REPROGRAMMING DNA METHYLATION IN BOVINE CELLS BY KNOCKING DOWN DNA METHYLTRANSFERASE-1 WITH RNA INTERFERENCE

A Thesis

by

TODD KEITH STROUD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Veterinary Physiology

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Approved by:

Chair of Committee,	Charles R. Long
Committee Members,	Robert C. Burghardt
	Charles R. Looney
	Mark E. Westhusin
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ABSTRACT

Reprogramming DNA Methylation in Bovine Cells by Knocking Down DNA Methyltransferase-1 with RNA Interference. (May 2009)

Todd Keith Stroud, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Charles R. Long

Embryos derived by somatic cell nuclear transfer (SCNT) produce few pregnancies that result in a live, healthy offspring. This has largely been attributed to the aberrant reprogramming of the somatic cell DNA used for cloning. In order to improve the efficiency of cloning there is a great deal of research needed to determine the role of proteins involved in early embryonic reprogramming. In addition, studies are needed to determine effects on somatic and embryonic cell development as a result of altering these proteins.

In this study we investigate the use of RNA interference in bovine somatic cells and embryos to knock down the expression of DNA methyltransferase-1 (*DNMT1*), an enzyme responsible for maintenance methylation in mammalian cells. We designed our experiments to test whether or not knocking down the DNMT1 gene would lead to a decrease in global methylation. It is our hypothesis that using somatic cells with reduced methylation may be advantageous for increasing the efficiency of cloning via somatic cell nuclear transfer.

To accomplish this task, we have designed an infectious non-replicating lentiviral vector capable of delivering a gene that produces a short hairpin RNA targeting the mRNA of *DNMT1*. The construct included a sequence coding for green fluorescent protein (GFP) that will allow us to identify cells expressing the hairpin as well as a region coding for neomycin resistance so we could select for a pure population of transgenic cells to use for analysis.

Infecting bovine fetal fibroblast cells with genes encoding shRNAs that target DNMT1 was successful. Quantitative real time PCR analysis of DNMT1 mRNA suggests that our shRNAs are capable of an 80% knockdown. The protein blot of indicates up to 90% knockdown of DNMT1. Cells transduced twice with a high titer virus showed the highest knockdown of both DNMT1 mRNA and the protein. Analysis of immunolabeled cytosine methylaiton showed a global decrease in DNA methylation as a result of the DNMT1 knockdown. However, double transduced cells with a high knockdown percentage of DNMT1 mRNA and protein became hypermethylated.

The second experiment was conducted to determine the effect of injecting small interfering RNAs (siRNAs) targeting DNMT1 into oocytes prior to parthenogenic activation. This experiment was designed to give us information on the survivability and epigenetic profile of early embryos with decreased DNMT1. Oocytes injected with siRNA targeting DNMT1 had little development past the 8-cell stage as compared to the sham injected oocytes. This treatment group also had decreased DNA methylation as determined by immunolabeling of methylated cytosine residues.

DEDICATION

This research is dedicated to my family and friends that embraced me through life's challenges and for their unending support regarding my life changing decisions. I also dedicate it to my old friends and teammates that chased the dream of playing Major League Baseball while I chose the path of education and science. A few of them made it, most failed, but we all had fun while it lasted.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Long, and my committee members, Dr. Burghardt, Dr. Looney, and Dr. Westhusin, for their guidance and support throughout the course of this research.

Thanks also go to my lab mates. Without their help in teaching me about molecular biology I am certain I would have never finished this project. I also want to extend my gratitude to Dr. Michael Golding for providing the basis for much of my research project. Thanks also to everyone that invited me to help with projects that involved hands on work with animals. The animals are the real reason I enjoy the work.

Finally, thanks to my mother and father for their encouragement and to my wife, Bridget for her patience and love.

NOMENCLATURE

DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase-1
DNMT3A	DNA methyltransferase-3A
DNMT3B	DNA methyltransferase-3B
dsRNA	Double stranded RNA
IVF	*In vitro* fertilized
IVP	*In vitro* produced
mRNA	Messenger RNA
miRNA	Micro RNA
RNA	Ribonucleic Acid
RNAi	RNA interference
SCNT	Somatic cell nuclear transfer
shRNA	Short hairpin RNA
siRNA	Short interfering RNA

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CHAPTER I

INTRODUCTION

Studies on somatic cell nuclear transfer (SCNT) opened the door for understanding the key mechanisms that shape embryonic differentiation, development, and cellular reprogramming. The desire to understand how an oocyte reprograms the DNA of a fully, differentiated adult cell into a single, totipotent cell capable of creating a fully functioning organism sparked a wide range of studies in the last decade. In addition, many studies were aimed at understanding reasons for the high percentage of failure among cloned embryos. Although we have learned a great deal from previous research, there are still many unanswered questions who's answers may improve cloning success rates.

Improving the efficiency of the cloning process is one of the most intensely investigated areas of assisted reproductive technology. One approach is to alter cloning protocols. Modifications to somatic cell culture media, embryo culture media, and handling procedures during micromanipulation increased the production of cloned bovine offspring by 5-10% as a function of embryo transfer (Gibbons, Arat et al. 2002). These changes to the cloning processes have helped, but have not accomplished the objective of high pregnancy rates and low fetal and peri-natal mortality when compared to *in vivo* and *in vitro* standards. The major limitation is that these changes do

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This thesis follows the style of Biology of Reproduction.

not directly address the problems involving the molecular processes of cloned embryo failure.

One molecular process that appears to play a very significant role in cloned embryo failure is the process of epigenetic modifications (Dean, Santos et al. 2001). Studies of gene expression and epigenetic modifications have led scientists to believe that improper epigenetic remodeling during the pre-implantion stage of cloned embryos has a dramatic effect on later stages of embryonic and fetal development as well as placentation (Armstrong, Lako et al. 2006).

The efficiency of cloning is a significant concern, not only for academic studies funded by government organizations, but for private industry as well. Private companies are producing mass numbers of cloned cattle and horses, as well as an increasing number of deer, goats, pigs, cats, and dogs. Currently, cattle cloning results in approximately one viable offspring per every seven to ten embryos transferred as compared to *in vivo* embryos (60-80%) or *in vitro* produced (IVP) embryos (40-50%) (Hasler 2001; Lane, Gardner et al. 2003; Farin, Piedrahita et al. 2006). Published information is not readily available for an accurate estimation of pregnancy rates from cloned embryos because university studies involving cattle cloning have relatively low numbers and usually involve too many treatment groups to show significant data (Amann 2005). Commercial production statistics remain relatively confidential with exception of information gathered by individuals checking for pregnancy on the recipients that received cloned embryos.

Pregnancy rates of transfered cloned embryos at 35 days are comparable to that of IVP embryos (40-50%), but large majority of these pregnancies abort before the end of the first trimester (Oback and Wells 2003). In addition, a significantly higher percentage of cloned animals are born abnormal or dead as compared with traditional *in vivo* or *in vitro* produced offspring (Campbell, McWhir et al. 1996; Bourc'his, Le Bourhis et al. 2001; Rideout, Eggan et al. 2001). This presents a problem, not only by increasing the total cost for cloned animals that do thrive, but it also diminishes the reputation of the cloning industry in the public eye. The actual cost to produce the cloned embryos is relatively low in a commercial situation where embryos are made in groups of several hundred at a time. However, with fluctuating costs of feed and fuel, it is a huge economic disadvantage for one to continue to board recipient cows that lose their fetus during mid-gestation or give birth to a stillborn or abnormal cloned calf.

In an effort to solve some of the issues previously described, we designed a set of experiments that may lead to improved efficiency of producing cloned offspring. This particular study focuses on the epigenetic parameters of somatic and embryonic cell development. More specifically, we investigated mechanisms that control heritable but potentially reversible changes in DNA methylation or chromatin structure. Previous studies showed that global levels of DNA methylation and expression of DNA methyltransferases are aberrantly regulated in embryos derived by SCNT when compared to embryos derived by *in vitro* fertilization or *in vivo* production (Dean, Santos et al. 2001; Kang, Koo et al. 2001; Rideout, Eggan et al. 2001; Liu, Yin et al. 2008). We sought to develop a mechanism that would allow us to create cloned

embryos that express normal levels of DNA methyltransferase protein and subsequently normal patterns of DNA methylation.

Epigenetics

Epigenetics is a field of science that emerged in the early 1980's involving the study of changes in gene expression. Initial epigenetic studies arose when scientists began trying to uncover the mysteries behind tumor development, X chromosome inactivation, and genomic imprinting (Lyon 1988; Wainscoat and Fey 1990; Deal 1995; Leighton, Saam et al. 1996). Epigenetic modifications do not involve a change in the DNA sequence of an organism's genome, rather they involve factors that prevent or allow access of specific DNA sequences to be transcribed. The result of these factors is an organization scheme that governs the timing of when genes are expressed. This determines the unique identity of the different types of cells in an organism's body.

A broad example of epigenetics at work is the differentiation of totipotent embryonic stem cells into pluripotent cells which eventually differentiate further into specific cell types such as muscle cells or neurons. Every cell type including oocytes and sperm cells are determined as a result of epigenetic modifications. The underlying mechanism behind how epigenetic regulation occurs is far from being completely understood. It is understood, however, that there are intrinsic and environmental influences that mediate epigenetic modifications (Jaenisch and Bird 2003). A significant portion of the research on intrinsic mechanisms focuses on DNA methylation and histone modifications (Dean, Santos et al. 2001; Kang, Koo et al. 2001; Rideout, Eggan et al. 2001; Liu, Yin et al. 2008).

DNA Methylation

DNA methylation is perhaps the most well understood mechanism of epigenetic regulation. Methylation of DNA results from the addition of a methyl group to the 5 position of cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides. These methyl groups, which are covalently bound to DNA, alter specific gene expression in one of two ways. They can either interfere with binding of transcription factors or create target sites for methyl-binding proteins which induce transcriptional repression by recruiting co-repressors, such as histone deacetylases or histone methylatransferases (Nan, Ng et al. 1998; Kiefer 2007). For these reasons DNA methylation in CpG islands of promoter regions is usually associated with gene silencing. The mechanism by which DNMT1 methylates cytosine residues can be seen in Figure 1.

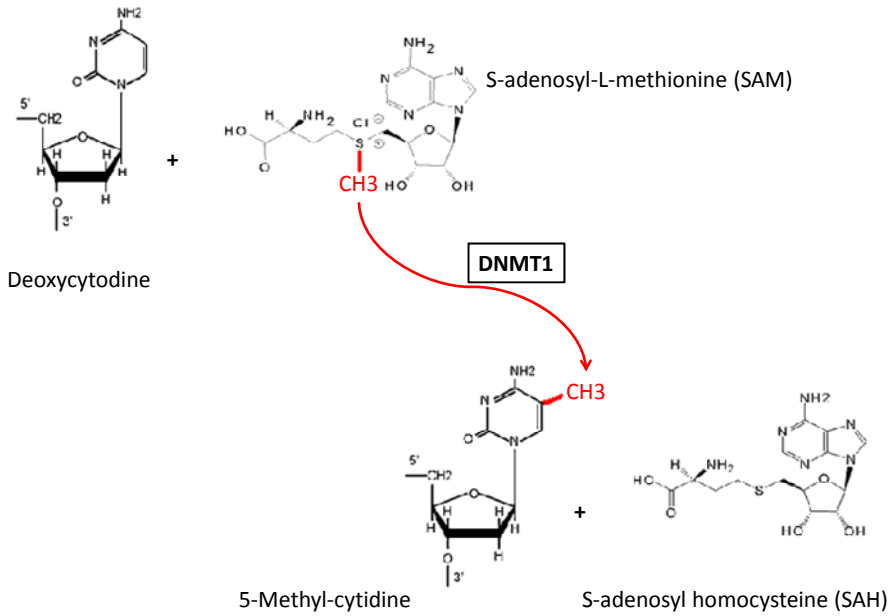


Figure 1. DNA methylation reaction catalyzed by DNA methyltransferase. Adapted from (Sulewska, Niklinska et al. 2007).

Clusters of CpG dinucleotides are referred to as CpG islands. At CpG islands DNA is modified by methylation in order to control gene expression. These islands are often found in the regulatory region of housekeeping genes in vertebrates and are typically protected from methylation so genes at these locations can be continually expressed. A few developmentally regulatory genes are protected in this manner during early embryonic development. These genes eventually become methylated when they are no longer required or when differentiation occurs (Deb-Rinker, Ly et al. 2005; Freberg, Dahl et al. 2007; Farthing, Ficz et al. 2008).

Chromatin Modifications

Epigenetic modifications of chromatin include posttranslational alterations to the N-terminal tail of histones. These alterations can include phosphorylation, acetylation, methylation, and ubiquitination (Collas, Noer et al. 2007). The nucleosome consists of 146 bp of DNA wrapped around an octamer of histones; H2A, H2B, H3, and H4. This is the basic structural unit of chromatin (Margueron, Trojer et al. 2005). Changes to histones lead to changes in chromatin structure. These structural changes affect the potential for DNA to interact with transcriptional proteins. Therefore, DNA that is tightly packed is more difficult to access and loosely packed DNA is more easily accessed.

DNA Methyltransferase Proteins

The DNA methyltransferase proteins are responsible for maintenance and *de novo* methylation of cytosine-guanine dinucleotides (5-methylcytosine) of mammalian

DNA. *DNMT1* is a large protein comprised of 1620 amino acid residues and is considered to be the maintenance methylation protein. During DNA replication, *DNMT1* adds a methyl group mostly to 5'-m^5CG-3' dinucleotides on the new strand of DNA to match that of the original strand (Bestor 2000). The DNMT1 protein can be subdivided into an N- and a C-terminal part. The C-terminal domain harbors the catalytic center and contains all the amino acid sequence motifs characteristic for prokaryotic DNA-(cytosine-C5)-methyltransferases (Jeltsch 2002). DNMT1 shows a preference for hemimethylated CG sites as they appear after DNA replication, then the activity of DNMT1 leads to the re-establishment of the original DNA methylation pattern on the daughter strand (Jeltsch 2006).

One of the protein interaction partners that bind to the N-terminal part of Dnmt1 during the s-phase of the cell cycle is proliferating cell nuclear antigen (PCNA). PCNA is the sliding clamp of eukaryotic DNA polymerases used during DNA synthesis. Because of this interaction, it is thought that DNMT1 is active at the replication fork during DNA replication (Leonhardt, Page et al. 1992). DNMT1 also interacts with retinoblastoma protein (Rb) at its amino terminal region (Robertson 2002).

Another notable quality of DNMT1 is its expression pattern and trafficking mechanism during preimplantation embryonic development. The oocyte specific form of this protein is called DNMT1o. This isoform was once thought to be the only one present in mouse embryos until implantation begins to occur. DNMT1o protein is present during the preimplantation phases but it is isolated to the cytoplasm and only accesses the nucleus during the 8-cell stage for a brief period (Chung, Ratnam et al.

2003). This brief activity is believed to be involved with maintaining imprinted genes. Messenger RNA of the somatic from of DNMT1 (DNMT1s) is present in the preimplanted mouse embryo as well. It was previously thought that in the mouse, DNMT1s mRNA does not get translated until implantation occurs; but it is now thought that DNMT1s protein is also present in preimplantation mouse embryos (Carlson, Page et al. 1992; Grohmann, Spada et al. 2005; Cirio, Ratnam et al. 2008; Kurihara, Kawamura et al. 2008). Reports indicate the absence of DNMT1o in bovine oocytes, and unlike in the mouse, the somatic form of DNMT1 is present (Golding and Westhusin 2003; Russell and Betts 2008).

DNMT3A and DNMT3B are other methyltransferases of interest and they are responsible for a significant portion of de novo cytosine methylation. DNMT3A and DNMT3B are of particular importance in the critical 8-16 cell stages of bovine embryonic development and in later stages in the mouse (Reik, Dean et al. 2001; Golding and Westhusin 2003). They establish the methylation pattern which is transmitted to the differentiating cells and tissues. Although the roles of DNMT3A and DNMT3B have been studied and described in embryonic cells, DNA methyltransferases that determine new methylation patterns in mature cells have not been well characterized (Majumder, Ghoshal et al. 2006; Sulewska, Niklinska et al. 2007). Previous studies in human cancer cells and mice involving the knockdown of DNMT1 and DNMT3B individually suggest that the lack of one protein initiates compensation by the other (Rhee, Bachman et al. 2002). A subsequent study utilized a double knockdown of these

two proteins using RNAi and indicated a higher instance of demethylation than when only one protein or the other was knocked down (Leu, Rahmatpanah et al. 2003).

Although it is known that DNMT proteins are responsible for methylating DNA, it is unknown how the methylation patterns are governed. It is thought that specific methylation or demethylation events in differentiating tissues could lead to changes in gene expression as needed (Reik, Dean et al. 2001). Bestor and colleagues showed that cytosine methylation plays an essential role in X-inactivation, genomic imprinting and genome stabilization. This discovery came after the induction of global genome demethylation caused by targeted mutations in the DNMT1 gene (Bestor 2000).

Cellular Reprogramming of DNA Methylation

There are very specific DNA methylation events that must occur during primordial germ cell differentiation and during embryonic differentiation for proper development to produce. Each process has previously been described. The dynamic process of demethylation and remethylation has been termed "epigenetic reprogramming." A schematic diagram of this is presented in Figure 2. Primordial germ cells (PGCs) in the mouse, derived from epiblasts, first arise at embryonic day 7.5 (E7.5) in the posterior primitive streak. By day E8.5 they begin to migrate to the genital ridge where they settle by day E11.5. The early PGCs are thought to have epigenetic modifications such as X chromosome inactivation, imprinted gene expression, and DNA methylation similar to that of their epiblast cell precursors. By the time the PGCs reach the genital ridge these patterns have been erased. The bulk of the demethylation occurs between days E11.5 to E12.5. Interestingly, the demethylation occurs in the presence of

DNMT1 suggesting an active demethylating mechanism although no true DNA demethylase has been described (Hajkova, Erhardt et al. 2002; Morgan, Santos et al. 2005).

Regions that are demethylated during PGC differentiation include imprinted genes. An imprinted gene is one whose expression depends on whether it was maternally or paternally inherited. Imprinted genes are normally always methylated in all cell types except for in primordial germ cells during this phase. Remethylation is necessary for resetting of imprints but it is not known how the patterns are reestablished.

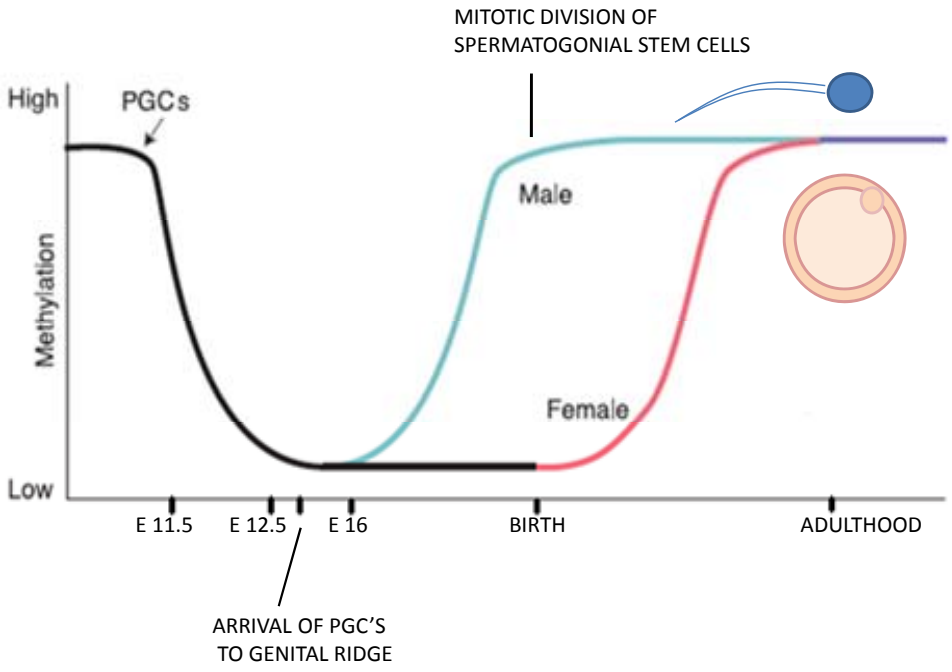


Figure 2. Diagram of PGC methylation during early development. The blue line represents sperm DNA and the pink line represents oocyte DNA. Adapted from (Reik, Dean et al. 2001).

The next reprogramming phase begins soon after male and female gametes undergo fertilization (Figure 3). Shortly following fertilization, asymmetric global demethylation of the male and female genome occurs. The male pronucleus requires major reconstruction after fertilization because of its highly condensed DNA. Protamines are removed and replaced by acetylated histones, followed by rapid demethylation of the paternal DNA.

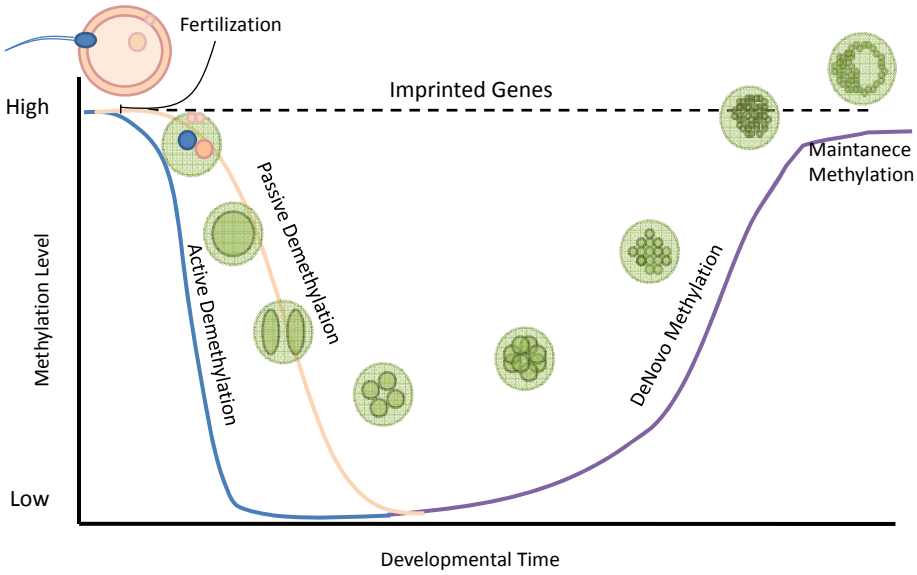


Figure 3. Diagram of bovine embryonic DNA methylation patterns during the preimplantation stages. The blue line represents the male genome, the pink line represents the female genome, and the dashed line represents imprinted genes. Adapted from (Reik, Dean et al. 2001).

The speed at which the paternal genome is demethylated could indicate an active process, although there has been no active demethylating enzyme discovered (Oswald, Engemann et al. 2000). The evidence of active demethylation in cloned embryos

suggests that, if there is an active demethylating enzyme, it is not sperm-dependant (Dean, Santos et al. 2001). Several theories have been proposed for mechanisms of active demethylation, but none provide a solid explanation with convincing data. Some sequences in the paternal chromosome are protected from demethylation. These include imprinted genes such as H19 and Ras Grf1 and some repeat sequences (Reik, Dean et al. 2001). There is some thought that the imprinted genes are maintained by DNMT3A and DNMT3B because during early embryo stages when imprinted genes are maintained, DNMT3A and DNMT3B are active while DNMT1 is not. The neighboring maternal genome is thought to passively demethylate after fertilization. This process progresses slowly, possibly due to the exclusion of DNMT1o (oocyte specific isoform) during the mitotic divisions of the preimplantation mouse embryo (Howell, Bestor et al. 2001). This period of demethylation may be essential in the formation of pluripotent cells or embryonic stem cells (Dean, Santos et al. 2001).

The demethylation and remethylation events appear to be conserved across eutherian mammals that have been studied thus far; however, the timing of the events is unique to each species. In the mouse *de novo* DNA methylation begins just after implantation in the expanded, hatched blastocyst (Monk, Boubelik et al. 1987). In contrast, the bovine embryo begins *de novo* DNA methylation at the 8-cell stage and is significantly remethylated by the 16-cell stage, by actions of DNMT3A and DNMT3B (Reik, Dean et al. 2001). The timing of methylation changes compared to the stage of embryonic development make the mouse embryo a difficult model for comparative studies with humans. A more ideal model would be to use bovine embryos due to their

similarities regarding the timing of DNA reprogramming, differentiation, and development.

Cloning Induced Problems: Epigenetic Malfunctions

It is widely accepted that many embryos derived by SCNT lack proper epigenetic reprogramming and have aberrant gene expression. It is not understood whether aberrant gene expression causes flaws in epigenetic reprogramming or vice versa. The interdependence of gene expression and epigenetic reprogramming make it difficult to determine cause relationships. Several studies describe the aberrant epigenetic reprogramming present in SCNT derived embryos. In general, cloned embryos have hypermethylated DNA during the same stages of development in which *in vitro* produced embryos are demethylated (Bourc'his, Le Bourhis et al. 2001; Dean, Santos et al. 2001). As described earlier, methylation, especially in CpG islands of promoter regions, is associated with gene silencing. A hypermethylated genome could be preventing vital genes from being expressed during important developmental stages. Evidence reported by Chung et al. suggests that the aberrant passive demethylation of cloned mouse embryos is a result of increased *DNMT1* expression during these developmental stages (Chung, Ratnam et al. 2003). The failure of cloned embryos to properly express the *DNMT* genes, and thus, accurately reprogram their somatic cell DNA could be the major cause of the high abortion rates, high birth weights and perinatal death (Kang, Koo et al. 2001; Liu, Yin et al. 2008). To further confound the issues behind epigenetic reprogramming in SCNT produced embryos, it is not known whether the majority of the problems are a result of oocyte inability to completely

reprogram the somatic DNA or if it is simply the lack of normal extrinsic factors in culture medium. It could also be due to physical abuse during micromanipulation. For example, if the DNMT1o isoform is only active in the mouse embryo nucleus for a brief period during the 8-cell stage, the injection or fusion process might cause a premature exchange of this protein across the cell membrane. A more simple theory would be that nuclei in cells transferred into oocytes during the cloning process already contain the somatic isoform of DNMT1 and, therefore, can never completely undergo passive demethylation. If this is the case, one could hypothesize that knocking down the expression of the DNMT1 protein prior to nuclear transfer might result in a one cell cloned embryo with the potential to undergo complete reprogramming. The following section is a description of how one might go about knocking down a gene such as DNMT1.

RNA Interference (RNAi)

The mechanism of RNAi was first described in 1998 by Andrew Fire and Craig Mello. While working with nematode worms they found that they could silence gene expression by introducing double-stranded RNA (dsRNA) into the cells of the organism (Fire, Xu et al. 1998). RNAi is a naturally occurring phenomenon that prevents mRNA from being translated into protein. Naturally occurring RNAi molecules are called micro RNA (miRNA). MiRNA were once thought of as meaningless degradation products from cleaved mRNA molecules, but are now known to have a considerable role in genes regulation.

Since their discovery, there has been an explosion of research in the field of RNAi in an attempt to try and understand its basic principles. In addition, researchers across the world are developing therapeutical techniques based on the RNAi mechanism that will undoubtedly lead to new methods of treatment for genetic disorders, cancer, and disease prevention (Paddison 2008).

Processing of dsRNA

As shown in Figure 4, polymerase II transcribed single stranded RNA are cleaved into short dsRNA. Double stranded RNA start out at variable lengths, but once processed, dsRNA are shortened and referred to as small interfering RNA (siRNA). When dsRNA enter the cytoplasm of a cell, whether naturally or artificially, the endoribonuclease Dicer processes them into ~21 nucleotide base pairs. The siRNA is then transferred to the RNAi-inducing silencing complex (RISC). RISC directs the destruction of one of the mRNA complementary strands of the siRNA. After the sense strand of the dsRNA is removed and degraded, the complementary strand binds to the target mRNA by means of base pair matching (Scherr and Eder 2007). The most potent of siRNA are those that match identically to the antisense strand of an RNA molecule (Fire, Xu et al. 1998).

SiRNA and miRNA Mediated Silencing

If base pairs of the siRNA strand are homologous to the target mRNA at each position RISC cleaves the target mRNA in the middle of the complementary region, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA and the remaining section of the target mRNA is then degraded via normal cytosolic pathways

(Lenz 2005). MiRNA are processed by the same enzymes and in the same manner as siRNA but they exhibit their silencing affect by a different mechanism. MiRNA bind to the mRNA with several non-matching bases that create loops. The RISC miRNA complex prevents processing of the target mRNA through the ribosome, therefore preventing translation of the mRNA into protein. Unlike siRNA that generally result in degradation, miRNA simply prevent translation by the ribosome (Hannon, Rivas et al. 2006; Scherr and Eder 2007).

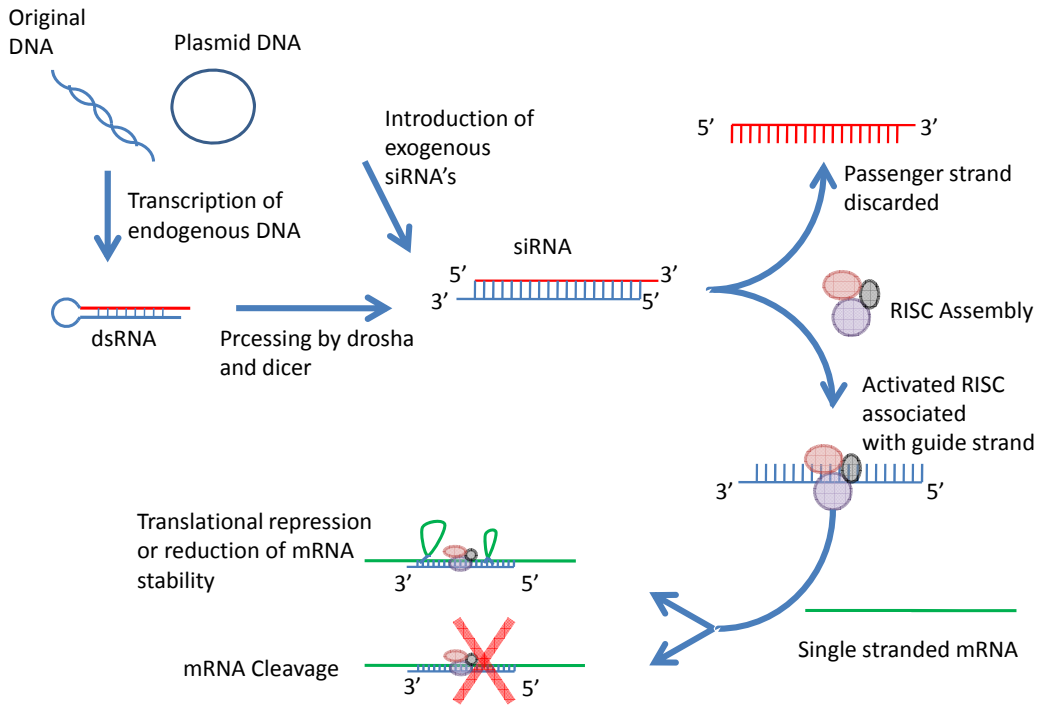


Figure 4. Overview of the RNAi pathway. When dsRNA enters the cell, it is processed by the enzyme complex Dicer and cleaved to yield siRNA. The siRNA then bind to the multiprotein complex RISC. Activation of the RISC is accompanied by the unwinding of the siRNA duplex. One strand of the siRNA then guides RISC to the target mRNA. The mRNA is then either cleaved by RISC nuclease activity or translation is blocked.

Short Hairpin RNA Delivery Methods

Methods exist to introduce exogenous interfering RNAs (in the form of shRNA) into cells to silence genes of interest. There are numerous methods for introducing interfering RNAs into cells and there are also several structural states that an RNAi molecule can assume when introduced. One method for introducing the RNAi pathway is by transfection of synthetic double stranded siRNA. These molecules tend to have a shorter half-life once introduced into the host cell than RNA molecules produced within the cell (Aagaard and Rossi 2007). These siRNA are only used in studies requiring short-term inhibition of target gene expression. Often, expression cassettes are used in order to harvest the power of eukaryotic polymerases, such as the U6 PolIII promotor. This promoter is commonly used because it has characteristics which make it suited to drive the expression of shRNA which is subsequently cleaved to a functional siRNA by dicer. Polymerase III promoters: (i) initiate from position +1 of the transcripts and (ii) the transcripts do not terminate with a poly-A tail but with a series of four to five thymidine residues, which results in a series of 3' U residues (Brummelkamp, Bernards et al. 2002).

Expression cassettes are often in the form of plasmid DNA that codes for the sense strand of siRNA of interest, followed by a spacer of non-coding DNA, then the equivalent of the antisense strand of the siRNA. When these sequences are transcribed, they fold back on themselves to form a double stranded RNA with a hairpin loop in the non-coding region. From this point they are processed by dicer and then RISC in the same fashion as naturally occurring miRNAs.

In order for plasmid DNA to be accessed by the promoter system, it must cross the cell membrane and reside in the cytoplasm. This can be accomplished in a number of different ways. Delivery of siRNAs is often achieved via by cationic liposome based strategies. In this system, the RNAi molecules are coated with lipid molecules and are allowed to pass through the hydrophobic plasma membrane lipid bilayer. Plasmid DNA and siRNA may also be injected directly into the cytoplasm of cells. This procedure is often limited to cells that are large enough to be penetrated with a micropipette such as oocytes or embryonic blastomeres.

Another mode of delivering expression cassettes into cells is by virally mediated integration of DNA into the host genome. This allows for more precise tissue targeting and longer, more stable expression of the siRNA. Expression cassettes that are integrated into viral vectors are designed with the same promoters and hairpin structures described earlier. Plasmid transfection and viral transduction are both effective, but lentiviral transduction has the advantage of stably integrating new genetic material into the genome making it possible to select and proliferate a pure and stable transgenic cell line (Lenz 2005).

Project Rationale

For this study we investigated the effects of knocking down DNMT1 in bovine somatic cells and preimplantation embryos. We hypothesized that by decreasing the amount of DNMT1 we would reduce global DNA methylation. In addition to a decrease in DNA methylation we hypothesized an increase in DNMT3A and DNMT3B to compensate for the loss of DNMT1.

We exploited the use of RNAi to knock down the expression of DNMT1 mRNA in bovine cells. In somatic cells we wanted to study the effect of long term knockdown of DNMT1 so we used a lentiviral approach for delivery of the shRNA. This approach would give use the best opportunity for collecting meaningful data because of stable expression of the transgene and the ability for us to select for a pure population of transgenic cells. Data indicating a decrease in DNA methylation would necessitate a follow up study utilizing a transient knockdown of DNMT1 in somatic cells for use in cloning. Even though that experiment is beyond the scope of the current study it is essential to our long-term goals. We predict that this would increase our ability to produce cloned embryos expressing an epigenetic profile more similar to that of *in vivo* or *in vitro* produced embryos. Thus, there would be less pregnancy loss and a higher survival rate of cloned offspring.

For this study, we also introduced siRNA into *in vitro* produced (IVP) zygotes and parthenote embryos to determine effects of decreased DNMT1 expression during early embryonic development. The approach for this part of the study was to inject siRNA targeting DNMT1 into either oocytes or one celled embryos so the silencing is elicited more explicitly during early embryonic cleavages. This would allow us not only to determine the necessity of DNMT1 in early bovine embryos, but also to predict what might happen if we transiently knocked down DNMT1 in somatic cells and subsequently used them for cloning.

CHAPTER II

VECTOR AND DNMT1 SHORT HAIRPIN RNA CONSTRUCTION

Short Hairpin RNA

The shRNA sequences were designed using a computer program on the Cold Spring Harbor website (http://www.cshl.edu/public/SCIENCE/hannon.html). A previous study in our lab suggested that two of the shRNA sequences effectively knocked down the expression of DNMT1 protein. These two shRNAs were labeled DNMT1 shRNA 3 and DNMT1 shRNA 4. For the remainder of this discussion they will be referred to as D1sh3 and D1sh4 respectively.

Vectors

The shRNA sequences were verified and cloned into the GIN-Zeo vector (Open Biosystems; Figure 5). GIN-Zeo is a lenti-viral vector containing a Green Fluorescent Protein (GFP) coding region under the influence of the CVM promoter. Driven off of the CVM promoter in the 5' to 3' direction are regions for expressing Green fluorescent protein (GFP), internal ribosome entry site (IRES), neomycin resistance, and finally the shRNA coding region. Located at the miR5 and miR3 sites where the shRNA coding region is cloned into the vector are EcoR1 and Xho1 restriction enzyme cut sites. At these locations, DNA sequences with complimentary sticky ends to the EcoR1 and Xho1 can be digested out or ligated in. The EcoR1-Xho1 regions are compatible with many plasmids which allows for these hairpins to be interchangeable.

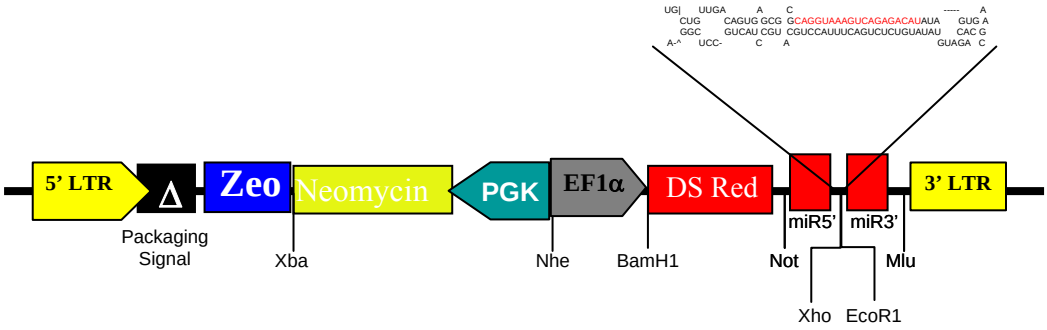
Figure 5. GIN-Zeo vector configuration. The GIN-Zeo plasmid consists of regions coding for GFP, IRES, a neomycin resistance gene, followed by the hairpin coding region all driven off of a CVM promoter.

Previous studies in our laboratory revealed that the control GIN-Zeo expression vector was not producing sufficient levels of luciferase specific shRNAs (luc-shRNA) to initiate mRNA degradation or translational repression in luciferase expressing controls. The reasons for this problem were never fully investigate, however, we hypothesized that due to the large amount of material expressed by the CVM promoter, it might not have been expressing all of the genes completely. More specifically, we may have been expressing the GFP and Neomycin genes, but not the hairpin. This would give us cells expressing GFP without the RNAi effect. We were concerned that if the GIN-Zeo vector containing the DNMT1 hairpin was equally insufficient, we would not be able to detect any knockdown at the mRNA or protein level. When efforts to quantify the efficiency in HEK 293 cancer cells and in bovine fetal fibroblasts revealed no measurable results, we made the decision to clone the hairpins into another vector.

The vectors chosen to replace GIN-Zeo were NEF-Red and PEF-Green (Figure 6A and 6B). Both are lentiviral vectors but are constructed quite differently. The NEF-Red plasmid contains two promoter regions that run in opposite directions. The PGK promoter initiates transcription in the 3' to 5' direction and expresses a Neomycin resistance gene. The EF1α promoter expresses dsRed and then the hairpin coding region

in the 5' to 3' direction. The concern for this plasmid is that only one promoter may be working efficiently. This could result in the plasmid efficiently expressing the dsRed and the hairpin, but would not allow for selection of a pure population of transgenic cells. The other vector, PEF-Green has the same limitations because it too runs off of

A.



B.

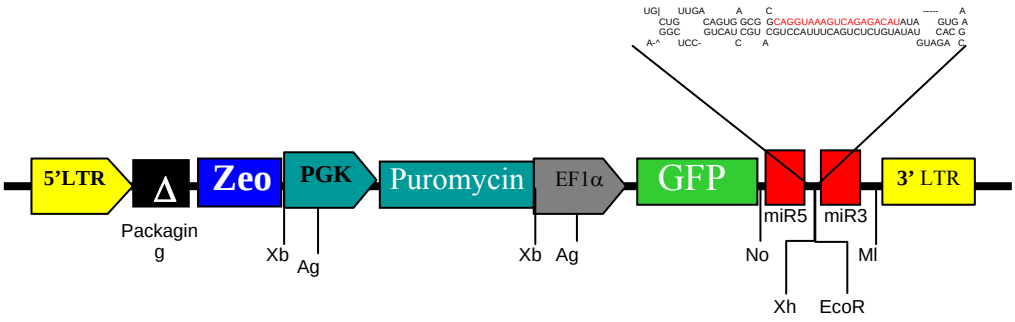


Figure 6. NEF-Red and PEF-Green vector configurations. NEF-Red vector configuration consists of a neomycin resistance gene driven off of a PGK promoter and DS Red and the hairpin expressing region driven off of an EF1α promoter (A). PEF-Green vector configuration consists of a region coding for puromycin resistance driven off a PGK promoter and regions coding for GFP and the hairpin driven off of an EF1α promoter (B).

two promoters, but they are unidirectional from 5' to 3'. It uses the PGK promoter to express a puromycin resistance, then it uses the EF1α promoter to express GFP and the

hairpin. Both the NEF-Red and the PEF-Green vectors also contain the EcoR1 and Xho1 sites at the Mir30 5' and Mir 30 3' locations where the hairpin is positioned.

Materials and Methods

Digestions

To extract the DNMT1 tatgeting constructs out of 2 ug GIN-Zeo plasmid we added sterile water, restriction endonuclease enzymes EcoR1 (New England Biolabs), and Xho1 (New England Biolabs) with the EcoR1 restriction endonuclease buffer. The mixture was placed on ice during preparation, and then heated to 37ºC for one hour. The digestion product was then run on a 1.2% agarose gel for 30 minutes. The shRNA expression constructs digested from the GIN-Zeo plasmid were visible under fluorescent light due to ethidium bromide staining and were cut out with a razor blade. A QIAquick Gel Extraction Kit (QIAGEN 28704) was used to purify the constructs and the products were measured to determine concentration by spectrophotometer.

Ligations and Transformations

Once adequate amounts of expression construct were obtained, they were ligated into the NEF-Red and PEF-Green plasmids which were previously cut using EcoR1 and Xho1 as indicated above. The ligation was performed using the Quick Ligase protocol (Invitrogen). Ligation products were transformed into Top 10 E. coli cells. Top 10 cells were thawed on ice then mixed with the newly constructed plasmids and left on ice to incubate for 30 minutes. They were then heat shocked for precisely 25 seconds at 42ºC and quickly returned to ice. Each individual tube received 100 ul of SOC medium and was place on the shaker for 90 minutes at 37ºC. The transformed bacteria were streaked

on agar plates containing the antibiotics ampicillin and Zeocin (Invitrogen 45-0430) for selection purposes and left overnight at 37ºC. The following day agar plates were checked for colonies.

Plasmid Cloning

Bacteria containing plasmids were grown in a low salt LB broth with ampicillin and Zeocin antibiotics overnight at 37ºC on a shaker. The following day plasmid DNA was extracted from the bacteria using QIAGEN Plasmid Mini, Midi, or Maxi Prep Kits (QIAGEN 27106, 12143, and 12162) depending on the amount of bacteria grown. Plasmid DNA was concentrated at the elution step in sterile water and stored at -20ºC.

Results

After several attempts to clone the DNMT1 targeting constructs into the NEF-Red and PEF-Green vectors we were unable to grow any bacterial colonies on agar plates that were confirmed to have the new plasmids. Multiple problem solving strategies were used at various points throughout the procedures with little or no success. The first step that was altered was the digestion. The first few attempts left us without a digestion band on the gel so we maximized the digestion with as much possible plasmid as the reaction would hold. This alteration actually did result in a product. The next troubleshooting process was designed to obtain a successful gel extraction. There were no significant alterations made in this procedure, it simply took several attempts to extract enough product out of the gel to get a reading on the spectrophotometer. Finally, we were prepared to attempt the ligation. This step proved to be most troublesome; it was never successful. The digestion, gel extraction, ligation and transformation

procedures were repeated several times without ever producing a single colony of bacteria. The ligation enzymes worked for hairpin cloning reactions that were unrelated to the DNMT1 project so we knew they were still good. We still ordered a new set of enzymes but got the same problem.

To address potential concerns with low quality digests of the GIN-Zeo plasmid, we amplified the shRNA coding region of the GIN-Zeo plasmid using a PCR amplification Kit (Invitrogen 12344-040). However, the sam problem persisted and no bacteria colonies were ever produced with the new plasmids.

Discussion

While the problem solving strategies for the hairpin expression construct cloning were going on, *in vitro* experiments using the GIN-Zeo plasmid were beginning to show signs of success. After spending four months trying to insert the hairpins into different vectors we made the decision to use GIN-Zeo for the remainder of the project. Subsequent chapters will describe in detail the procedures, results, and conclusions of how the GEN-Zeo plasmid performed for this project.

CHAPTER III

KNOCKDOWN OF DNMT1 IN BOVINE SOMATIC CELLS

Previous studies have evaluated the effects of RNAi mediated silencing of DNMT1 mRNA in immortalized cell lines and primary cell lines (Leu, Rahmatpanah et al. 2003; Adams, Pratt et al. 2005; Oridate and Lotan 2005). Of these studies, only Adams et al studied bovine primary cell lines, perhaps due to the difficulty of success using RNAi strategies in such cell lines. The bovine cells showed only a 15.4% knockdown of DNMT1 mRNA whereas Adams showed a 56.5% knockdown in primary murine cells. It is interesting to note that the knockdown in mRNA did not correspond to a detectable knockdown in protein as determined by Western blot analysis (Adams, Pratt et al. 2005). Perhaps the delivery system was not optimal or the siRNAs did not silence the mRNA for long enough to see a high degree of knockdown. For their study, Adams et al transfected siRNAs directly into cells without using an expression vector. In this study, we attempted both transient transfections of plasmids and stable integration of expression constructs producing DNMT1 hairpins to elicit a longer silencing effect and allow for negative selection of non-transfected cells. With this approach we could express the hairpin transiently or produce a permanent DNMT1 knockdown.

The first objective for this part of the project was to investigate the effectiveness of multiple protocols to achieve high transfection and transduction efficiencies in bovine primary fibroblast cells. In order to induce an observable knockdown, our needs were twofold: to transfect hairpins into a very high percentage of cells in a population prior to antibiotic selection; and to get multiple copies of the hairpin expressing plasmids into

each cell. Fulfilling these requirements would allow us to detect meaningful reductions in mRNA levels from a real time PCR reaction as well as reductions in protein via western blot analysis.

After determining the effect of the shRNAs on DNMT1 transcript levels, we would determine the effect on DNA methylation. To our knowledge there are no studies in the bovine upon which to base our predictions. If the bovine mechanism is similar to the mouse model, alternate DNA methyltransferases such as DNMT3A or DNMT3B would compensate for the loss of DNMT1 by taking over the role of maintenance methylation. In this case we hypothesized no detectable change in global DNA methylation (Rhee, Bachman et al. 2002). For this reason, we included the mRNA expression of DNMT3A and DNMT3B on our RT PCR panel as well.

In primary mouse cells the DNMT1 knockout and high knockdown is lethal (Egger, Jeong et al. 2006). One could assume the same is required in bovine cells as well. If the bovine cells are able to survive with very low levels of DNMT1 this could result from either a total compensation by DNMT3A and DNMT3B or from another undefined mechanism.

To determine whether shRNA expression produced a knockdown of DNMT1 and to observe the effects of DNMT1shRNA expression, we evaluated the following:

- transfection/transduction rates
- cell morphology/proliferation
- PCR for mRNA levels (DNMT1, 3a, 3b)
- Western blot for DNMT1
- 5-meC staining of somatic cell nuclei

To properly control for these experiments we used a non-targeting shRNA with a hairpin targeting GDF8 (myostatin). This gene is only expressed in muscle cells so there is no target mRNA in the fibroblast and epithelial cells that were used in these experiments.

There was one potential pitfall to this experiment. If our hairpins targeting bovine DNMT1 were not 100% homologous to the DNMT1 mRNA sequence of our cell line, we would not have expected to observe a knockdown at the mRNA level, but would have expected knockdown at the protein levels. This is because the silencing mechanism would be acting through translational inhibition rather than mRNA degradation. Had this been the case, analyzing the effectiveness of the DNMT1 targeting shRNAs would have relied on the results of a western blot. This was undesirable because prior attempts in our lab at identifying the bovine DNMT1 protein using commercially available antibodies were difficult to repeat.

Materials and Methods

Bovine Fetal Cell Isolation and Culture

Bovine fetuses were collected from pregnant slaughtered cows and placed on ice in plastic bags in physiological saline solution (0.9% NaCl) for transport to the laboratory. In all, we collected three male and one female fetus approximately day 37 to 80 post fertilization. To remove the majority of neuronal and intestinal tissue the heads were removed and body cavity eviscerated prior to cell isolation. The rest of the fetus was minced in 1 x PBS without Ca++ or Mg++ into pieces between 5 and 10 mm in diameter. The PBS-tissue mixture was poured into a 50 mL conical tube until the tissue

settled to the bottom. To wash the tissue, the liquid portion was decanted and fresh PBS was added back to the tube. This was repeated three more times. After the fourth wash, 15 ml of 1 x trypsin in 1 x PBS Ca++ Mg++ free were added to the tube and left in the incubator for 15 minutes at 37°C and 5% CO2. The tubes were removed inverted five times to mix the tissue and 15 more mLs of 1 x trypsin (Sigma T4174) were added to the tubes. Typically fibroblast and epithelial cells are of the first to dissociate from the tissue. The tubes were placed back in the incubator for 30 minutes and inverted 5 times every 10 minutes to ensure proper mixing.

The supernatant was removed, placed in a 15 ml tube, and centrifuged for 10 minutes at 3,000 RPMs to create a pellet of epithelial and fibroblast cells. The trypsin reaction was repeated on the tissue one more time. After centrifugation, the supernatant was discarded and the cell pellets were re-suspended with complete medium consisting of DMEM-F12 (Gibco 12500-062) containing 10% fetal bovine serum (HyClone SH30088.03) and 50 µg/ml gentamycin (Gibco 15750-078). The cell suspensions were then placed in T-25 flasks and placed in the incubator with 5% CO2 and air at 37°C. Remaining tissue was placed into T-25 flasks with complete media in incubators with individual pieces of tissue no closer than 2 cm from one another. When primary fibroblast cell cultures became nearly confluent, they were exposed to 1.0% trypsin solution to remove the adhered cells from the plastic flask. After each passage, cells were cryopreserved in micro tubes by adding 10 % DMSO to approximately 1.0 x 10^6 cells. The tubes were placed in the -80°C freezer in methanol. Each batch was labeled according to the cell line and passage in which they were frozen.

Transient Transfection

Gene Jammer transfection reagent (Stratagene 204130) was used for transient transfections of plasmids into HEK 293 and bovine cells. Cells were grown to 60% confluence in 30 mm dishes prior to transfections. The recommended protocol was used; however, we experimented with different ratios of DNA to Gene Jammer to find the optimal combination. G418 (Gibco 10131-035) was added to cultures 36-48 hours post transfection to select for transgenic cells.

Viral Transduction

Production of recombinant lentiviral particles was performed using a three plasmid system via calcium phosphate transfection into the HEK 293T packaging cell line. HEK 293 T cells were grown to 80% confluence in 100 mm round cell culture dishes and passaged 24 hours prior to transfection. Eight milliliters of complete medium was added one hour before the transfection. Reagents were mixed at the following ratios for 100 mm plates: 36.0 ul of 2.0M CaCl2, 5.0 µg of the coat protein VSVG, 10.0 µg delta, 10.0 µg of plasmid DNA, then add sterile water for a final volume of 300 µl. An equal volume of 2X Hepes Buffered Saline (HBS) was added to the DNA/CaCl2 solution drop-wise, flicking the tube with a finger between drops. The 600 µl of transfection solution was added drop-wise to the 100 mm plates, then 1000 x Polybrene (Sigma H9268) was added at a volume of 1 µl/ml of complete media. Cultures were placed in incubators at 37°C in 5% CO_2 and for 24 hours and media was replaced with the addition of 1000 x sodium butyrate at a volume of 1 µl/ml in complete media. After transduction, cells were allowed to grow for 36 hours before the addition of G418 at 10

mg/ml. Cells that were not used for analysis were cryopreserved at passage one and two post transfection (i.e. passage three and four overall).

Some cells were transduced a second time. After the second transduction, cells were either analyzed or cryopreserved for future use. These cells were analyzed or frozen at either passage six or seven overall. Double transduction was carried out in both male and female cell lines and for shRNA 3 and shRNA 4. The male cell line that was transduced twice with lentiviral vector containing the shRNA 4 expression construct took several additional days (as compared to other cell lines) to grow enough cells to analyze. There were also only enough cells to cryopreserve one vial with 0.25 million cells while other cells were frozen in groups of approximately one million cells.

Immunocytochemistry

Transgenic cells were subpassed by standard techniques and plated to a 10 mm 4-well Nunc dish (Nunc 176740) with glass coverslips (Fisherbrand 12-545-83 15CIR-1D) at the bottom. The coverslips were previously treated with 4 M HCl and rinsed thoroughly with Millipore water so the cells would strongly adhere to them. Cells were placed in flasks at a concentration so that within 16-18 hours they were at 80-90% confluency. Using the 4-well Nunc dish to culture the cells minimized the amount of antibody needed for each reaction. When cells reached 80-90% confluency they were washed twice with 1 x PBS and fixed with -20°C methanol for 5 minutes. Cells were washed in 1 x PBS and permiabilized for 30 minutes at room temperature with 0.2% Triton (Sigma Aldrich X100-100ml) in 1 x PBS. Permeablization medium was then removed and 3 molar HCl diluted in ddH2O with 0.1% Triton was added to denature the

DNA and allow binding of the primary antibody to methylated DNA. The HCl was removed after 10 minutes and a 100 mM Trizma Hydrochloride buffer (Sigma T3253), pH 8.5, was added for 30 minutes to neutralize the HCL. Blocking buffer with 3.0% BSA (Sigma 9022) and 0.1% Triton 100X in 1 x Ca++ and Mg++ free PBS (Sigma D1283) was incubated on the cells for four hours at room temperature to block non-specific binding of antibodies. The polyclonal primary antibody to 5-methylcytidine (Eurogentec MMS-900P-B) was diluted 1:200 in blocking buffer and 150 µl was added to each Nunc well and incubated for 30 minutes at 37°C. One well was always an IgG control that received no primary antibody. Primary antibody was washed off by rinsing with blocking buffer six times for five minutes. An Alexa 488 (Invitrogen A11001) secondary antibody was diluted 1:200 in 1 x PBS with 0.1% Tween 20 (Sigma P9416-100ml) and incubated for 30 minutes at 37°C. Secondary antibody was washed three times with 1 x PBS with 0.1% Tween 20 for five minutes each time. Cells were incubated for 20 minutes at 37°C with RNase A (Sigma R-6148) diluted to 25 µg/ml in 1x PBS. Cells were then incubated for 30 minutes at 37°C with propidium iodide (PI) diluted 25 µg/ml in 1 x PBS. The cover slips with adherent cells were mounted on glass slides with a 50/50 solution of FloroGuard anti-fade reagent (Bio-Rad 170-3140) and 40% glycerol in PBS and sealed with clear fingernail polish.

Imaging

Fluorescent imaging was performed on a Nikon Eclipse 3500 microscope on the 20 x objective with a numerical aperture of 0.45 using a Roper Scientific camera. Fluorescent images were taken of the 5-methylcytodine antibody (Alexa 488) and the PI.

All images in a single study group were taken on the same day and were taken at the same exposure time to avoid mechanical inconsistencies. Image analysis was carried out using NIS Elements 3.0 software. Mean intensity measurements of the 5-methylcytodine antibody (Alexa 488) fluorescent output and the PI fluorescent output were gathered from each individual cell.

Real Time PCR

RNA extraction was done via the RNeasy mini kit. Cell cultures were washed in 1 x PBS twice before adding lysis buffer RLT (Qiagen S13-26-36-46). RNA was used in a reverse transcription reaction to create cDNA using an Iscript Kit (Bio-Rad 170-8891). Samples were used for a real time PCR reaction using primers designed for bovine DNMT1, DNMT3A, and DNMT3B. The internal control gene was bovine GAPDH. Runs were done with triplicates and were repeated 3 times to give nine data points for each gene in each cell line.

Western Blot

Cells were grown 100% confluent in 30 mm dishes in complete medium and rinsed with 1 x PBS just prior to adding 300 µl of protein lysis buffer. After five minutes the lysate was centrifuged in 1.5 ml micro tubes for 30 minutes at 13,000 RPMs and 4°C. The supernatant containing the protein was removed and stored in a micro tube at --20°C.

Proteins were separated by molecular weight using gel electrophoresis, and transferred onto a PVDF membrane. The membrane was blocked using a 2.0% solution of skim milk in TBST for 1 hour. The DNMT1 goat polyclonal IgG primary antibody

(Santa Cruz Biotech sc-10221) was diluted 1 μg/ml in the 2.0% skim milk/TBST solution and applied to the membrane. It was then incubated overnight at 4°C on a rocker. The membrane was washed three times with TBST and the secondary donkey ant-goat IgG horse radish peroxidase antibody (Santa Cruz Biotechnology sc-2020) was diluted 1:20,000 in 2.0% skim milk/TBST solution and rocked for 10 minutes at room temperature. The membrane was washed three more times with TBST and then incubated for 5 minutes on the rocker under foil with equal parts of Super Signal West Pico Stable Peroxide and Luminol Enhancer which make up the chemiluminescent detection solution (Pierce 34080). The membrane was imaged and analyzed using the AlphaInnotech imaging system.

Statistics

Average intensity measurements from each cell were computed from fluorescent output of 5 methylcytosine labeling. Nuclear area was also recorded from each cell. The mean was calculated for each treatment group and compared to others using a T-test with α set at 0.05. Real time PCR expression profiles were combined from each run and compared using a T-test with α set at 0.05. Protein blot band densities were compared using T-test with α set at 0.05.

Results

The most efficient combination of DNA and Gene Jammer for both the HEK 293 cells and the bovine fibroblasts was 6 μg of DNA to 9 μl of Gene Jammer to achieve a

35-45% transfection efficiency. Percentages were estimated visually 36-48 hours after transfection by observing for the presence of GFP via fluorescence microscopy. Cells were observed to express GFP from the GIN-Zeo plasmid for approximately 7 days after the transfection. Selection of transiently transfected cells to achieve a pure population of transgenic cells was difficult to achieve for two reasons. Low transfection rates required that more than 55% of the cell population be destroyed via G418 treatment. Therefore,

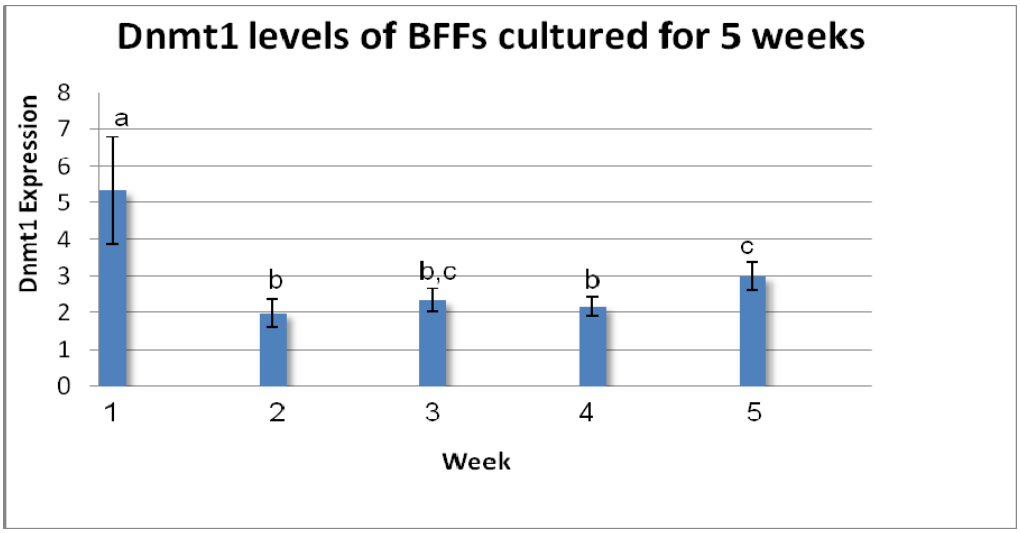


Figure 7. Mean DNMT1 mRNA expression in normal bovine fetal fibroblasts. Cells were cultured for 5 weeks and RNA samples were harvested once per week to determine DNTM1 expression. Bars with similar letters were not significantly different ($p < 0.05$).

the remaining cells would require time to multiply and grow to a cell density that we could use for analysis. Alternatively, by the time the neomycin took effect and the cell population had recovered, plasmid expression had weakened. For these reasons, no reliable data were collected.

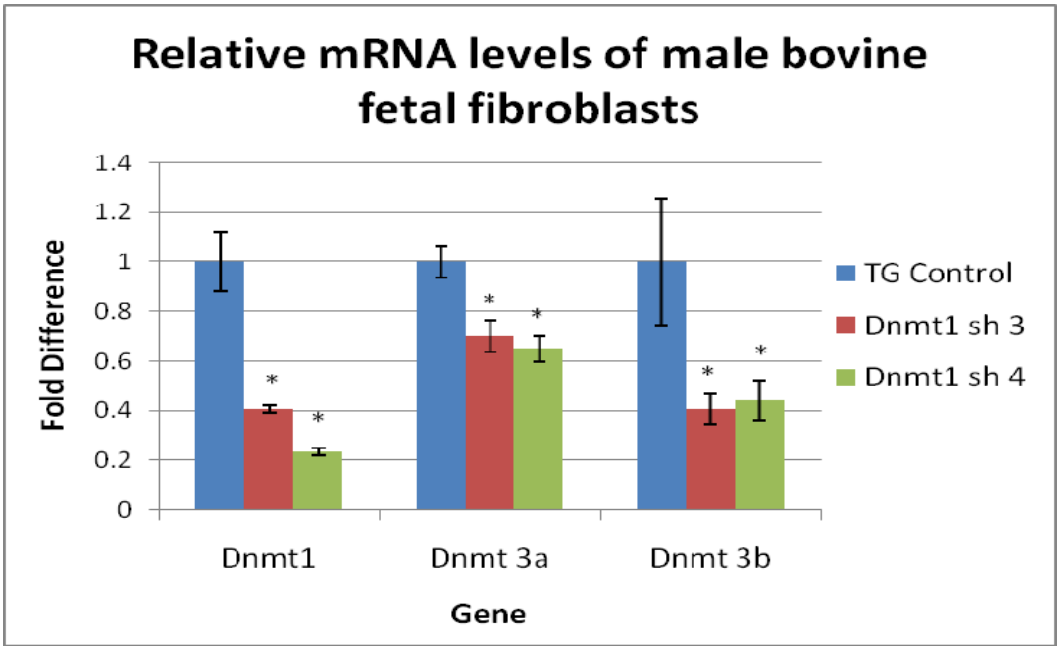


Figure 8. Mean real time PCR expression profile of DNMT1, DNMT3A, and DNMT3B in male bovine cells. The analysis includes a control hairpin targeting GDF8 (blue) and two different hairpins targeting DNMT1 (red and green). Bars with * indicate a significant difference from the control ($p < 0.05$).

Bovine cells that were transduced with lentivirus became 80-90% transgenic within 36-48 hours after transduction. Cells were then selected 3-5 days with neomycin to obtain pure cultures expressing DNMT1 hairpins and the non-targeting control hairpin. These cells were used for analysis of mRNA, protein, and global methylation.

Before analyzing the transgenic cells, the expression pattern of DNMT1 was measured in normal bovine cells to account for any *in vitro* culture affects that may have occurred. Cells cultured longer than one week *in vitro* had lower DNMT1 expression, but after the first week of culture there was no further decrease (Figure 7). As a result of

this, viral transductions were done on primary cells that were of the same passage to avoid misinterpretation of expression data.

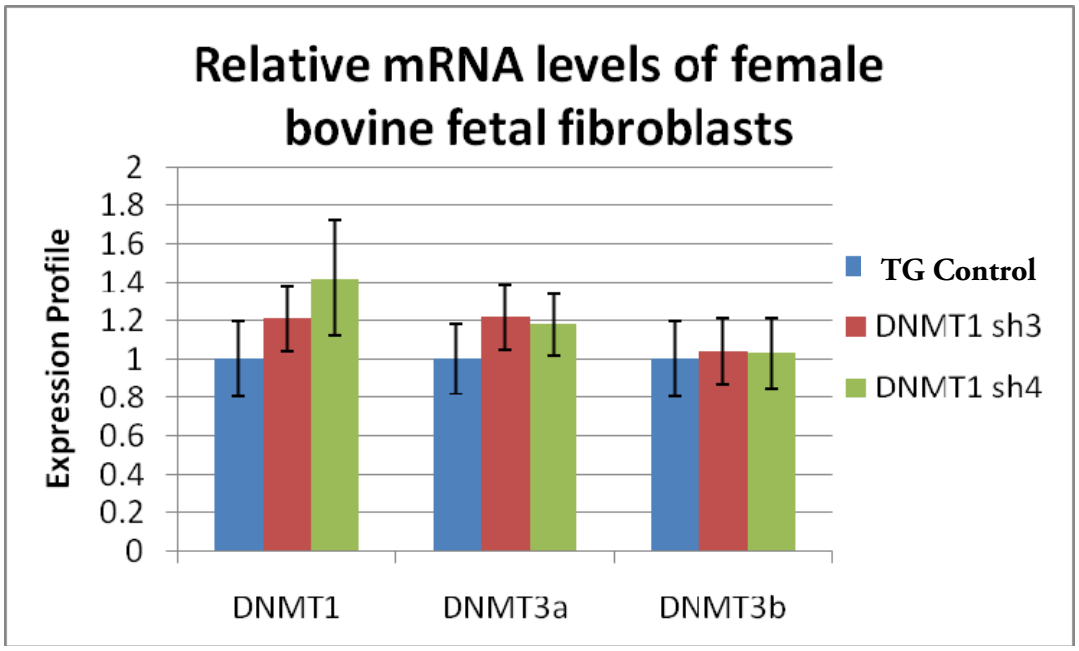


Figure 9. Mean real time PCR expression profile of DNMT1, DNMT3A, and DNMT3B in female bovine cells. The analysis includes cells expressing a control hairpin targeting GDF8 (blue) and two different hairpins targeting DNMT1 (red and green). There were no significant differences of any group compared to controls ($p > 0.05$). Data are presented as expression level compared to the internal control, GAPDH.

Real time PCR showed a significant decrease in DNMT1, DNMT3A, and DNMT3B mRNA in male bovine cells expressing the DNMT1 targeting shRNA compared to cells expressing the non-targeting shRNA to GDF8 (Figure 8). Expression of DNMT1 mRNA was 59.3% lower for D1sh3 and 76.7% lower for D1sh4 compared to the control. Expression levels of DNMT3A and DNMT3B were also significantly lower in these cells. Female bovine cells did not show a significant decrease in any of the three tested genes (Figure 9). The female data is presented different than the male data

because the male data consisted of three separate triplicate experiments combined into a single formula to calculate differences. The female data is from a single triplicate experiment which displays the expression profile. This was necessary due to difficulty in producing usable data for the female cells.

Western blot analysis showed a decrease in DNMT1 protein in both the male and female cell lines from each of the two shRNAs when compared to the control (Figure

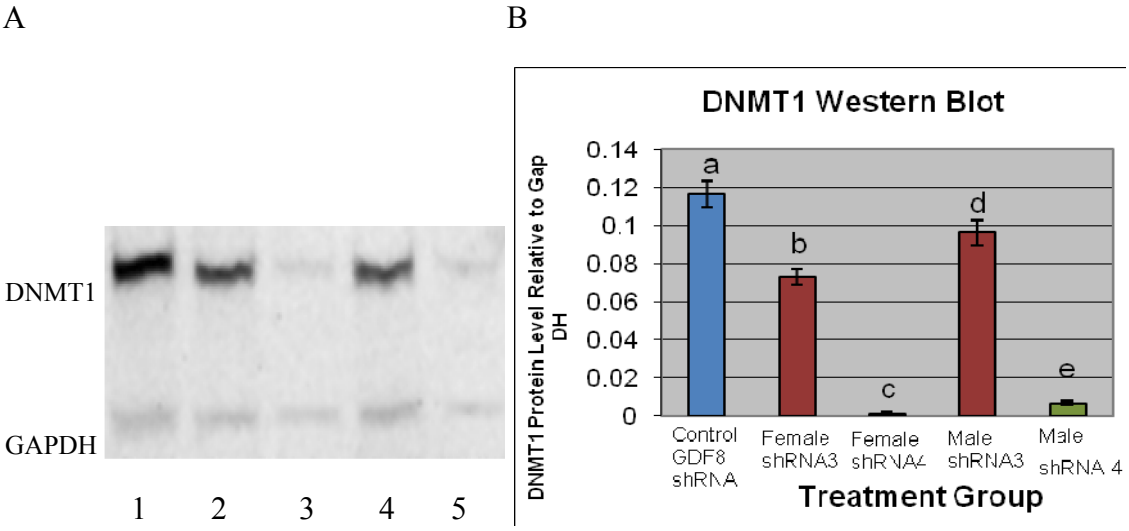


Figure 10. Protein blot analysis. Western blot showing DNMT1 protein bands for the following treatment groups: GDF8 male control cells (1), D1sh3 female cells (2), D1sh4 female (3), D1sh3 male (4), and D1sh4 male cells (5) (A). Quantification of DNMT1 band density compared to GAPDH (B). Bars with similar letters are not significantly different ($p < 0.05$).

10A). Quantitative analysis of band density is diagrammed in Figure 10B. Density measurements of the bands showed that all cell lines were significantly different from the control. Immunocytochemistry results indicate that DNA methylation was altered as a result of the DNMT1 shRNAs. Figure 11 is a display of pictures taken of male bovine

control and treated cells. Figure 11A shows normal methylation from untreated cells and is similar to the GDF8 control cells in Figure 11B. The cells pictured in Figure 11C were infected with D1sh3. These cells had a significantly decreased amount of methylation as can be observed via immunolabeling. Interestingly, cells infected twice with the D1sh4 revealed a significant increase in global methylaiton (Figure 11D).

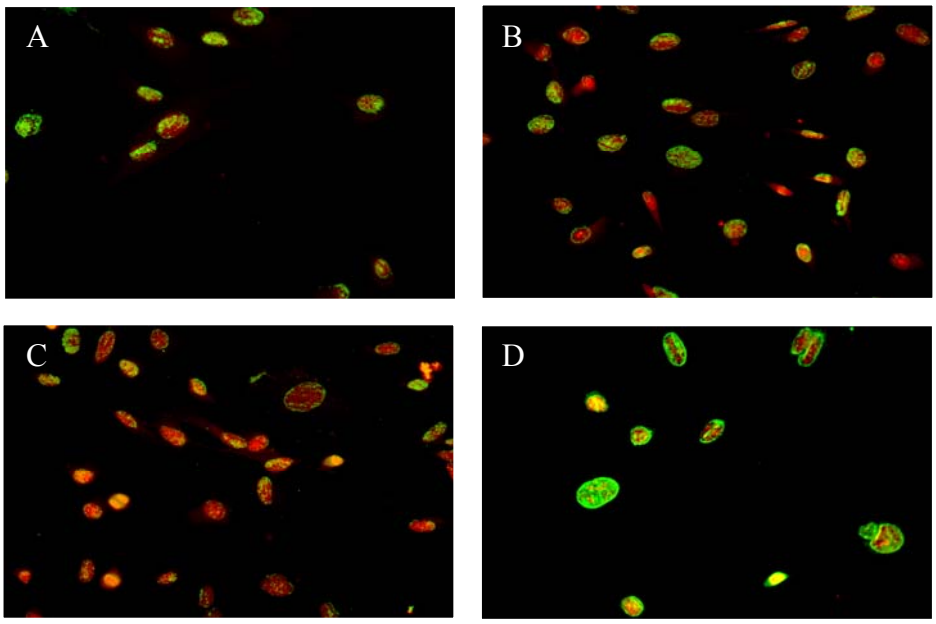


Figure 11. Male bovine cells labeled with 5-methylcytosine. Alexa 488 secondary antibody (green) and propidium iodide nuclear stain (red). Pictured here are untreated control cells (A), GDF8 non-targeting controls (B), D1sh3 treated cells (C), and D1sh4 double infected cells (D). All pictures were taken at the same magnification.

Fluorescent intensity measurements were recorded from the fluorescent output of labeled DNA methylation for both the male and female cell lines. The results were compared using a Student-T test to determine statistical differences. Figure 12A and B and Table 1 show the measurement results. The female cells showed a slight drop in

methylation in both treated groups while the male cells showed a drop as a result of D1sh3 and an increase as a result in D1sh4 treatment.

In addition to changes in methylation, gene, and protein expression, male and female cells infected with D1sh4 showed morphology changes including: multinucleated cells, nuclear vacuoles, increased cellular and nuclear size, and an overall decrease in cell division speed. Figure 13 displays a few of these cellular morphology changes. Of the double infected cells expressing the D1sh4 hairpin, greater than 25% had multiple nuclei (Fig 13D) and nuclear size was increased by an average of 19% ($p < 0.05$) as detected by fluorescent labeling of the nucleus.

A

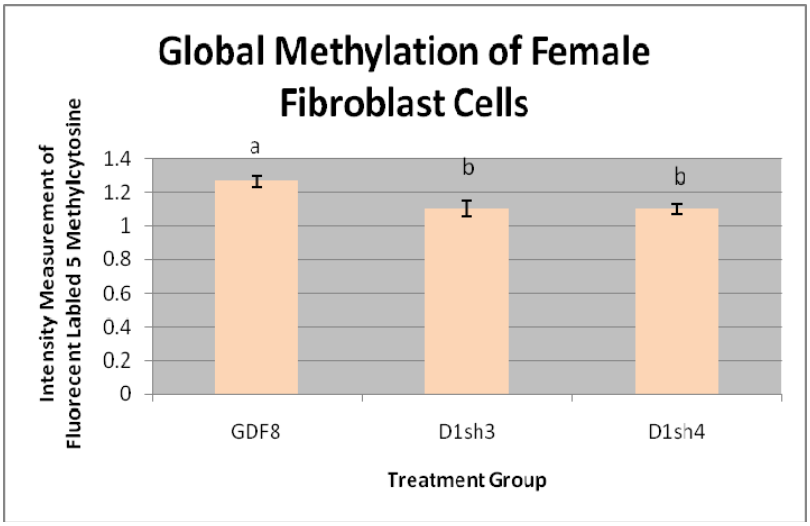


Figure 12. Measurement of global methylation of female (A) and male (B) fibroblast cells. 5 methylcytosine fluorescence was ratioed to PI ($p < 0.05$).

B

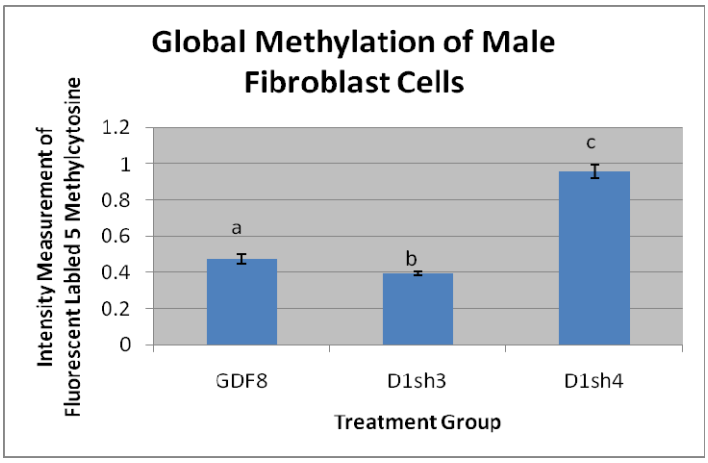


Figure 12 Continued.

Table 1. Intensity measurement of 5 methylcytosine per treatment group. Groups with like letters are not significantly different ($p < 0.05$).

Intensity Measurement of Fluorescent Labeled 5 Methylcytosine						
Hairpin	Female Fibroblast Cells			Male Fibroblast Cells		
	n	avg	SE	n	Avg	SE
GDF8	102	1.26a	0.047	145	0.48a	0.023
Dnmt1 sh3	100	1.10b	0.032	127	0.39b	0.010
Dnmt1 sh4	85	1.09b	0.031	134	0.95c	0.037

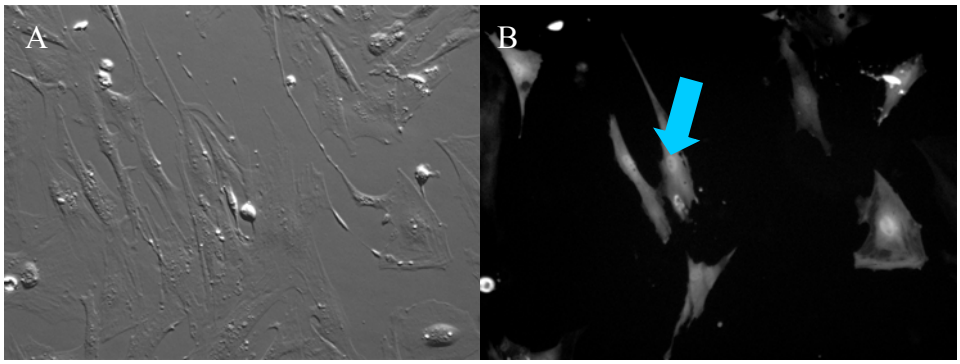


Figure 13. Brightfield image (A) and fluorescent image (B) showing GFP expression of control GDF8 cells. Brightfield (C) and GFP image (D, E) of D1sh4 double infected cells. The blue arrow points at a normal nucleus, the green arrow points at the oversized nucleus, the red arrows point at vacuole structures, and the white arrow indicates multiple nuclei. All pictures were taken with a 20x objective.

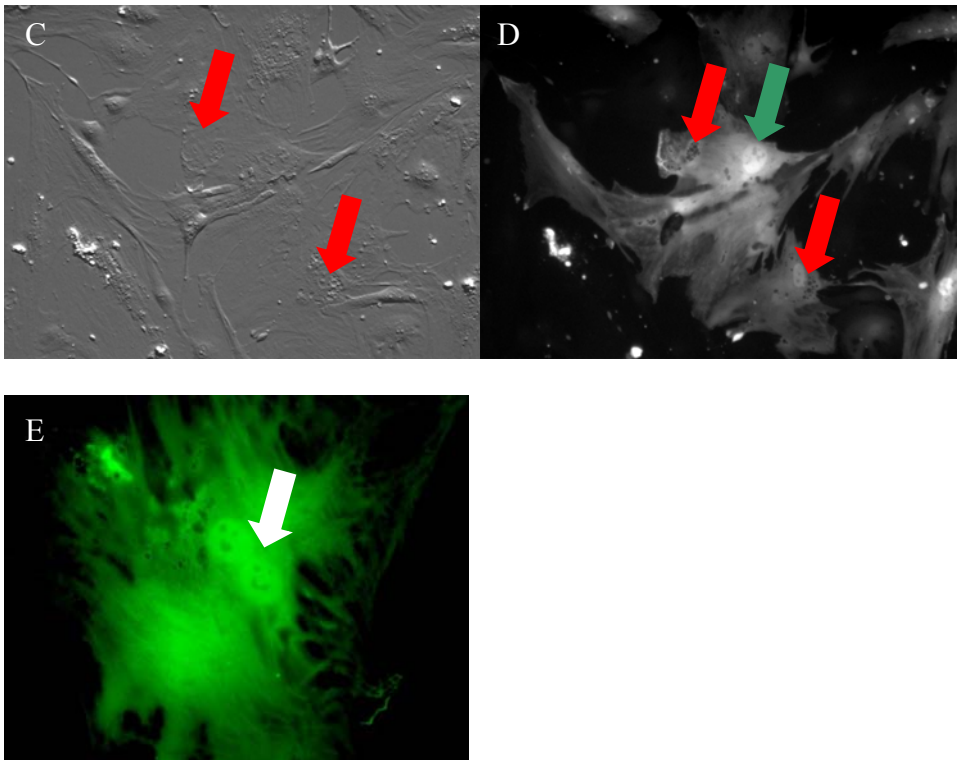


Figure 13 Continued

Conclusions

Real time PCR data indicated a decrease in DNMT1 mRNA in the male cell line with each shRNA, however, D1sh4 resulted in a slightly greater knockdown. There was no detectable change in the female cell line. The hairpins may be acting to degrade the DNMT1 mRNA in the male cells, but not the female cells. The data also suggests that DNMT3A and DNMT3B decreased in samples that showed a knockdown of DNMT1. This increase in 3A and 3B are not representative of previously published work in the mouse and may indicate species differences in regulation of DNA methylation.

At the protein level, there were decreases in DNMT1 protein in both the male and female cell lines from each of the two hairpins. Furthermore, it is apperant that D1sh4 elicited a much higher degree of knockdown at the protein level than D1sh3 did. Since the female cells showed a knockdown at the protein level but not the mRNA level it is possible that the hairpins only blocked translation and did not cause mRNA degradation.

Conclusions regarding the methylation status of treated cells are difficult to establish. In the male cell lines, 59.3% knockdown resulted in a decrease in overall DNA methylation while a 76.7% knockdown induced hypermethylation. Double infection appeared to only affect the male cell line when the D1sh4 hairpin was used. The hypermethylation was coincidental with a drastic knockdown at the DNMT1 protein level as reported. We have not been able to make any formidable conclusions as to why the female cell lines with a high protein knockdown did not exhibit the same hypermethylation and morphological traits. The morphological traits of the hypermehtylated cells indicate apoptotic signaling and senescence. We conclude that this is a result of severely aberrant gene expression due to massive global gene silencing.

Discussion

Interestingly a 59.3% knockdown at the mRNA level resulted in a decrease in overall DNA methylation while a 76.7% knockdown induced hypermethylation. One might hypothesize that overcompensation of DNMT3A or DNMT3B would have aberrantly methylated the DNA, but as we have shown, the transcription of these genes decreased as detected by RT PCR of mRNA levels. The real time data that suggests this

decrease in DNMT3A and DNMT3B as a result of DNMT1 knockdown is contradictory to knockdown data in mice that suggests compensation of these two genes as a result of DNMT1 knockdown (Rhee, Bachman et al. 2002). More work must be done to investigate the mechanism in which these cells became hypermethylated.

Another interesting finding was that the female cell line showed no knockdown at the mRNA level, yet it showed an even higher knockdown at the protein level than the male cell line. Although possible, it is unlikely that both hairpins were homologous to the male mRNA but not to the females mRNA. This would explain the miRNA-like silencing observed in the female cells. It is unlikely that the mRNA sequences were different in the male and the female cells in this experiment because they were isolated from fraternal twins of the same uterus. Thus, genetics did not likely play a role in this phenomenon. If there is a sex specific difference for why the hairpins acted in different ways for this experiment there will need to be a significant amount of research to determine this mechanism. Reports showed epigenetic differences between male and female bovine blastocysts produced in vitro (Bermejo-Alvarez, Rizos et al. 2008). This information supports our findings in somatic cells. This experiment needs further repetition to verify results.

The morphology changes seen here indicate that the male cells showed signs of senescence and even apoptosis. Similar studies on human and mouse cells describe DNA replication defects as a result of DNMT1 knockdown (Unterberger, Andrews et al. 2006). Unterberber et al proposed that a decrease in DNMT1 availability may cause an S-phase arrest as a result of induction of stress response genes, a mechanism that may be

in place to prevent additional loss of methylation. This could explain the decrease in cell proliferation, but it still would not explain the observed hypermethylation. Without any comparable studies in bovine cells, there are few solid conclusions we can make from our data.

Our stated hypothesis was correct because at some level of DNMT1 knockdown we did observe induced global hypomethylation. In the future these cells can be used in studies for SCNT to determine their potential as nuclear donors. It would be interesting to find out what genes compensate for the absence of DNMT1 at the 8-cell stage, the time when this gene is usually first expressed in bovine embryos (Dean, Santos et al. 2001; Golding and Westhusin 2003).

After determining that the hairpins do induce gene silencing, a follow up study would be more fitting if it were designed with a transient effect. Somatic cells would need to be transiently transfected with plasmids and then used for SCNT. The transient expression of hairpins would last for about seven days. Therefore, if the nuclear transfer was done three days post transfection, the effect of the shRNAs would be low by the time the embryos reached the 8-cell stage. Normal DNMT1 expression would continue for the rest of development. Another benefit of this experimental design is that the resulting offspring would not technically be transgenic because there is no integration of foreign DNA into the genome.

CHAPTER IV

DNMT1 KNOCKDOWN IN PREIMPLANTATION BOVINE EMBRYOS

Although most previously published works have studied gene function in somatic cell types, our interest is in the early embryo. Therefore we designed experiments to examine the result of DNMT1 knockdown in *in vitro* derived embryos. Experiments using in vitro produced bovine embryos have many difficulties one must overcome that may not be present with mouse embryos or with somatic cell cultures. The oocytes are expensive, one must have a reliable production system in order to achieve high success rates, and physical manipulation to the oocytes or embryos can be deleterious.

The original goal for this project was to micro-inject synthetic DNMT1 siRNA into one cell *in vitro* produced zygotes, 20-22 hours after fertilization. Due to an extremely low rate of in vitro fertilization (IVF) embryo production in the non-injected controls, we elected to alter our embryo production method and utilize parthenogenetic oocyte activation. This resulted in a more uniform set of embryos without the variation from paternal effects.

Oocyte activation does not require sperm and is a very efficient alternative for producing embryos *in vitro*. Parthenote embryos differ from IVF embryos because rather than an oocyte being fertilized by a sperm to acquire a full set of DNA, the second polar body re-associates with the nucleus to make a 2N set of chromosomes. The components required for activation are similar to that of nuclear transfer. For this reason, we found this to be an acceptable model for this experiment.

Materials and Methods

IVF Embryo Production and Injection

Mature oocytes were shipped by Ovitro Biotechnology, Inc. from Hereford, TX to College Station, TX. Oocytes were removed approximately 22 hours after being placed in maturation medium and washed through two plates of TL Hepes (Lonza 04-616F) at 37°C. Oocytes were then moved in groups of 50 with a 20 µl pipette into a 4-well dish containing 420 µl of fertilization media. Semen was thawed for 30 seconds at 37°C and spun through a 45/90% Percoll (Sigma P1644) gradient at 400 X g for 20 minutes. The semen pellet was removed from the gradient, counted for concentration, and diluted to 1.5 million sperm per ml in fertilization medium. Sperm, phenolalanine/hypotaurine (PH), and heparin (10 µl/ml final concentration) were added (20µl each) to the fertilization plates for a final volume of 500 µl per well. The fertilization plates were incubated for 22 hours in 5% CO2 and air at 38.5°C. The following day zygotes were stripped of cumulus cells and the non-injected controls were held in TL Hepes. The treated embryos were placed in TL Hepes and injected with either a non-targeting control siRNA or an siRNA targeting DNMT1 at 10-100 picoliters per embryo. The siRNAs were diluted prior to injection to 50 mM in sterile water. Both siRNAs were injected with Cy3 for a visual conformation that the injection successfully made it into the cytoplasm. The zygotes were subsequently placed in G1^{TM} -Version 3 media (Vitrolife 10091) for 3 days then G2^{TM} -Version 3 media (Vitrolife 10092) for culture from the 8-cell to the blastocyst stage.

Oocyte Injection and Activation

Mature oocytes were stripped of cumulus cells 22 hours after being placed in maturation media. Oocytes to be injected were then placed in a 200 mM solution of sucrose in Hanks/Hepes medium with 10% FBS and gentamycin. The sucrose solution shrinks the cytoplasm and makes it easier to penetrate with the micro pipette. Each siRNA was diluted to 50 mM in sterile water. Treatment groups for the oocyte injections were the same as the IVF zygotes. Oocytes were each injected with approximately 10-100 picoliters siRNA until a slight expansion of the vitelline membrane was visible. The expanding vitelline membrane is an indication that the siRNA was not simply being injected subzonally into the perivitelline space. Injections were done in groups of 50 oocytes at a time. After injection, oocytes were washed in TL Hepes and moved into a series of four ionomycin washes. The total duration of these washes was four minutes. Oocytes were then washed in TL Hepes and moved immediately into a DMAP solution (Calbiochem 476493) in M199 Earls (Gibco 12340) with 10% FBS and incubated at 38.5°C and 5% CO_2 and air. Four to six hours later the oocytes were washed in fresh M199 and moved to G1 media for 84 hours followed by G2 for 72 hours. When the parthenotes were moved from G1 to G2 they were at the 8-16 cell stage.

Immunocytochemistry

Parthenotes at the 8-16 cell stage (84 hours post activation) and blastocyst stage (168 hours post activation) of each treatment group were harvested for immunostaining.

They were removed from culture, washed twice with 1 x PBS and fixed with -20°C methanol for 5 minutes. Embryos were washed in 1 x PBS and permiablized for 30 minutes at room temperature with 0.2% Triton in 1 x PBS. Embryos were removed from permiablization medium and placed in 3 molar HCl diluted in ddH2O with 0.1% Triton to denature the DNA. This made binding of the 5-methylcytidine primary antibody to methylated DNA possible. Embryos were removed from the HCl dilution after 13 minutes and placed in 100 mM Trizma Hydrochloride buffer, pH 8.5, for 30 minutes to neutralize the HCl. The embryos were incubated in blocking buffer with 3.0% BSA (Sigma 9022) and 0.1% Triton in 1 x PBS Ca++ and Mg++ free for four hours at room temperature to block non-specific binding of antibodies. Primary antibody to 5-methylcytidine was diluted 1:200 in blocking buffer and embryos were incubated in 150 µl per group for one hour at 37°C. One well was always a control that received no primary antibody. Primary antibody was washed off by washing embryos through blocking buffer six times for five minutes each. An Alexa 488 secondary antibody was diluted 1:200 in 1 x PBS with 0.1% Tween 20 and embryos were incubated for one hour at 37°C. Secondary antibody was washed three times with 1 x PBS with 0.1% Tween 20 for five minutes each time. Embryos were incubated for 20 minutes at 37°C in RNase A diluted to 25 µg/ml in 1x PBS. Embryos were then transferred to a solution of PI diluted 25 µg/ml in 1 x PBS and incubated for 30 minutes at 37°C. Embryos were placed on a glass slide with 20 µl of a 50/50 solution of anti-fade and glycerol. Slides were covered with a coverslip and sealed with clear fingernail polish.

Imaging

Images were taken using a BioRad Radiance 2003 multiphoton/confocal microscope and a 60X water immersion objective using Laser Sharp 2000 imaging software. A z-series was taken from individual embryos with 5 microns between each section. There were approximately 20-35 images per embryo depending on their shape and size. Each section contained an image of the 5-methylcytosine label and one for the PI nuclear stain. Mean intensity measurements of the embryo images were taken using NIS Elements 3.0 software. Using the software, the z-series was reconstructed into a 3-D image. The images were rotated on a 3-D axis and frozen in a position that allowed an accurate measurement for the highest number of blastomeres at one time as possible. The intensity measurements of 5-methylcytosine were divided by the intensity of the PI stain for each individual 3-D blastemere to give us the ratio of methylated DNA to total DNA for each cell in an embryo.

Real Time PCR

RNA extraction was done via the RNeasy kit. Groups of ten embryos were washed in PBS twice before being placed in lysis buffer RLT. RNA was used in a reverse transcription reaction to create cDNA using Iscript. Samples were used for a real time PCR reaction using primers designed for bovine DNMT1, DNMT3A, and DNMT3B. The internal control gene was bovine GAPDH. Real time PCR was performed in triplicate and were repeated 3 times to give nine data points for each gene in a set of embryos.

Statistics

Average intensity measurements were computed from fluorescent output of 5 methylcytosine labeling from each individual blastomere from each embryo. The average intensity of each blastomere per treatment group was compared to those in other treatment groups using a T-test with α set at 0.05. Intensity of blastomeres from each embryo were also averaged together to obtain an average intensity per embryo. Embryos from each treatment were averaged and compared using a T-test with α set at 0.05. Embryonic development rates from each treatment group were compared using a Chi-Square with α set at 0.05.

Results

The production of IVF embryos in summarized in Table 2. As earlier stated, these production numbers were insufficient for our project goals. Our focus was then aimed at making parthenote embryos.

Table 2. Production of IVF embryos.

Treatment	N	Cleaved	% Cleaved	8-cell	% 8-cell from cleaved	8-cells removed	# of cleaved after removal	blastocysts	% blast
no siRNA	302	195	64.57	35	17.95	20	175	15	8.57
non-Target siRNA	202	29	14.36	5	17.24	3	26	2	7.69
DNMT1 siRNA	196	41	20.92	3	7.32	3	38	0	0.00

Parthenote embryo production can be viewed in Table 3. The non-injected control group had a higher cleavage rate (83.5%) than the injected controls (48.8%) which had a higher cleavage rate than the DNMT1 targeting shRNA injected group

Table 3. Production of parthenote embryos. Groups with similar letters were not significantly different as determined by Chi-Square with α set at 0.05

Treatment	n	Cleaved	% Cleaved	8-cell	% 8-cell from cleaved	8-cells removed	# of cleaved after removal	Blastocysts	% blast
no siRNA	182	152	83.5 a	112	73.7 a	46	106	40	37.7 a
non-Target siRNA	375	183	48.8 b	124	67.7 a,b	53	130	38	29.2 a
DNMT1 siRNA	525	182	34.6 c	107	58.79b	46	136	8	5.8 b

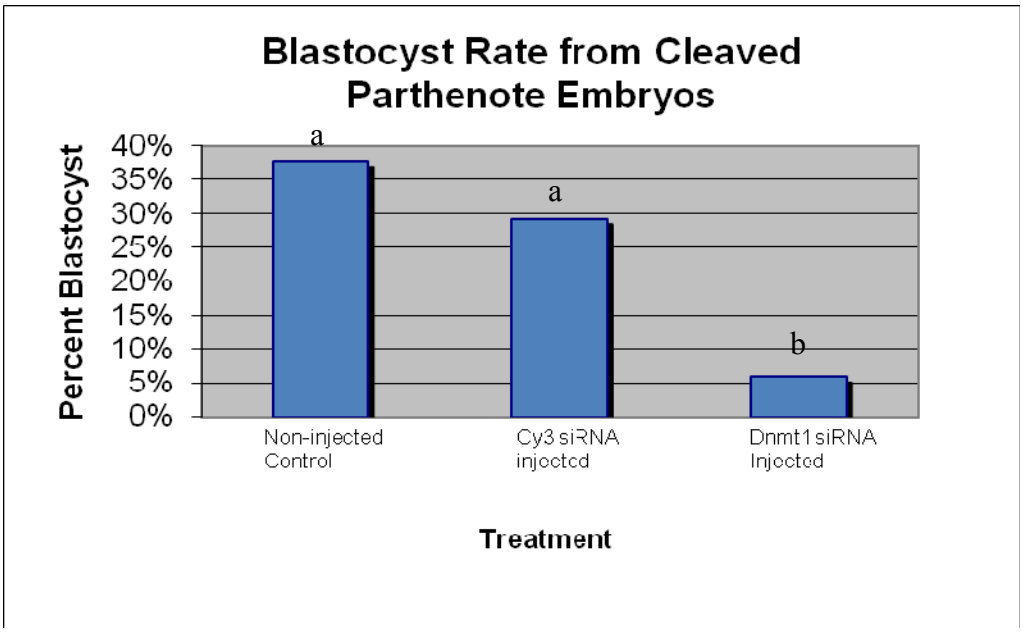


Figure 14. Blastocyst rate from cleaved parthenote embyros. Significance was determined by Chi-square with α set at 0.05.

(34.7%). Of the embryos that cleaved, the two control groups had a similar development rate to 8-cells (73.7% and 67.8%) and the DNMT1 injected embryos (58.7%) were different than the non-injected controls but not the non-targeting control group. Rate of development to blastocyst was similar in the two controls (37.7% and 29.2%) but

significantly lower in the DNMT1 injected group (5.9%). Figure 14 shows a diagram of parthenote blastocyst production for each treatment group.

Since there were very few embryos in the DNMT1 targeting siRNA injected group that developed beyond 8 cells we were only able to collect data on the methylation status of embryos with less than 8 cells or with 8 or more cells. Data analyzing the methylation status of individual blastameres across treatment groups can be seen in Figure 15. The data is broken down between embryos that have less than eight cells and those with eight or more cells. In the group with less than eight cells, blastomere methylation from the non-injected control was the same as the DNMT1

A

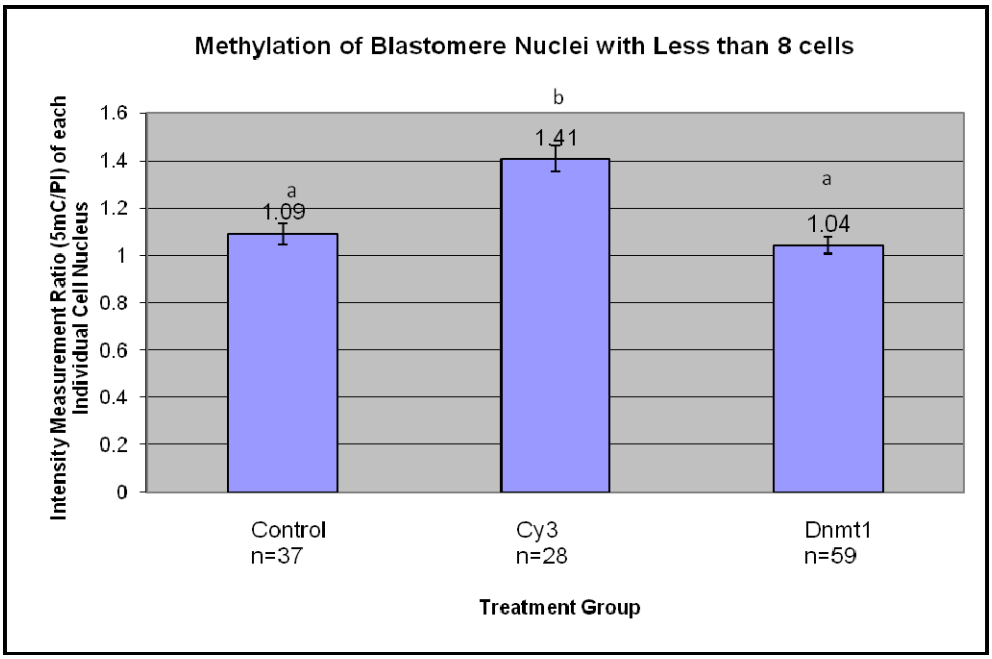


Figure 15. Methylation of parthenote DNA. Fluorescent intensity measurement of blastomere nuclei from embryos with less than 8-cells (A). Fluorescent intensity measurement of blastomere nuclei from embryos with less than 8-cells (B). Columns with different letters were significantly different ($p < 0.05$).

B

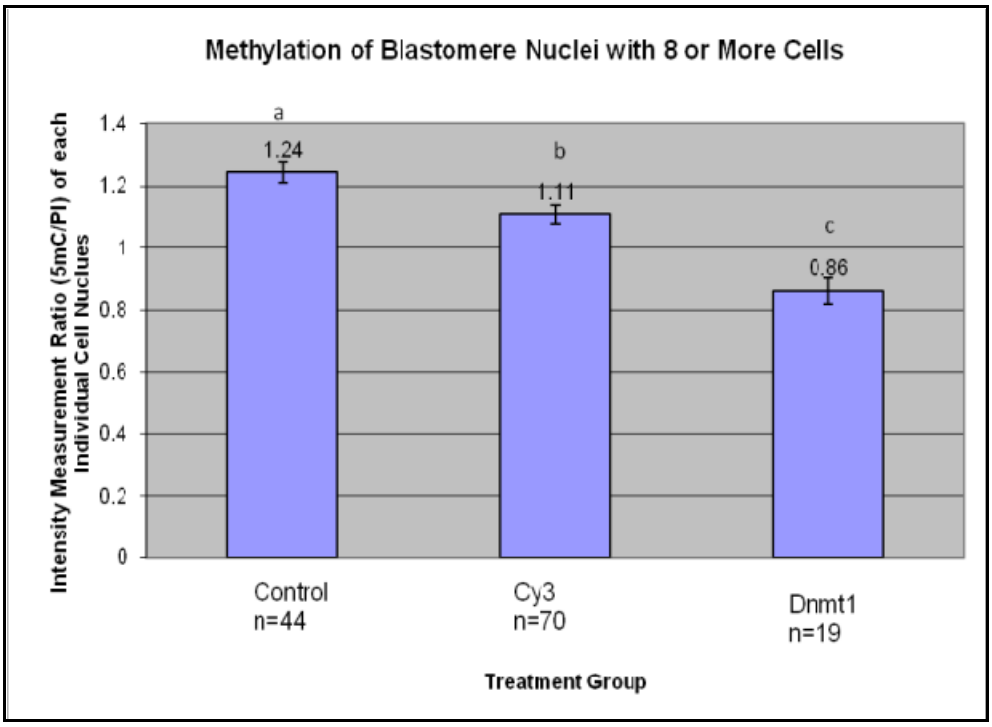


Figure 15 Continued

siRNA injected embryos. However, the Cy3 injected group had significantly higher methylated DNA. In the group with eight or more cells, the least methylated group was the DNMT1 siRNA injected embryos and the most methylated was the non-injected controls with the Cy3 injected group being in the middle. All three groups were significantly different as determined by t-test with alpha set at 0.05. Representative images from each group can be seen in Figure 16.

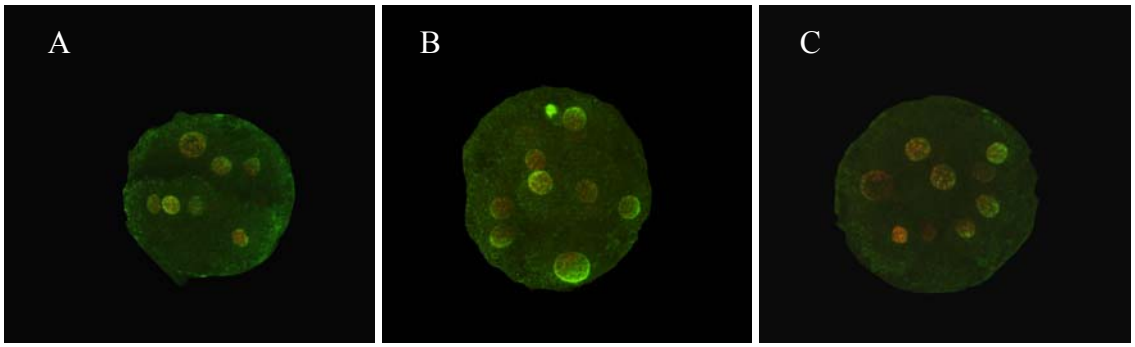


Figure 16. Images of embryos positioned for optimal visualization of nuclei. Red represents PI and green represents methylated cytosine residues. Non-injected control 8-cell embryo (a), Cy3 injected control 9-cell embryo (b), DNMT1 injected 9-cell embryo (c).

Conclusions

The data in Figure 10 shows a decrease in the number of cleaved embryos coming from the two injected group compared to the non-injected group. This was expected because of trauma from the injection process. There were fewer embryos injected with siRNA targeting DNMT1 that cleaved than in the control injected group. We did not expect to observe a decrease at this point, but since we did, we have to consider the possibilities. The hairpin could have had an effect at this point, although unlikely. Alternatively, there could have been variation in the micromanipulator's ability to consistently inject.

After cleavage, the number of embryos that continued to develop to the 8-cell stage was less variable. The embryos injected with siRNA targeting DNMT1 did, however, have slightly lower development to the 8-cell stage than the non-injected controls. Despite this small decrease, there is little evidence of a high degree of difference in control and treated embryos to this point. The developmental effects of the DNMT1 targeting siRNA become apparent at or soon after the 8-cell stage. Only 5.9%

of the embryos in the DNMT1 targeting shRNA injected group survived past the 8-cell stage where 29.2% of the control injected embryos developed into blastocysts. This is evidence that the bovine embryo requires DNMT1 for development past this point. Due to variation in the total amount of hairpin that was injected into the oocytes it is possible that the few that survived did not get enough siRNA to elicit a deleterious knockdown.

The embryos that were used for ICC showed a decrease in methylation in the DNMT1 injected group. With the evidence shown in the fibroblast experiments, we can speculate that the decrease in methylation is a result of DNMT1 silencing, however, without RT PCR or western blots to verify a decrease in the mRNA or protein this is merely speculation. Additional experiments will have to be done to collect more embryos for mRNA and protein isolation.

Discussion

The experiments described in this chapter with early bovine embryos are difficult to compare with other research due to the relatively small amount of work with bovine embryos in this field. Previous work done in sheep IVF embryos injected with DNMT1 siRNA showed a striking similarity (Taylor, Moore et al. 2008). They too had very little development past the 8 cell stage indicating the same limitation for ovine embryos to develop further in the absence of DNMT1. Taylor reported a high knockdown at the mRNA level. It is very likely that we had a similar knockdown in the bovine parthenotes. If the sheep study and our bovine study had a high enough knockdown to simulate a knockout, the results are not surprising since in the mouse a knockout is lethal (Li, Bestor et al. 1992).

We have demonstrated that exposure to siRNAs targeting DNMT1 influences both DNA methylation and embryonic developmental capabilities in the early bovine embryo. Our data suggests that by the 8 cell stage DNMT1 must be present in order for differentiation and development to continue. To our knowledge no data like this has been reported in the bovine.

Future studies will involve determining if there is a threshold amount of siRNA that will induce the lethal phenotype. It will be interesting to find out if it is possible to knockdown DNMT1 just enough to slightly reduce methylation, but without the lethal side effect. The ultimate goal would then be to utilize this technology with SCNT to aid the oocyte in reprogramming the genome of the donor cell nucleus. The experiment would involve injecting the same siRNA into the oocyte around the same time as reconstruction so the somatic cell is exposed to the siRNA. This method would be valuable because the siRNA would elicit its effect transiently and then it would be degraded allowing the embryo to continue with normal development.

CHAPTER V

SUMMARY

The goals of this experiment were three fold. The first goal was to construct a vector that could appropriately transfect and transduce bovine fetal fibroblasts and express short hairpin RNA targeting bovine DNMT1. The second goal was to collect DNMT1 gene expression and DNA methylation data from bovine fetal fibroblast cells expressing shRNAs targeting DNMT1. The final goal of this experiment was to evaluate changes in DNA methylation of *in vitro* produced embryos after being injected with siRNA targeting DNMT1. Our general hypothesis was that the interfering RNAs targeting DNMT1 would cause a decrease in DNMT1 gene and protein expression, as well as a decrease in global DNA methylation.

The findings from our study on bovine fetal fibroblasts expressing DNMT1 targeting shRNA indicated knockdown of DNMT1 at both the mRNA and protein level. The cells also exhibited a decrease in overall DNA methylation with the exception of one cell line that became hypermethylated. This cell line had the lowest expression of DNMT1 mRNA and protein of all the treatment groups and did not grow and proliferate normally while others did. These data indicate that the fibroblast cells can tolerate a certain degree of DNMT1 knockdown before there is an adverse affect on the cells. Data indicated that the hairpins did not knock down DNMT1 mRNA in the female cells as it did in the male cells, but both male and female showed a decrease in DNMT1 protein and DNA methylation. More data is required to make logical conclusions about these results. In general, our hypothesis was correct with regards to the fetal fibroblast

study with the exception of data collected on DNMT3A and DNMT3B gene expression. We expected one or both of these genes to up-regulate to compensate for loss of DNMT1, but we actually observed a decrease in these two genes when DNMT1 was knocked down. This is evidence that the bovine methylation mechanism is much different than that of the mouse.

The study on embryo production yielded interesting results. The most interesting result was the lack of embryonic development past the 8-cell stage from embryos injected with siRNA targeting DNMT1. This cease in development is evidence that bovine DNMT1 may be essential for embryonic development past the 8-cell stage. It was also determined that the embryos injected with the siRNA targeting DNMT1 had decreased global DNA methylation. This supports data from the fibroblast experiment. Real time PCR and protein blot data need to be collected to finish and help explain the results of this experiment.

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