOT	145	2,465	70	80	75.00±8.12
SUVR	145	2,465	10	60	50.00±8.01

The mean and standard deviation of VOL, CONC, MOT and SUVR were 4.50±2.49, 1.33±3.31, 75.00±8.12, and 50.00±8.01, respectively.

4.2.2.3 Sperm flow cytometric parameters

Using flow cytometer technique, the three additional PMI, PAS and DFI of the sperm were recorded. The number of bulls (N), number of inseminations (n), range (minimum and maximum), means, and standard deviation (SD) observed in the population are shown in table 10:

Table 10: Number of bulls (N), number of inseminations (n), mean, standard deviations and range of sperm flow cytometric parameters

Sperm flow cytometric parameters	Number of bulls (N)	Number of inseminations (n)	Min	Max	mean± SD
PMI	177	990	52	56	54.18±2.43
PAS	177	990	22	28	25.54±3.87
DFI	177	990	3	5	4.30±1.41

The mean and standard deviation of the sperm flow cytometric parameters, PMI, PAS, and DFI were 54.18±2.43, 25.54±3.87, and 4.30±1.41, respectively.

4.2.3 Results of genotyping

A) Single loci

The genotype and allele frequencies of the diallelic loci within the bull population were calculated and shown in table 11 and table 12.

Table 11: The genotype frequencies and number of bulls (N) genotyped at the loci DNMT1, DNMT3a, and DNMT3b

Locus	Genotype	Number of bulls (N)	Frequency (%)
DNMT1	CC	30	10.0
	CT	133	44.3
	TT	137	45.7
DNMT3a	CC	84	27.4
	CT	160	52.4
	TT	62	20.2
DNMT3b	AA	51	16.7
	AG	175	57.6
	GG	77	25.7

The frequencies of heterozygote CT and homozygote TT variation of DNMT1 were 44.3 and 45.7%, respectively. The homozygote CC was rare with only 10% in the bull population. For DNMT3a and DNMT3b, the heterozygote CT and AG variation were most frequent. The homozygote CC, TT and AA, GG ranged between 16.7 and 27.4% (Table 11). The allele frequencies found in the bull population at the loci DNMT1, DNMT3a, and DNMT3b are shown in table 12.

Table 12: Alleles, and allele frequencies found in the bull population at the loci DNMT1, DNMT3a, and DNMT3b

Locus	Allele	Frequency (%)
DNMT1 (N=300)	C	32.1
	T	67.9
DNMT3a (N=306)	C	53.6
	T	46.4
DNMT3b (N=303)	A	45.5
	G	54.5

In table 12, allele T of DNMT1 was very frequent, nearly 70%. The allele T and G of DNMT3a and DNMT3b were almost equal, about 50% in the bull population.

The genotype and allele frequencies of the single loci within the embryo population with different development stages were calculated and the results are shown in table 13 and table 14.

Table 13: The number of genotyped embryos (N) and genotype frequencies (Freq) at the loci DNMT1, DNMT3a, and DNMT3b

Locus	Genotype	Early cleavage		Late cleavage		Blastocyst	
		N	Freq (%)	N	Freq (%)	N	Freq (%)
DNMT1	CC	4	2.5	11	12.6	12	15.0
	CT	19	23.8	19	21.8	25	31.3
	TT	59	73.7	57	65.5	43	53.8
DNMT3a	CC	18	22.5	37	31.9	17	18.9
	CT	36	45.0	42	36.2	49	54.4
	TT	26	32.5	37	31.9	24	26.7
DNMT3b	AA	15	17.9	16	12.3	17	18.5
	AG	44	50.0	55	42.3	49	53.3
	GG	29	33.9	59	45.4	26	28.3

The homozygote genotype TT of DNMT1 was most frequent in the early cleavage, late cleavage, and blastocyst embryo groups (Table 13). The frequency of homozygote TT was 73.7, 65.5, and 53.8%, while the heterozygote CT was moderate with frequencies 23.8, 21.8, and 31.3%, respectively. The heterozygote CT and AG of DNMT3a and DNMT3b were most frequent in the early cleavage, late cleavage, and blastocyst embryo groups. The frequencies were 45.0, 36.2, 54.4% and 50.0, 42.3, 53.3%, respectively (Table 13). The allele frequencies found in the genotyped embryos at the loci DNMT1, DNMT3a, and DNMT3b are shown in table 14.

Table 14: Alleles frequencies at the loci DNMT1, DNMT3a, and DNMT3b

Locus	Allele	Early cleavage	Late cleavage	Blastocyst
DNMT1(N=249)	C	14.3	23.5	30.6
	T	85.7	76.5	69.4
DNMT3a (N=286)	C	45.0	50.0	46.1
	T	55.0	50.0	53.9
DNMT3b (N=310)	A	41.10	33.4	45.0
	G	58.9	66.6	55.0

As shown in table 14, allele T and G of DNMT1 and DNMT3b were 85.7, 76.5, 69.4% and 58.9, 66.6, and 55.0% in early cleavage, late cleavage, and blastocyst group, respectively. For DNMT3a, the allele frequency C and T were similar.

B) Combined loci

In this experiment, the interaction effect of DNMT1, DNMT3a, and DNMT3b were also observed in the bull population as shown in table 15.

Table 15: The genotype frequencies (Freq) and number of bulls (N) at the combined loci DNMT1xDNMT3axDNMT3b

Genotype	N	Freq (%)	Genotype	N	Freq (%)
CCxCCxAA	1	0.46	CTxCTxGG	11	4.91
CCxCCxAG	1	0.46	CTxTTxAA	5	2.23
CCxCTxAA	3	1.34	CTxTTxAG	9	4.01
CCxCTxAG	7	3.12	CTxTTxGG	3	1.34
CCxCTxGG	4	1.78	TTxCCxAA	4	1.78
CCxTTxAA	2	0.89	TTxCCxAG	10	4.46
CCxTTxAG	9	4.02	TTxCCxGG	3	1.34
CCxTTxGG	2	0.89	TTxCTxAA	16	7.14
CTxCCxAA	8	3.57	TTxCTxAG	34	15.17
CTxCCxAG	11	4.91	TTxCTxGG	7	3.12
CTxCCxGG	4	1.78	TTxTTxAA	3	1.34
CTxCTxAA	18	8.03	TTxTTxAG	8	3.57
CTxCTxAG	39	17.41	TTxTTxGG	2	0.89

As shown in the table 15, the frequency of CTxCTxAG was the highest (17.41%) while CCxCCxAA and CCxCCxAG were both the lowest (0.46%) in the bull population.

4.2.4 Results of associations

4.2.4.1 NRR

A) Single loci

Association of the different genotypes with NRR were analyzed using model 1. The statistical results were given as least square means and their standard errors. The representations of the effects of DNMT1, DNMT3a, and DNMT3b on the phenotypes of the bulls are shown in table 16. Analysis of variance revealed a significant association of DNMT1, DNMT3a, and DNMT3b with the NRR in the population.

Table 16: Effect of DNMT1, DNMT3a, and DNMT3b genotypes on the Non return rate (NRR) (%)

Locus	No. of inseminations	Genotype	LSM±SE
DNMT1	24,959	CC	70.50±0.03[a]
	21,7085	CT	75.77±0.03[b]
	12,6650	TT	67.96±0.04[c]
DNMT3a	93,909	CC	72.65±0.04[a]
	228,338	CT	74.25±0.04[b]
	46,447	TT	67.36±0.05[c]
DNMT3b	42,543	AA	71.62±0.06[a]
	166,972	AG	73.23±0.07[b]
	159,179	GG	74.26±0.05[c]

a, b, c: ($P<0.001$)

Table 16 shows the estimates of NRR for bulls depending on the DNMT1, DNMT3a, and DNMT3b loci. For both the DNMT1 and DNMT3a loci, the NRR of heterozygous bulls is higher than those the homozygous genotypes.

B) Combined loci

The interaction effect of the three genes DNMT1, DNMT3a, and DNMT3b was analysed in the bull population as shown in table 17. The results of the statistical analysis revealed a significant association of the combined genotypes DNMT1xDNMT3axDNMT3b with NRR.

Table 17: Effect of combination of DNMT1, DNMT3a, and DNMT3b genotypes on NRR[1] (%)

Genotype	No.of inseminations	LSM ± SE
CTxTTxGG	4,787	88.23±0.12
TTxCTxGG	13,266	79.73±0.07
TTxTTxAG	10,278	76.33±0.08
TTxCCxAG	21,855	75.03±0.08
CTxCTxGG	100,975	74.37±0.07
TTxCTxAG	50,841	73.35±0.04
TTxCCxGG	7,351	71.18±0.10
CCxCTxAG	8,198	69.40±0.12
CTxTTxGG	1,466	68.04±0.19
TTxTTxAA	5,729	64.23±0.15
TTxTTxGG	3,142	62.52±0.15

Significant difference (P<0.001)

The Estimate of NRR for bulls depending on the DNMT1xDNTM3axDNMT3b ranged from 62.52 to 88.23%. Bulls with genotype TTxTTxGG and CTxTTxGG had the lowest and highest NRR, respectively (Table 17).

4.2.4.2 Sperm quality traits

A) Single loci

The effects of the candidate genes DNMT1, DNMT3a, and DNMT3b on VOL, CONC, MOT, and SUVR were estimated according to the model 2. The results of the statistical analysis revealed that DNMT3a and DNMT3b were significantly associated with MOT. However, DNMT1, DNMT3a, and DNMT3b did not have a significant effect on VOL, CONC, and SUVR. No association was found of DNMT1 with MOT (Table 18).

Table 18: Effect of DNMT1, DNMT3a, and DNMT3b genotypes on sperm quality (%)

Locus	Genotype	No.of inseminations	VOL	CONC	MOT	SUVR
DNMT1	CC	248	4.65±0.31	1.25±0.17	72.74±0.43	48.34±0.54
	CT	979	4.79±0.34	1.36±0.16	72.60±0.53	48.79±0.54
	TT	1,238	4.83±0.44	1.53±0.16	72.82±0.56	49.11±0.56
DNMT3a	CC	636	4.63±0.45	1.29±0.15	72.09±0.53[a]	47.69±0.74
	CT	1,368	4.90±0.34	1.39±0.16	72.10±0.44[a]	48.06±0.65
	TT	461	5.02±0.50	1.69±0.15	73.04±0.23[b]	48.78±0.59
DNMT3b	AA	414	4.60±0.56	1.41±0.12	73.00±0.62[b]	48.43±0.66
	AG	1,534	4.90±0.67	1.39±0.14	72.23±0.53[a]	47.88±0.54
	GG	517	5.00±0.55	1.55±0.13	72.00±0.47[a]	48.58±0.33

a, b: ($P<0.05$)

Table 18 shows estimates of VOL, CONC, MOT, and SUVR for the bull depending on the DNMT1, DNMT3a, and DNMT3b genotypes. Significant effects of the DNMT3a and DNMT3b gene on MOT were observed in the bull population. Bulls with homozygous genotype TT and AA of DNMT3a and DNMT3b had higher MOT than those of other genotypes, respectively.

B) Combined loci

In addition, the interaction effect of the three genes, DNMT1, DNMT3a, and DNMT3b was analysed. The statistical analysis revealed a significant association of the combined genotypes DNMT1xDNMT3axDNMT3b with MOT and SUVR in the bull population as shown in table 19.

Table 19: Effect of combination of DNMT1, DNMT3a, and DNMT3b genotypes on MOT and SUVR (%)

Genotype	No.of inseminations	LSM ± SE	
		MOT	SUVR
CCxCCxAG	17	56.78±2.81[a]	50.48±2.84[cd]
CCxCTxAG	66	72.65±1.76[b]	41.44±1.81[c]
CTxCCxGG	96	69.72±1.22 [b]	50.32±1.23[d]
CTxCTxAA	68	70.96±1.40 [b]	49.46±1.41[d]
CTxCTxAG	277	73.14±0.75 [b]	49.27±0.76[d]
CTxTTxAG	145	73.33±1.08 [b]	49.48±1.10[d]
TTxCCxAG	148	72.16±0.94 [b]	50.27±0.95[d]
TTxCTxAA	185	73.36±1.04 [b]	48.45±1.06[d]
TTxCTxAG	504	72.21±0.63 [b]	48.83±0.63[d]
TTxCTxGG	119	70.96±1.15 [b]	49.17±1.17[d]
TTxTTxAG	81	71.94±1.23 [b]	50.24±1.23[d]

a, b: (P<0.01); c, d: (P<0.05)

The estimates of MOT and SUVR ranged from 74.61 to 56.78% and 41.44 to 50.48%, respectively. The bulls with genotype CCxCCxAG and CCxCTxAG had the lowest MOT and SUVR, respectively. The bulls with genotype TTxCCxGG and CCxCTxAG had the highest MOT and SUVR, respectively.

4.2.4.3 Sperm flow cytometric parameters

The effects of the candidate genes DNMT1, DNMT3a, and DNMT3b on PMI, PAS, and DFI were estimated according to the model 2. The results of the statistical analysis revealed that only DNMT1 was associated with PAS. Bulls with the homozygous genotype CC had higher PAS than those with CT and TT. No association was found of DNMT3a and DNMT3b with PAS. No association was found among DNMT1, DNMT3a, and DNMT3b with MPI and DFI (Table 20). In addition, the interaction effect of the three genes, DNMT1, DNMT3a, and DNMT3b on PMI, PAS, and DFI, was not observed.

Table 20: Effect of DNMT1, DNMT3a, and DNMT3b genotypes on PMI, PAS and DFI

Locus	Genotype	No.of inseminations	PMI	PAS	DFI
DNMT1	CC	76	40.29±1.63	31.21±1.44[a]	4.94±0.43
	CT	424	49.55±1.63	25.73±1.46[b]	4.38±0.53
	TT	490	48.42±1.54	26.50±1.56[b]	4.65±0.46
DNMT3a	CC	262	45.07±1.64	28.66±1.55	5.06±0.53
	CT	557	49.07±1.53	26.13±1.56	4.47±0.44
	TT	171	50.43±1.65	24.48±1.15	4.33±0.43
DNMT3b	AA	166	51.13±1.65	22.84±1.62	4.30±0.42
	AG	541	47.35±1.67	28.08±1.64	4.41±0.53
	AG	283	48.15±1.55	25.28±1.63	5.17±0.47

a, b: ($P<0.05$)

4.2.4.4 Embryonic development

The effect of candidate genes DNMT1, DNMT3a, and DNMT3b on embryonic development early cleavage, late cleavage, and blastocyst were estimated according to the model 3. The chi-square results showed a significant association DNMT1 with embryonic development as shown in Table 21. Early cleavage with genotype CC was lower in frequency compared to both the late cleavage and blastocyst groups. No association of DNMT3a and DNMT3b with embryonic development was observed.

Table 21: Effect of DNMT1, DNMT3a, and DNMT3b genotypes on embryonic development

Locus	Genotype	Genotype frequency (%)		
		Early cleavage	Late cleavage	Blastocyst
DNMT1	CC	2.5[a]	12.6[b]	15.0[b]
	CT	23.8	21.8	31.3
	TT	73.8	65.5	53.8
DNMT3a	CC	22.5	31.9	18.9
	CT	45.0	36.2	54.4
	TT	32.5	31.9	26.7
DNMT3b	AA	17.9	12.3	18.5
	AG	50.0	42.3	53.3
	GG	33.9	45.4	28.3

a, b: ($P<0.05$)

5 Discussion

DNA methylation is a major epigenetic modification involved in genomic imprinting that causes parental-origin-specific monoallelic expression of mammalian genes. Imprinted genes are established in the parental germline and then maintained throughout embryonic development. The imprinting genes play important roles in diverse biological phenomena such as embryonic development, placental formation and fetal growth (Reik and Walter 2001). The first evidence that DNMT1 is essential for maintenance of imprinting genes comes from gene knockout mice performed by Li et al. (1993). Up to now, the functional study of DNMT1 gene on embryonic development and subsequent influence on imprinted gene expression has been limited in bovine preimplantation. In addition, the effect of DNMT1, DNMT3a and DNMT3b (DNMTs) genotypes on bull fertility traits and embryonic development has not been studied. In this study, we demonstrated that suppression of DNMT1 affects the embryonic development during preimplantation period and enhances IGF2 gene expression. Moreover, we show associations between sequence variants of DNMTs with bull fertility traits and early embryonic development.

5.1 Expression of DNMT1 mRNA during early preimplantation

During bovine preimplantation development, the genomic methylation pattern is erased during the first few cleavage divisions and then reasserted during the 8- to 16-cell transition (Dean et al. 2001). The specific DNMT enzymes responsible for dynamic genomic methylation are currently unknown but studies in mouse suggest the ability of DNMT1o and DNMT1s proteins synthesized at the same time to substitute for one another's maintenance function of methylation imprints in the embryonic development (Cirio et al. 2008). In human, both the DNMT1o and DNMT1s mRNA are expressed earlier during oogenesis and persisted in early preimplantation development (Huntriss et al. 2004). In this study, unlike mouse and human, we demonstrated that bovine DNMT1 mRNA contains the coding sequence necessary to produce the DNMT1o splice variant but could not be amplified. Therefore, the DNMT1s is an isoforms that expresses during bovine preimplantation stage embryos. Our finding was in accordance with Golding and Westhusin (2003) who found only the DNMT1s mRNA to be expressed during early bovine embryonic development. Thus, it is possible that bovine DNMT1o is transcribed and translated during the earliest stage of oocyte development and utilized during this

time to carry out the allele specific imprints observed in the mouse (Cirio et al. 2008). Alternatively, the splice variant of the DNMT1s, the DNMT1b which is shown to be consistent with a tissue-specific mode of regulation and possess the functional domains necessary for maintenance and *de novo* methyltransferase activity (Russell and Betts 2008) replaces the DNMT1o activity. The misexpression of the DNMT1o isoforms must be considered regarding direct and indirect effects on epigenetic programming during normal bovine development (Golding and Westhusin 2003).

The relative abundance of DNMT1 mRNA was detected throughout bovine preimplantation developmental stage. The expression was highest at mature oocyte stage and lowest at blastocyst stage (Figure 10). This result was similar with the previous studies in bovine using RT-PCR analysis (Golding and Westhusin 2003; Russel and Betts 2008).

5.2 Effect of suppression of DNMT1 mRNA on early embryonic development

Bovine embryonic development in the early preimplantation stages is supported by mRNA and protein transcribed from the maternal and embryonic genome. Until the major round of embryonic transcription during the 8- to 16-cell stage in bovine embryos, the development is largely dependent on the transcripts and protein formed by the oocyte (Memili and First 2000). DNMT1 regulates the cell cycle (Detich et al. 2001) which is associated with the protein proliferation (Chuang et al. 1997) concurrently occurring during DNA replication (Araujo et al. 1998). Unterberger et al. (2006) demonstrated that DNMT1 depletion in the DNA replication leads to overall arrest of replication. Antisense knock down of DNMT1 was reported resulting in inhibition of DNMT1 leading to inhibition of initiation of DNA replication (Araujo et al. 1998; Knox et al. 2000; Milutinovic et al. 2003) and subsequently effecting cell proliferation (Fournel et al. 1999), cell growth (Liu et al. 2003) and 50% reduction in cell numbers (Egger et al. 2006).

The DNA methylation inhibitor 5-azacytidine and its derivatives have been studied. Khan et al. (2006) suggested 5-azacytidine induces cell cycle arrest involving all G0/G1, and G2 phases in cell lines. Other studies of 5-azacytidine in rats (Doerksen and Trasler 1996; Doerksen et al. 2000) and mice (Kelly et al. 2003, Oakes et al. 2007a) have shown that the

treatment resulted in a disruption of spermatogenesis by lowering sperm counts and survivability. In *Xenopus*, antisense knocks down of DNMT1 results in developmental arrest (Kaito et al. 2001) and abnormal embryonic development (Stancheva and Meehan 2000, Stancheva et al. 2001). In addition, DNMT1 knockdown and 5-AZA had no effect on genomic methylation (Unterberger et al. 2006).

In the present study, the quantitative expression profiling results throughout the preimplantation embryonic stage evidenced that DNMT1 is activated from the maternal genome. The transcript abundance sharply increases after maturation and is reduced until 8-cell stage. The detectable amount of DNMT1 transcript was very low between morula and blastocyst stages. Therefore, microinjection of SpsiRNA DNMT1 and 5-AZA is targeting transcripts from the maternal genome and minor embryonic genome activation at 2- to 4-cell stages. Consequently, injection of SpsiRNA against DNMT1 and 5-AZA at the zygote stage has resulted a reduction in the proportion 8-cell embryos at 72 hr pmi compared with the water injected and uninjected control groups. Similarly, the total blastocyst rate was lowered in 5-AZA compared to the other treatment groups. It is suggested that the normal development of zygote to term is dependent on the major maternally derived mRNA and protein accumulation. The blastocyst rate was not affected by microinjection of SpsiRNA. This can be explained in terms of suppression efficiency and availability of protein at blastocyst stage.

The reduction of expression of target DNMT1 (80%) dramatically resulted in reducing protein accumulation at 8-cell stage in the group injected with SpsiRNA compared with those injected with 5-AZA, water and uninjected control groups. By introducing of RNAi to mammalian cells, the amount of target mRNA and protein was effectively decreased 24-48 and 48-72 hr pmi, respectively (Bonetta 2004). However, there was no difference in protein expression at blastocysts in microinjection of SpsiRNA groups. This could be explained that moderate suppression of the target mRNA (50%) at blastocyst stage was not enough to reduce amount of protein accumulation and subsequently leads to embryonic development normality. This might be due to the protein's half-life, its abundance, and the regulation of its expression (Elbashir et al. 2002). In studies showing the successive knockdown of DNMT1 protein such as in human colorectal cancer cells, transfection was carried out every day and the cultures were split every other day (Robert

et al. 2003; Ting et al. 2004). Alternative splicing isoforms of DNMT1, the DNMT1b which reportedly has been expressed throughout bovine preimplantation (Russell and Betts 2008) may compensate to assist in normal embryonic development. However, its function has not yet been known.

5-AZA has been known as an anti-cancer agent that irreversibly binds DNMT1, DNMT3a and DNMT3b protein (Gabbara and Bhagwat 1995), resulting in trapping in 5-AZA-containing DNA but not causing disappearance of the protein (Unterberger et al.2006). Therefore, in the present study, microinjection of 5-AZA has no effect on protein degradation at 8-cell and blastocyst stages.

The lowered development of 8-cell embryos and total blastocyst in the present study is in agreement with relevant studies where DNMT1 mutant mouse showed reduced rate of cell division (Jackson-Grusby et al. 2001), increased embryonic death, failure to progress beyond the first trimester (Li et al. 1992) and the late gestation period (Howell et al. 2001) and delayed development of multiple organs at mid-gestation (Toppings et al. 2008). Spermatogonial cells treated with 5-AZA showed complete inhibition of differentiation into spermatocyte stage (Raman and Narayan 1995), blocked testicular cord formation and sertoli and leydig cell differentiation (Mizukami et al. 2008) and subsequently reduced sperm motility, fertilization ability, early embryo development to the blastocyst stage and sequence-specific DNA methylation (Oakes et al. 2007a). Furthermore, 5-AZA has halted cell proliferation and growth and subsequently reduced percentage of blastocysts rate in bovine embryonic development (Enright et al. 2005; Khan et al. 2006)

5.3 Effect of suppression of DNMT1 mRNA on apoptosis level

In the present study we have investigated post microinjection of SpsiRNA DNMT1 and 5-AZA using TUNEL staining. 5-AZA has been extensively studied, and its ability to damage DNA by activating the G1 checkpoint regulator protein 53 (p53) (Karpf et al. 2001) has been shown. Furthermore, 5-AZA treatment results in inhibition of cell proliferation due to p53-dependent activation of $p21^{Waf1/Cip1}$ (Karp et al. 2001, Zhu et al. 2004). The 5-AZA also induces apoptosis, either in a p53-dependent (Schneider-Stock et al. 2005) or p53-independent (Nieto et al. 2004) manner. In previous reports, antisense knock down DNMT1 resulted in negative effect on cell proliferation (Fournel et al. 1999), reduced in cell growth (Liu et al. 2003) and total cell numbers (Egger et al. 2006). In the present study, microinjection of 5-AZA reduced proportion of 8-cell embryos at 72 hr pmi and total blastocyst rate until Day 8 pmi, whereas microinjection of SpsiRNA DNMT1 reduced proportion of 8-cell embryos at 72 hr pmi. It is due to the fact that activity of DNMT1 knockdown and 5-AZA has no effect on genomic methylation (Unterberger et al. 2006), the subsequent influence on apoptotic cell development has to be confirmed.

The effect of suppressing DNMT1 on the level of cellular DNA fragmentation as a characteristic feature of apoptotic cells was performed at blastocyst stage. Microinjections of DNMT1 SpsiRNA and 5-AZA resulted in significant increase of apoptosis index (API) compared to water injected and uninjected controls. The total cell numbers were slightly lowered in embryos injected with SpsiRNA and 5-AZA compared with water injection and uninjected control. However, these differences were not statistically significant. Thus, microinjection of SpsiRNA DNMT1 may cause DNA damage, which could result in reduced proportion of 8-cell and total blastocyst rate by inhibiting cell proliferation activators. This finding was in agreement with a previous report where a DNMT1 mutation has shown to increase API in primary mouse fibroblast 6 days post infection (Jackson-Grusby et al. 2001). Antisense induced knock down of DNMT1 resulted in a DNA damage caused by apoptosis dependent of p53 in mouse germline somatic cells (Takashima et al. 2009).

Alternatively, it is possible that the decrease of 8-cell and total blastocyst rate results from antisense knock down of DNMT1 causing re-expression of tumor suppressor genes;

cyclin-dependent kinase inhibitor 2A (*p16*^{ink4A}), cadherin 1 (*CDH1*), ras association (RalGDS/AF-6) domain family 1 A (*RASSF1A)* (Suzuki et al. 2004), programmed cell death 4 (*PDCD4*) and prostaglandin E synthase (*PTGES*) (Fan et al. 2007). Studies in *Xenopus* embryos also supported that antisense knock down of DNMT1 provide a signal via p53 that induces apoptotic cell development (Stanchev et al. 2001). It is also consistent with the report where 5-AZA treatment has resulted in an increase of apoptotic cells in human cell lines (Khan et al. 2006; Kiziltepe et al. 2007).

5.4 Effect of suppression of DNMT1 mRNA on expression of imprinted genes

DNA methylation plays an important role to control the imprinting gene expression in which only one allele of a specific gene is expressed, depending on its parental origin (Li et al. 1992 and Li et al. 1993). The first evident study in frog, however, showed that antisense knock down DNMT1 resulted in demethylation and induction of premature gene activation (Stancheva and Meehan 2000; Stancheva et al. 2001). Similarly, conditional knock out of DNMT1 caused demethylation and increasing global gene expression in mouse fibroblasts cells (Jackson-Grusby et al. 2001). The 5-AZA which interferes with the activities of DNMT1 protein leads to genomic hypomethylation and reactivates silenced tumor suppressor genes (Fan et al. 2007; Suzuki et al. 2004). Knock out DNMT1 resulted in higher expression of IGF2 due to biallelic expression (Biniszkiewicz et al. 2002). It is known that the bovine clone calves exhibit abnormally biallelic expression of imprinted genes, such as Xist, H19, IGF2, and IGF2R, but relatively normal expression in normal cloned calves. These facts indicate that DNA methylation plays an important role in normal gene expression during early preimplantation development (Xue et al. 2002; Yang et al. 2005).

In this study, we showed that DNMT1 silencing increased the expression of imprinted genes, IGF2, IGF2R and IGFBP-4 at blastocyst stage. However, the increment of IGF2R and IGFBP-4 was not statistically significant due to high standard deviation. The higher expression of IGF2 in this study was also relevant in previous reports of tissue of cloned calves (Humpherys et al. 2001; Li et al. 2007), overgrown fetuses and placentae of cloned mice (Ogawa et al. 2003). Thus, the higher expression might be due to the biallelic expression revealed with bisulfite analysis (Gebert et al. 2006; Li et al. 2007)

IGF2R is a well-characterized negative regulator of IGF2 that binds and down regulates IGF2 activity by endocytosis and degradation (Ludwig et al. 1996). Both hyper- and hypo-methylated alleles in the IGF2R were found in Day 9.5 cloned mice fetuses that had developed normally (Ogawa et al. 2003). Strongly reduced levels of DNA methylation were observed in the differentially methylated regions (DMR) of IGF2R in the second intron of cloned sheep (Young et al. 2003).

5.5 Association of sequence variants of DNMT1, DNMT3a, and DNMT3b with bull fertility traits

We have shown that suppression or inhibition of DNMT1 using SpsiRNA or DNMT1 inhibitor 5-AZA resulted in a reduction of proportion of 8-cell and total blastocyst rate, increment of API and enhancement of imprinted gene expression. Firstly, we demonstrated that the DNMT1 gene is important for normal embryonic development during the preimplantation period. However, the effect of exonic/intronic mutation of DNMT isoforms on embryonic development or phenotype variations needs to be investigated.

For this study, DNMT1, DNMT3a and DNMT3b sequence variants have been associated with bull fertility traits; NRR, sperm quality, sperm flow cytometric parameters, and embryonic development. The fertility traits have a low heritability (h^2 ~0.007-0.049) but show high genetic variation. Therefore higher numbers of animals are required to be analyzed in order to eliminate the effects by environment and single loci (Long and Langeley, 1999). In contrast, sperm quality traits have moderate to medium heritability (h^2 for ejaculation volume, concentration and motility: 0.09, 0.16, and 0.22, respectively) (Kealey et al. 2006). The bull fertility traits of NRR and sperm quality traits have been successively applied as the parameters for genetic improvement of cattle population. However, both methods do not provide insight details of membrane, acrosome and sperm chromatin structure that may have a major impact on fertilizing ability of the sperm. Data analysis from Computer-Assisted Semen Analyzer (CASA) in combination with fluorescent staining assessed by flow cytometry are more precise than data from conventional methods (Christensen et al. 2005; Garner et al. 1994; Januskauskas et al. 1996). A simple approach is an evaluation of plasma membrane, acrosome, and DNA

integrity, which can be assessed with various fluorescent probes (Anzar et al. 2002; Celeghini et al. 2007; Graham 2001; Silva and Gadella 2006).

Hardy-Weinberg equilibrium is performed to test whether the genotype frequencies in the population remain constant or are in equilibrium from generation to generation without influence from genetic selection, mutations, limited population size, genetic drift and gene flow (Wigginton et al. 2005). In this study, the candidate genes DNMT1 and DNMT3a loci were in Hardy-Weinberg equilibrium, while the DNMT3a locus was not in the Hardy-Weinberg equilibrium in the bull population. In the embryo population, only the DNMT3a locus was in Hardy-Weinberg equilibrium. The reason why the loci do not obey Hardy-Weinberg equilibrium is unknown; however, it may reflect the fact that selection and random genetic drift cause a change in allele frequency.

Obviously, male fertility has an influence on the reproductive performance and productivity in commercial herds. The development of highly polymorphic genetic markers and extensive linkage maps now makes it possible to dissect genetic variation for quantitative traits and to identify the chromosomal regions with genes contributing most to variation (Fries 1999). As mentioned, efforts to identify quantitative traits loci (QTL) for fertility traits (Ben Jemaa et al. 2008; Holmberg and Andersson-Eklund 2006; Schrooten et al. 2000), milk yield (Khatkar et al. 2004) and work ability traits (Schrooten et al. 2000) are already well done. Several studies have reported significant association between SNPs with genes that control production traits (Khatib et al. 2007; Leonard et al. 2005; Weikard et al. 2005) as well as the fertility traits (Lin 2005; Lin et al. 2006a; Lin et al. 2006b; Wimmers et al. 2005). In the current study, SNP of DNMT1, DNMT3a and DNMT3b were found to be associated with bull fertility traits including NRR and sperm quality traits, sperm quality traits and sperm flow cytometric parameters.

The impact of nucleotide difference is variable and elusive, but it is clearly dependent upon the location of the polymorphism in the genome (Shen et al. 1999). Nucleotide differences in regions upstream of the protein-encoding gene regions influence the binding of promoter repressors, resulting in differential regulation of transcription. However, the polymorphisms at intron/exon boundaries have been reported to probably affect exonic or intronic splicing enhancer or silencer position, modifying the polypeptide

and its structure of protein (Nissim-Rafinia and Kerem 2002). So far, the role of intronic splicing in regulation the expression level of genes or tissue specific expression pattern has been noted in several reports (Garifulin et al. 2007; Greenwood and Kelsoe 2003; Jiang et al. 2000; Pagani and Baralle 2004; Van Laere et al. 2003; Virts and Raschke 2001).

The diallic loci of DNMT1, DNTM3a and DNMT3b, and their interaction on multiple loci of DNMT1xDNTM3axDNMT3b were for the first time shown to affect the NRR (56 days). The silent mutation at position Ala1197Ala of DNMT1 and intronic splicing effect of DNMT3a and DNMT3b might have an impact on mRNA and protein expression and its structure of the corresponding gene. It is due to the fact that, during embryonic development, DNA methyltransferase enzyme family (DNMTs) play important roles in DNA methylation. DNMT1 primarily maintains established methylation patterns during DNA replication, whereas patterns are established by the *de novo* methyltransferase DNMT3a and DNMT3b (Li et al. 1992; Okano et al. 1999). In our study, therefore, multiple combinations of loci of DNMT1xDNTM3ax DNMT3b supported the polygenic influence on complex traits of NRR. The highest NRR value was 88.23% in the combination of DNMT1xDNMT3axDNMT3b with CTxTTxGG.

The SNPs of Ala1197Ala DNMT1, 308446C>T DNMT3a and 337339A>G DNMT3b located on *Bos taurus* autosomes (BTA) BTA7, BTA11 and BTA13, respectively. They were not in accordance with the QTLs showing significant effects on NRR (56 Days) that mapped on BTA9 (Holmberg and Andersson-Eklund 2006; Schrooten et al. 2000) and BTA18 (Kuhn et al. 2003). This enforces that the bull fertility trait of NRR is controlled by several genes located on different chromosomes so that the SNP associated with NRR found in this study might be additional markers potentially linked to these complex traits.

In this study, it was for the first time detected that the diallelic loci of DNMT3a and DNMT3b are associated with sperm motility and multiple loci of DNMT1xDNMT3axDNMT3a are associated with sperm motility (MOT) and survivability after thawing (SUVR). Although the exonic or intronic splicing effects of DNTM1, DNMT3a, and DNMT3b polymorphisms on sperm quality traits are not clear. DNMTs are known to play an important role for DNA methylation in the process of male

germ cell development during the spermatogenesis (Davis et al. 1999; La Salle and Trasler 2006; Oakes et al. 2007b). Inactivation of the DNMTs through gene-targeting results in male infertility (Bourc'his et al. 2001; Kaneda et al. 2004). Demethylation with 5-AZA causes insufficient of DNMT proteins (Gabbara and Bhagwat 1995) in rats (Doerksen et al. 2000; Doerksen and Trasler 1996) and mice (Kelly et al. 2003; Oakes et al. 2007a) resulting in disruption of spermatogenesis by reducing sperm motility and increasing levels of abnormalities in testicular histology, which is linked to lower survivability of sperm after thawing. The multiple loci of DNMT1xDNMT3axDNMT3b with TTxCCxGG (74.61%) and CCxCCxAG (50.48%) were the highest for MOT and SUVR in the population under study.

This experiment revealed for the first time that DNMT1 locus is significantly associated with plasma membrane integrity (PMI). Thus, maintenance of the sperm fertilizing potential depends on the integrity and functionality of different cellular structures. Plasma membrane integrity is crucial to sperm survival inside the female reproductive duct (Celeghini et al. 2007). The acrosome is filled with hydrolytic enzymes which are necessary for penetration of sperm to zona pellucida (Silva and Gadella 2006). Plasma membrane and sperm acrosome integrity were reported to be positively correlated with sperm motility (Hua et al. 2006). The integrity of sperm DNA is also important for the accurate transmission of genetic information and further development of embryos (Agarwal and Said 2003; D'Occhio et al. 2007). Inhibition of DNMTs with 5-AZA caused failure of the acrosome reaction, capacitation, sperm-egg recognition, and chromatin quality (Oakes et al. 2007b) and subsequently lowered the ability of sperm to successfully fertilize the oocyte. It is shown in this study that the DNMT1 locus with genotype CC (31.21%) had higher PMI than genotype CT and TT (25.73 and 26.5%).

5.6 Association of DNMT1, DNMT3a, and DNMTT3b sequence variants with embryonic development

The purpose of this study was to correlate the single nucleotide polymorphism of candidate genes with the embryonic development in terms of cleavage time and blastocyst rate. The bull sperm selected for fertilization of oocytes aspirated from slaughterhouse ovaries were heterozygote for all three genes. Zygotes developing to 2-cell stage within

30 hpi are consisdered as early cleaved (Ward et al. 2001). The early development of embryos is controlled by many genes. In mice, the *Ped* (preimplantation embryo development) gene has been identified involving in controlling cell division and embryo survival (Warner et al. 1998) during preimplantation. In human, Oct-4 (Abdel-Rahman et al. 1995) and gap-junction connexin 43 (Hardy et al. 1996) were involved in embryonic development normality. In this study, suppression of DNMT1 affected early embryonic development. Additionally, sequence variants of either single or combined loci of DNMT1, DNMT3a and DNMT3b were found to be associated with bull fertility traits and sperm flow cytometric parameters. Therefore, the association of DNMT1 sequence variants with the embryonic development in terms of cleavage time and blastocyst rate was reliable supported.

5.7 Future prospect

In this study, we demonstrated that suppression of DNMT1 affected the embryonic development during the preimplantation period and enhanced IGF2 gene expression. Moreover, we showed the relationship between sequence variants of DNMT1 and both DNMT3a, and DNMT3b with bull fertility traits and early embryonic development. In the future research, the candidate gene approach (DNMT1, DNMT3a and DNMT3b) to study association with bull fertility traits and/or sperm flow cytometric parameter should be confirmed using different bull population. Additionally, the genotypes dependent of embryonic development should be quantified the mRNA expression levels among the development groups.

6 Summary

DNA methyltransferase enzymes (DNMTs) are believed to be involved in DNA methylation which is the well-characterized epigenetic modulator that has been shown to have essential functions in germline and embryonic genome imprinting. The first study was conducted to investigate the consequences of suppressing and inhibiting DNMT1 on the development, the levels of apoptosis and the expression of imprinted genes in preimplantation bovine embryos. *In vitro* produced zygotes were categorized into four groups, namely: those injected with SpsiRNA (n = 800), 5-AZA (n = 864), nuclease free water (n = 850) and uninjected control (n =755). The mRNA expression data were generated using the RT-PCR based on relative standard curve method employing GAPDH as a normalizer. The apoptotic index (API) was calculated by dividing the number of apoptotic cells by the total cell numbers. The proportions of different stages of embryos were assessed 48 and 72 hr post microinjection (pmi) while blastocyst rate was assessed at day 8 pmi. The proportions of 2-, 4- and 8-cell embryos at 48 pmi were not significant among treatment groups. However, the proportion of the 8-cell embryos was lower ($P<0.05$) in SpsiRNA (16.3±4.5) and 5-AZA injected groups compared to water injected and uninjected control at 72 hpi. The lowest total blastocyst rate ($P<0.05$) was observed in 5-AZA treatment group compared to SpsiRNA and water injected and uninjected control. Microinjection of SpsiRNA has reduced the target mRNA by 80% and 50% in 8-cell and blastocyst stage embryos, respectively, compared to uninjected control. The protein expression level was also reduced at 8-cell stage embryos as confirmed by western blotting. Injection of 5-AZA had no significant effect on mRNA and protein expression. The highest API ($P<0.05$) was found in SpsiRNA and 5-AZA injected groups compared to water injected and uninjected control. The microinjection of SpsiRNA and 5-AZA at zygote stages has increased the expression of IGF2 by 1.67 and 1.55 times at blastocyste stage embryos. However no effect on the expression of both IGF2R and IGFPB-4 was found.

The second study was conducted to elucidate the association of single polymorphisms (SNP) of DNMT1 (rs41256891; C>T), DNMT3a (rs41569254; C>T) and DNMT3b (rs41700758; A>G) isoforms with bull fertility traits including; NRR (56 days) and sperm quality traits namely, sperm volume per ejaculate, sperm concentration ($x10^9$/ml), sperm motility, and survivability after thawing. Moreover, association study was done with

sperm flow cytometric parameters including positive acrosome status (PAS), plasma membrane integrity (PMI) and DNA fragmentation index (DFI). For this, sperm DNA from 310 breeding German Holstein bulls obtained from Rinder-Union West eG (RUW) station were genotyped at those loci. Analysis of variance revealed association of DNMT1 with NRR ($p<0.0001$) and PAS ($p<0.01$), while DNMT3a and DNMT3b were found to be associated with NRR ($p<0.0001$) as well as sperm motility ($p<0.01$). In addition, interaction analysis of variance among DNMT1 x DNMT3a x DNMT3b showed significant association with NRR ($p<0.0001$), sperm motility ($p<0.001$) and survivability after thawing ($p<0.001$). However, SNPs of all the three isoforms have no significant association with sperm volume per ejaculate, sperm concentration ($x10^6$/ml), PMI and DFI.

In addition, the SNP of DNMT1, DNMT3a and DNMT3 sequence variant were correlated with the embryonic development in terms of time at first cleavage, late cleavage and blastocyst rate. Fore this, 350 embryos were produced *in vitro* from oocytes aspirated from slaughterhouse ovaries and fertilized with bull spermatozoa heterozygote for all three genes. The results showed that the DNMT1 locus was significantly correlated with embryonic development *($p<0.05$).*

In conclusion, suppression and inhibition of DNMT1 resulted in lower proportion of 8-cell embryos, reduced blastocyst rate, increased apoptotic index and affected the expression of some imprinted genes. In addition, the SNP of DNMT1, DNMT3a and DNMT3b sequence variants revealed association with bull fertility traits and were correlated with embryonic development. This gene evidently plays a critical role in bovine preimplantation development and associates with bull fertility traits and embryonic development. Following validation of this result in an independent population, there is a great potential to use these loci as markers of fertility to enhance embryonic development.

7 Zusammenfassung

Die Funktion der DNA Methyltransferase (DNMTs) wird in der DNA-Methylierung vermutet, welches eine gut charakterisierte epigentische Regulationseinheit ist und eine essentielle Rolle in Keimbahnprozessen und embryonalem genomischen Imprinting spielt. Die erste Studie wurde durchgeführt, um die Konsequenzen eines repressiven und hemmenden Einflusses von DMNT1 auf die Entwicklung und Grad der Apoptose und Expression genomisch geprägter Gene von preimplantativen bovinen Embryonen zu untersuchen. *In vitro* erzeugte Zygoten wurden zufällig in vier Gruppen aufgeteilt, die mit drei unterschiedlichen Injektionen behandelt wurden: der Injektion (a) mit Smartpool siRNA (SpsiRNA) (n=800), (b) mit 5aza-2'-deoxycytidine (5-AZA) (n=864) und (c) mit Nuklease freiem Wasser (n=850). Gruppe 4 verblieb als unbehandelte Kontrolle (n=755) bestehen. Die Daten der mRNA Expression wurden durch RT-PCR generiert, die dann mit der Relativen-Standard-Kurven-Methode auf Grundlage von GAPDH normalisiert wurden. Der Apoptose Index (API) wurde mittels der Division der Anzahl apoptotischer Zellen durch die Anzahl Gesamtzellen berechnet. Das Verhältnis der jeweiligen Entwicklungsstadien der Embryonen in den Behandlungsgruppen wurde 48 und 72 hr post Mikroinjektion (pmi) beurteilt, wohingegen die Bastozystenrate erst 72 hr pmi festgestellt wurde. Der Anteil der 2-, 4- und 8- Zell Embryonen 48 hr pmi war zwischen den Behandlungsgruppen nicht signifikant verschieden. Dagegen war der Anteil 8- Zell Embryonen 72 hr pmi in den Gruppen SpsiRNA (16.3 ± 4.5) und 5-AZA geringer ($P<0.05$) als in der mit Wasser behandelten Gruppe oder der unbehandelten Kontrollgruppe. Die geringste Blastozystenrate ($P<0.05$) wurde in der Behandlungsgruppe 5-AZA im Vergleich zur SpsiRNA, der Wasser und unbehandelten Gruppe beobachtet. Die Mikroinjektion von SpsiRNA reduzierte die Expression der Target mRNA um 80 bzw 50% in Embryonen des 8-Zellstadium im Vergleich zur unbehandelten Kontrolle. Zusätzlich war die Proteinexpression in dieser behandelten Gruppe reduziert, welches mit Hilfe von Western Blotting bestätigt werden konnte. Die Injektion von 5-AZA hatte keinen signifikanten Effekt auf die Expression von mRNA und Proteinen. Der API war in den Behandlungsgruppen SpsiRNA und 5-AZA, verglichen mit der Gruppe die mit Wasser injiziert wurde und der unbehandelten Gruppe, höher ($P<0.05$). Der Einfluss der Mikroinjektion von SpsiRNA und 5-AZA in die Zygote

steigerte die Expression von IGF2 auf 1.67 bzw. 1.55 in Blastozysten. Es konnte allerdings kein Effekt auf die Expression von IGF2R und IGFPB-4 beobachtet werden.

Im zweiten Abschnitt dieser Arbeit wurde der Einfluss der genetischen Polymorphismen (single nukleotide polymorphisms (SNP)) von DNMT1 (rs41256891; C>T), DNMT3a (rs41569254; C>T) und DNMT3b (rs41700758; A>G) auf zwei unterschiedlichen Merkmalskomplexe geprüft. Zum einen wurde die Fruchtbarkeit von Deutschen Holsteinbullen an Hand der Parameter Non-Return-Rate nach 56 Tagen (NNR), Spermienqualität (Volumen je Ejakulates, Spermienkonzentration, Motilität, Überlebensfähigkeit der Spermien nach dem Auftauen), sowie Durchfluss-zytometrieparameter des Spermiums (Plasma Membran Integrität (PMI), Akrosomen Integrität (PAS), DNA Integrität (DFI) untersucht. Des Weiteren standen Merkmale der Embryonalentwicklung in den Stadien der 1. Zellteilung, der späten Zellteilung und in Blastocysten im Mittelpunkt. Zu diesem Zweck wurden genomische DNA aus 310 Spermienproben von Zuchtbullen der Rinder Union West eG (RUW) und DNA Proben von 350 Embryonen an den entsprechenden Genorten genotypisiert. Die Varianzanalyse wies eine Assoziation des SNP in DNMT1 mit NRR ($P<0.0001$) und PAS ($P<0.01$) vor, während DNMT3a und DNMT3b einen signifikanten Einfluss auf NNR ($P<0.0001$) und Spermienmotilität ($P<0.01$) hatten. Zusätzlich zeigte eine kombinierte Genort Varianzanalyse von DNMT1 x DNMT3a x DNMT3b einen signifikanten Effekt auf NNR ($P<0.0001$), Spermienmotilität ($P<0.001$) und Überlebenfähigkeit nach dem Auftauen ($P<0.001$). Allerdings konnte kein Einfluss der SNPs in den drei Isoformen auf Volumen je Ejakulates und Spermienkonzentration, sowie PMI und DFI nachgewiesen werden.
Zusätzlich konnte eine Korrelation der genetischen Polymorphismen von DNMT1, DNMT3a und DNMT3b auf die embryonale Entwicklung zum Zeitpunkt der ersten Zellteilung, der späten Zellteilung und der Blastozystenrate beobachtet werden. Zu diesem Zweck wurden *invitro* Eilzellen aus Schlachthof Ovarien aspiriert und mit Bullen Spermatozoen, die heterozygot für alle drei Gene waren, befruchtet. So konnten 350 Embryonen erzeugt werden, die über die unterschiedlichen genetischen Eigenschaften verfügten. Das Ergebnis aus diesem Versuch zeigte, dass der Genort DNMT1 einen signifikanten Einfluss ($P<0.05$) auf die embryonale Entwicklung hat. Verfügten Embryonen über den Genotyp CC, so war der Anteil an Blastozysten höher (15.0), als zum Zeitpunkt der späten Teilung (12.6) oder der ersten Teilung (2.5) ($P<0.05$), während

kein signifikanter Unterschied zwischen den Genotypen CT und TT in den jeweiligen Entwicklungsstufen festgestellt werden konnte.

Abschließend hat ein repressiver und hemmender Effekt von DNMT1 eine schlechtere Entwicklung der Embryonen, vor allem im 8-Zellstadium und Blastocysten, zur Folge und ändert das Expressionsverhalten einiger genomisch geprägter Gene. Zusätzlich zeigten die Polymorphismen in DNMT1, DNMT3a und DNMT3b einen signifikanten Einfluss auf Fruchbarkeitsmerkmale und die embryonale Entwicklung. Das Gen DNMT1 spielt sichtbar eine Rolle in der bovinen Preimplantation. Des Weiteren lässt es sich mit Merkmalen der Bullenfruchtbarkeit und embryonalen Entwicklung assoziieren. Dies könnte ein Hinweis auf einen nützlichen, genetischen Marker zur Verbesserung dieser Merkmale sein, welcher durch weitere unabhängige Studien bewiesen werden sollte.

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