CHARACTERIZATION AND ANALYSIS OF THE BOVINE EPIGENOME DURING PREIMPLANTATION EMBRYO DEVELOPMENT IN VITRO

A Thesis

by

GAYLE LINGER WILLIAMSON

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

August 2011

Major Subject: Genetics
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Approved by:

Chair of Committee, Charles R. Long
Committee Members, Mark E. Westhusin, Bhanu P. Chowdhary
Intercollegiate Faculty Chair, Craig Coates

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ABSTRACT

Characterization and Analysis of the Bovine Epigenome during Preimplantation Embryo Development In Vitro. (August 2011)

Gayle Linger Williamson, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Charles Long

During early mammalian embryogenesis, the embryonic genome undergoes critical reprogramming events that include changes in both DNA methylation and histone modifications necessary to control chromatin structure and thus, gene expression. Improper reprogramming of the epigenome during this window of development can lead to a vast number of imprinting anomalies, which are increased in children and livestock conceived in vitro. In the bovine, which closely resembles human preimplantation development, epigenetic changes occur from fertilization through the blastocyst stages. In particular, and concurrent with embryonic genome activation (EGA), de novo DNA methylation begins at the 8-cell stage. In order to explore the roles of histone-modifying enzymes during this crucial period of development, we characterized the transcript expression of several enzymes key enzymes across in vitro bovine preimplantation development using quantitative real-time PCR. Two of the 7 genes analyzed (Suz12 and Lsh) exhibited notable increases at the 8-16 cell stages, with
basal levels observed both before and after this. These increases coincided with both EGA and de novo DNA methylation. We further explored their roles in bovine preimplantation embryos by knocking down expression via the use of gene-specific targeting siRNAs. Independent suppression of either Suz12 or Lsh via cytoplasmic microinjection of targeting siRNAs resulted in lower development rates ($p < 0.0001$), and poorer embryo quality of the morulas and blastocysts that survived. In addition, Suz12 suppression led to reductions in both H3K27 ($p < 0.0001$) and H3K9 ($p = 0.07$) trimethylation, and an increase in DNA methylation levels ($p < 0.0001$), as compared to the null-injected controls. Lsh suppression did not change H3K27, but led to a reduction in H3K9 trimethylation ($p = 0.006$) and an increase in DNA methylation ($p < 0.0001$). Clearly our data demonstrate that these epigenetic modifiers play a critical role in formation of the embryonic epigenome, but further research would be necessary in order to fully characterize gene activities during this developmental window.
DEDICATION

I wish to dedicate this thesis to my family, without whose love and support I may not have come this far.

To my parents:
Thank you for always encouraging me to pursue my interests, even when you may not have understood them, and for many times providing the means to get there.

To Grandmother Weydell:
Thank you for your many life lessons and stories, and for your value and provision of my education.

And to my husband, Geoffrey:
Thank you for your continued love and patience, especially through these trying graduate years.

Je t’aime toujours...
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Most importantly, I wish to thank the members of my committee: Dr. Charles Long for the opportunity to pursue this research and the guidance in doing so, and Drs. Mark Westhusin and Bhanu Chowdary for your advice and encouragement along the way.

To my mentors, Dr. Rudolfo Aramayo and Dr. Paul Harms: thank you for instilling in me the passion to pursue the science you introduced me to.

I would also like to extend my thanks to the various people who contributed to this work: Dr. Edward Rucker for teaching me qPCR, Dr. Kevin Wells for teaching me improved microinjection techniques, Dr. Kimberly Tessanne and Jane Pryor for their assistance with IVF and embryo microinjection, Alison Wilkerson for running the TaqMan qPCRs, and Raven Landry for handling all of the ICC.

Furthermore, I would like to thank all of the current and past members of the Reproductive Sciences Lab (Dr. Duane Kraemer, Dr. Carol Hanna, Dr. Mike Golding, Dr. Paulette Suchodolski, Kim Green, Mike Peoples, Lisbeth Ramirez-Carvajal, Gisele Montano and Greg Burns) for their guidance and support throughout this project.

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<tr>
<td>5meC</td>
<td>5-methylcytosine</td>
</tr>
<tr>
<td>ac</td>
<td>Acetylated</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>ARTs</td>
<td>Assisted reproductive technologies</td>
</tr>
<tr>
<td>AS</td>
<td>Angelman syndrome</td>
</tr>
<tr>
<td>BWS</td>
<td>Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-Guanine (dinucleotides)</td>
</tr>
<tr>
<td>DMR</td>
<td>Differentially methylated region</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>dpc</td>
<td>Days post-coitum</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
</tr>
<tr>
<td>ET</td>
<td>Embryo transfer</td>
</tr>
<tr>
<td>H3K4</td>
<td>Histone 3 lysine residue 4</td>
</tr>
<tr>
<td>H3K9</td>
<td>Histone 3 lysine residue 9</td>
</tr>
<tr>
<td>H3K27</td>
<td>Histone 3 lysine residue 27</td>
</tr>
<tr>
<td>HAC</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone de-acetylase</td>
</tr>
<tr>
<td>HMG</td>
<td>Histone modifying gene</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatic protein 1</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post-fertilization</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>ICR</td>
<td>Imprinting control region</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>MBPs</td>
<td>Methyl CpG-binding proteins</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney (cells)</td>
</tr>
<tr>
<td>me2</td>
<td>Dimethylated</td>
</tr>
<tr>
<td>me3</td>
<td>Trimethylated</td>
</tr>
<tr>
<td>PGCs</td>
<td>Primordial germ cells</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>siRNAs</td>
<td>short interfering RNAs</td>
</tr>
<tr>
<td>TEs</td>
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<td>Xi</td>
<td>Inactivated X-chromosome</td>
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CHAPTER I

INTRODUCTION

ABNORMALITIES ASSOCIATED WITH ASSISTED REPRODUCTIVE TECHNOLOGIES

The use of assisted reproductive technologies (ARTs) dates to at least the late 1800s when embryo transfer (ET) was performed for the first time in rabbits (Heape 1891). In the time since, ART use has expanded to include additional technologies such as multiple ovulation embryo transfer (MOET), artificial insemination (AI), in vitro maturation and fertilization (IVM and IVF, respectively), intracytoplasmic injection (ICSI), embryo splitting, somatic cell nuclear transfer (cloning), gender preselected semen and others (Willett et al. 1951, Elsdon et al. 1976, Iritani & Niwa 1977, Newcomb et al. 1978, Willadsen 1986, Prather et al. 1987, Robl et al. 1987, Smith 1988a, Smith 1988b, Bondioli et al. 1990, Gray et al. 1991, Barnes et al. 1993, Johnson et al. 1994, Campbell et al. 1996, Wilmut et al. 1997, Betteridge 2003). In livestock, these technologies evolved as ways to maximize the use of and/or preserve superior genetics. Approximately 15% of all bovine embryos produced around the world are now produced in vitro (Thibier 2003, Mapleton & Hasler 2005). The widespread research and use of superovulation, AI, ET and IVF in cattle and other animals led to application

This thesis follows the style of Reproduction.

The use of ARTs involves *in vitro* manipulation of oocytes and embryos at a time when essential epigenetic changes are occurring, any deviation from which can inhibit developmental competence. In livestock, it has been observed that *in vitro* produced embryos result in increased rates of prenatal morbidity and mortality, as well as fetal overgrowth that can lead to dystocia during parturition, when compared to embryos conceived via AI or ET (Young *et al.* 1998, Lonergan *et al.* 2003, Summers & Biggers 2003, Lawrence & Moley 2008, Sinclair 2008). There are an increasing number of reports (reviewed in Owen and Seagars 2009) which suggest an increased risk of imprinting abnormalities associated with the use of ARTs in humans. Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) are among the most common examples of imprinting abnormalities that have been linked with ARTs. The
incidence of BWS for children conceived via ARTs has been reported to be as high as nine times that of naturally conceived children (Halliday et al. 2004). With an estimated incidence of 1 in 13,700 live births in the general population, clinical manifestations of the syndrome are highly variable but include fetal overgrowth and hypoglycemia, macroglossia, hemihyperplasia, facial nevi and an increased risk for embryonal tumors, yet normal intellect (Amor & Halliday 2008). BWS can result from either genetic or epigenetic alterations of two imprinting control regions (ICRs) on chromosome 11p15, although approximately 70% of cases result from epimutations within these regions (Lawrence & Moley 2008). One of these ICRs, DMR1 (differentially methylated region 1), regulates methylation of two genes: IGF2 (normally maternally methylated) and H19 (normally paternally methylated). The other ICR, DMR2 (differentially methylated region 2), regulates expression of three genes: KCNQ1OT1 (LIT1), KCNQ1, and CDKN1C. Under normal conditions, maternal methylation of the DMR2 ICR silences the maternal LIT1 allele, thereby allowing only paternal expression of LIT1. In particular, loss of maternal methylation of the DMR2 imprinting center is the most common cause among reported cases of BWS in ART-conceived children, and similarly, is responsible for 50-60% of sporadic cases of BWS (Huntriss & Picton 2008, Lawrence & Moley 2008, Sinclair 2008, Owen & Segars 2009).

Risk for AS has also been reported to be increased in association with ARTs, especially when mothers underwent ovarian stimulation (Huntriss & Picton 2008). With an estimated prevalence of about 1 in 15,000 in the general population, AS is
characterized by microcephaly and severe mental retardation, frequent laughter with congenial affect, ataxia, seizures and speech impairment (Amor & Halliday 2008, Lawrence & Moley 2008). It results from loss of maternal expression of the UBE3A gene at chromosome 15q11-13 and can be caused by several mechanisms (one being imprinting defects that silence the maternal allele). Other imprinting defects have also been reported, due to changes in methylation status at other DMRs (Huntriss & Picton 2008, Lawrence & Moley 2008, Sinclair 2008, Owen & Segars 2009).

In particular, a recent study demonstrated a clear link between superovulation (compared to spontaneous ovulation) and a reduction in methylation of several imprinted loci (Snrpn, Peg3, H19 and Kcncot1) in mouse blastocysts (Market-Velker et al. 2010b). The same group also assessed the effect of culture media on embryo development, comparing the methylation status of these same imprinted loci in mouse blastocysts that had been cultured in several different medias, to that of blastocysts produced in vivo. Not surprisingly, none of the medias tested were able to maintain methylation levels of the imprinted loci well enough to successfully duplicate an in vivo environment, in terms of methylation status of imprinted genes (Market-Velker et al. 2010a). The evidence clearly suggests the need for a better understanding of epigenetic events occurring during early development, so that we may improve current in vitro systems to meet those requirements.
**EPGENETICS**

Epigenetic modifications are heritable traits of gene expression that result without any alteration of the DNA sequence, involving changes in DNA methylation, histone modifications and RNA interference. These normal alterations can “help lead to the selective utilization of genome information through the activation or inactivation of functional gene transcription during gametogenesis, embryogenesis and cell differentiation” (Teitell & Richardson 2003, Turek-Plewa & Jagodzinski 2005). Epigenetic modifications can be affected by diet, age, development, or exposure to pharmaceutical drugs or other chemicals in the environment (2005). In general, exposure to an unsuitable or inadequate environment can result in abnormal epigenetic changes occurring within cells, ranging from mild (temporary alteration) to severe (inherited to offspring) effects. Downstream epigenetic contributions can directly affect the stability of chromatin structure, control of tissue-specific gene expression, replication timing, genomic imprinting, embryonic development and female X-chromosome inactivation (Geiman & Robertson 2002, Chow & Brown 2003, Ehrlich 2003, Robertson et al. 2004, Rousseaux et al. 2004, Ting et al. 2004, Turek-Plewa & Jagodzinski 2005).

**DNA Methylation**

DNA methylation has been widely characterized in many organisms, including mammals, as a way to regulate gene expression (Drahovsky & Morris 1972, Gama-Sosa
et al. 1983, Kautiainen & Jones 1985, Gardiner-Garden & Frommer 1987, Kaslow & Migeon 1987, Monk et al. 1987, Kafri et al. 1992, Li et al. 1992, Li et al. 1993, Sasaki et al. 1993, Okano et al. 1999, Jones & Takai 2001, Reik & Dean 2001, Thompson et al. 2001, Geiman & Robertson 2002). It has also been linked to the control of many other cellular functions, including genomic imprinting, X-chromosome inactivation and chromatin structure (Woodcock et al. 1986, Kaslow & Migeon 1987, Caiafa et al. 1991a, Caiafa et al. 1991b, Kafri et al. 1992, Li et al. 1993, Sasaki et al. 1993, Feil & Khosla 1999, Rein et al. 1999). In multicellular eukaryotes, DNA methylation occurs by the addition of a single methyl group to the 5-carbon position of a cytosine base, forming 5-methylcytosine (5meC), predominantly within the context of C-G (CpG) dinucleotides (Bestor & Tycko 1996). In mammalian genomic DNA, it has been estimated that approximately 70-80% of cytosine residues occurring within CpG dinucleotides are methylated (Ehrlich et al. 1982, Bird 2002). Within GC-rich (60-70% GC content) DNA regions, 0.2-5 kb DNA fragments with clusters of CpG dinucleotides are termed “CpG islands,” and are typically located near the promoter or within the first exon of numerous genes (Takai & Jones 2003, Law & Jacobsen 2010). The formation of 5meC occurs non-randomly in the genome and can create regions of hyper- and hypomethylation, further leading to the formation of heterochromatin (DNA is less accessible to transcriptional machinery), or euchromatin (DNA is more accessible). Certain regions, such as repetitive and transposable elements, are hypermethylated and transcriptionally inactive; other regions, such as CpG islands in promoters of

DNA methylation, which is associated with a repressed chromatin state, can inhibit gene expression either directly or indirectly. Direct inhibition involves the modification of cytosine bases, which then inhibits the binding of transcriptional activators to their cognate DNA sequences (Watt & Molloy 1988). With indirect inhibition, proteins that recognize the 5meC mark (methyl CpG-binding proteins, or MBPs) can initiate the modification of surrounding chromatin by recruiting co-repressors (Nan et al. 1998, Yoon et al. 2003).

DNA methyltransferases (DNMTs) are the enzymes responsible for catalyzing the conversion of cytosine to 5meC and can be generally classed as either de novo or maintenance methyltransferases (see Figure 1.1 and Table 1.1). Both contain a C-terminal catalytic region as well as an N-terminal regulatory region (Turek-Plewa & Jagodzinski 2005). The primary de novo methyltransferases include DNMT3A and DNMT3B, and are responsible for the introduction of cytosine methylation at previously unmethylated CpG sites, thereby establishing new methylation patterns on DNA. Maintenance methyltransferases, namely DNMT1, preferentially attach methyl groups to hemimethylated DNA during replication, ensuring that the DNA methylation pattern
is faithfully copied to the newly synthesized DNA strand following replication. While their catalytic regions are all very similar, their regulatory regions diverge significantly, consistent with their different functions. The eukaryotic DNMT family also includes $DNMT1o$, $DNMT1p$, $DNMT1s$, $DNMT2$, and $DNMT3$-like ($DNMT3L$) (Geiman & Robertson 2002, Hermann et al. 2004a, Kelly & Trasler 2004, Turek-Plewa & Jagodzinski 2005, Klose & Bird 2006, Law & Jacobsen 2010).

![Figure 1.1 DNA methylation and demethylation in mammals. Dnmt3a and Dnmt3b introduce methyl groups to unmethylated DNA de novo. During DNA replication, Dnmt1 recognizes methyl groups on hemimethylated DNA and copies the mark to the daughter strand. In the absence of Dnmt1, passive demethylation occurs and is replication cycle-dependent. Although DNA demethylases have not been well characterized, they represent a mechanism of active removal of methyl groups not coupled with DNA replication (Reik & Walter 2001).]

Forming the core of the replication fork, $DNMT1$ methylates newly bio-synthesized DNA strands directly after the replication round, using the parent strand as the template from which to copy the proper methylation pattern. It displays 5- to 40-fold
higher activity in vitro for hemimethylated DNA than for unmethylated DNA and also exhibits very weak de novo methylation activity, presumably stimulated by DNMT3A (Bestor 2000, Fatemi et al. 2002, Hermann et al. 2004b). It has further been shown to be involved in the mismatch repair system as well (Wang & James Shen 2004). The DNMT1 gene is quite unique, in that it possesses sex-specific promoters (Mertineit et al. 1998) and a multi-potential first exon. Unique sequences of the first exon are formed as a result of alternative splicing, and lead to the tissue-specific isoforms DNMT1o, DNMT1p and DNMT1s (Ko et al. 2005). Dnmt1o has been shown in mice to be present in growing oocytes and stored in the cytoplasm of mature MII oocytes and preimplantation cleavage stage embryos. It briefly translocates to the nucleus at the 8-cell stage, then returns to the cytoplasm at the 16-cell stage (Ratnam et al. 2002, Huntriss et al. 2004). However, this isoform has not been demonstrated to show any activity in other species. DNMT1p is present in pachytene spermatocytes, but its sequence interferes with translation machinery and prevents the synthesis of DNMT1

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<th>Localization</th>
<th>Interacting, chromatin-associated proteins</th>
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<td>Deacetylate chromatin, transcriptional repression, facilitate DNA methylation?</td>
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| | | | DNMT3L | Establishment of maternal methylation imprints |

Table 1.1 Summary of the properties of the three catalytically active mammalian DNMTs (Geiman & Robertson 2002).
protein. Thus, although the transcript is abundantly present in spermatocytes during spermiogenesis, there is no functional protein activity (Trasler et al. 1992). DNMT1s is present in somatic cells (Carlson et al. 1992, Mertineit et al. 1998, Ratnam et al. 2002, Chung et al. 2003, Turek-Plewa & Jagodzinski 2005).

Both de novo methyltransferases (Okano et al. 1998), DNMT3A and 3B share a high degree of primary structure homology, but are actually encoded by different genes on different chromosomes. Most active during early embryogenesis, transcript levels taper off upon the differentiation of ES cells and remain low in adult tissues (Xie et al. 1999). DNMT3A demonstrates a preference for sites flanked by promoters, and while it is highly specific for CpG methylation, it can also methylate cytosine at CpA and CpT dinucleotides. DNMT3B is specialized for the methylation of CpG dinucleotides within repeat sequences of chromosome pericentric satellite regions (Hermann et al. 2004a, Turek-Plewa & Jagodzinski 2005). DNMT3L, a non-catalytic stimulatory factor, is known to interact with both DNMT3A and unmethylated histone 3 lysine 4 (H3K4) tails. A model was proposed to explain these interactions in primordial germ cells (PGCs) following the wave of demethylation of imprinted genes and transposable elements (TEs), in which DNMT3L binds unmethylated H3K4 tails and recruits the DNMT3A isoform DNMT3A2 to imprinted loci in order to re-establish parent of origin specific DNA methylation imprints (Jia et al. 2007, Ooi et al. 2007, Otani et al. 2009, Law & Jacobsen 2010).
Histone Modifications

Histones are protein macromolecules that assist in the organization and compaction of DNA. These octomeric proteins are made up of 2 molecules each of H2A, H2B, H3 and H4 and form the nucleosome, around which approximately 147 bp of DNA are wound (Luger et al. 1997). However, histones do not just serve as “spools” to neatly organize the DNA; chemical modification of histone tails can result in changing the degree of DNA compaction (heterochromatin or euchromatin), which then allows or prevents transcriptional machinery from binding. “Histone modifications of nucleosomes distinguish euchromatic from heterochromatic chromatin states, distinguish gene regulation in eukaryotes from that of prokaryotes, and appear to allow eukaryotes to focus recombination events on regions of highest gene concentrations” (Holmquist & Ashley 2006). Thus, in addition to DNA methylation, histone tail modifications serve as an additional level of epigenetic control over gene expression. Modifications of various amino acid residues within histone tails can be in the form of acetylation, methylation, phosphorylation or ubiquitination. In general, acetylation of lysine residues results in a more relaxed DNA compaction state, increased accessibility of transcriptional proteins to the DNA and thus an increase in gene expression. Methylation of lysine residues can lead to tighter compaction of the DNA, inaccessibility of transcriptional activators and gene silencing, or a more relaxed state, depending on the residue that has been methylated (Nielsen et al. 2001a, Nielsen et al. 2001b, Peters et al. 2002, Vakoc et al. 2006, Izzo & Schneider 2010). Furthermore, lysines can be mono-, di- or tri-
methylated, which also can lead to different downstream effects, even when occurring on the same residue (Birney et al. 2007, Koch et al. 2007, Izzo & Schneider 2010).

Histone modifications have been widely explored during mammalian preimplantation development (Schultz & Worrad 1995, Dennis et al. 2001, Erhardt et al. 2003, Yan et al. 2003a, Yan et al. 2003b, Sarmento et al. 2004, de la Cruz et al. 2005, Santos et al. 2005, de la Cruz et al. 2007, Pasini et al. 2007, Puschendorf et al. 2008, Ross et al. 2008), and several of these studies have demonstrated relationships between altered histone modifications, DNA methylation and downstream heterochromatic spreading (Dennis et al. 2001, Erhardt et al. 2003, Yan et al. 2003b, de la Cruz et al. 2007, Pasini et al. 2007, Puschendorf et al. 2008). These alterations could potentially lead to profound changes in phenotype, including cancer, imprinting diseases and other developmental abnormalities (Cuthill 1994, Geiman et al. 2001, Khosla et al. 2001a, Khosla et al. 2001b, Tycko & Morison 2002, Ehrlich 2003, De La Fuente et al. 2006, Xi et al. 2007). It would be no surprise to discover a strong link between changing histone modifications and embryonic reprogramming, any deviation from which could lead to the increase in imprinting-type anomalies seen in mammals following in vitro exposure during the oocyte and/or embryo stage. Several candidate genes involved in methylation of H3K4, H3K9, H3K27 and H4K20 would be interesting for further investigation during this window of preimplantation development. These modifications have been characterized in a wide variety of applications relating to general gene activation/repression in facultative and constitutive heterochromatic
regions (Peters et al. 2002, Plath et al. 2003, Schotta et al. 2004, Zhang et al. 2004, Bernstein et al. 2005, Morillon et al. 2005, Botuyan et al. 2006, Vakoc et al. 2006, Cao et al. 2008, Huen et al. 2008, Pesavento et al. 2008, Yang et al. 2008); however, it is less well-known exactly how they contribute specifically to embryonic genome activation during the reprogramming period. In mouse 2-cell embryos, differential histone modifications exist between the maternal and paternal genomes. In particular, H3K27me3 marks are enriched on maternal, but not paternal chromosomes (Santenard et al. 2010). Additionally, H4K20me3 marks begin disappearing from the maternal chromatin, and do not reappear until the blastocyst stage (Kourmouli et al. 2004). In mouse embryos between the 4-cell and morula stages, approximately equal levels of H3K9, H3K27 and H4K20 are evident between blastomeres (Erhardt et al. 2003). Once blastomeres are committed to the formation either the trophectoderm (TE) or inner cell mass (ICM), differential histone modification marks are seen once again (Tachibana et al. 2002, Erhardt et al. 2003, Dodge et al. 2004). However, one must keep in mind that differences in the timing of the reprogramming events exist between species (described later), so what is known about histone modifications during mouse development may not be applicable to humans or the bovine model. Further exploration in this area would be most helpful in determining whether or not histone modifications regulate, or at least contribute to, reprogramming in early bovine and human embryos.
RNA Interference

Prior to the discovery of the RNA interference (RNAi) mechanism, researchers stumbled upon the puzzling phenomenon that when exogenous transgenes were introduced into petunias in order to enhance color intensity (by increasing the activity for an enzyme involved in the production of specific pigmentation in petunia flowers), it instead resulted in either the partial or complete loss of color. This meant that the introduced transgenes were able to affect the expression of endogenous genes, by decreasing the activity of the pigmentation enzyme, and resulting in partially or completely white flowers (Napoli et al. 1990). This phenomenon was termed “co-suppression” and later renamed post-transcriptional gene silencing (PTGS) (Van Blockland et al. 1994).

Although it had been observed previously, Andrew Fire and Craig Mellow, were credited with the actual discovery of the RNAi mechanism, when they concluded that specifically double-stranded RNA (dsRNA) resulted in silencing of the targeted gene (Fire et al. 1998). Later, it was determined that the introduction of dsRNA into mammalian cells, in the form of synthesized siRNA molecules, also resulted in the desired gene-silencing response (Elbashir et al. 2001a, Elbashir et al. 2001b). This discovery established that the RNAi pathway also operates in mammalian cells.

RNAi can act in most eukaryotes via three distinct pathways: by degrading mRNA (PTGS), by repressing translation and by targeting specific loci for heterochromatinization (Holmquist & Ashley 2006). Primarily of interest here, however, is the use of siRNA technology as a mechanism to suppress expression of
specifically targeted genes without generating a complete knockout. Following introduction into a cell via transfection or microinjection, these small ~21-bp siRNAs are processed, become single stranded and mediate post-transcriptional silencing of their specific targets by mRNA degradation (Bartel 2004, Holmquist & Ashley 2006).

**THE ROLE OF THE EPIGENOME DURING EARLY DEVELOPMENT**

During oocyte growth and maturation, the oocyte must achieve both cytoplasmic and nuclear/meiotic maturation in order to be competent for fertilization and further development. At birth, the female’s (mouse, bovine and human) oocytes are poised at prophase I of meiosis within primordial follicles (Picton *et al.* 1998). Follicular waves within each reproductive cycle result in the recruitment of primordial follicles to grow and develop. Unlike the male’s gametes at their corresponding stage of spermatogenesis, oocytes at the beginning of follicular recruitment have not yet re-acquired all the necessary (maternal) imprints. These maternal imprints are re-established gradually during follicular growth from the primary to the antral stages (Huntriss & Picton 2008, Lawrence & Moley 2008). In addition to the acquisition of maternal imprints and oocyte growth (size) during follicular recruitment, the oocyte also begins storing maternal mRNA. Very little transcription occurs during the first few cleavage divisions following fertilization, so the newly formed embryo must rely on the maternally stored mRNAs the oocyte accumulated prior to fertilization to sustain development until transcriptional activation of the embryonic genome is initiated.
Once the oocyte has acquired all the maternal mRNAs and other cytoplasmic organelles it will need, the oocyte is considered to be cytoplasmically mature. However, the oocyte must still achieve meiotic competence via nuclear maturation; the LH surge causes spontaneous resumption of meiosis, which continues until arrest at the metaphase II stage in preparation for fertilization (Rodriguez & Farin 2004).

Unlike the oocyte, which is designed to contribute all the cytoplasm with necessary components for the resulting zygote, the sperm’s main role is to serve as an efficient DNA-delivery vehicle. For this reason, a sperm’s DNA is packaged with protamines, allowing tighter compaction of the DNA than could be accomplished with histones. Following fertilization and prior to syngamy, however, sperm chromatin undergoes decondensation as the protamines are replaced with histones from the oocyte (Mann & Bartolomei 2002).

In most mammalian species, the parental genomes become differentially demethylated soon after fertilization. In general, the paternal genome is actively demethylated prior to the first cleavage division, while the maternal genome is passively demethylated (replication-dependent) over the first few cell divisions (see Figure 1.2). Though the mechanism is currently unknown, imprinted genes and some repeat sequences somehow manage to escape this erasure and reestablishment of DNA methylation (until later reprogramming during gametogenesis). Stage-dependent on species, the embryonic genome is then remethylated with the proper marks it will
need. In the cow, remethylation occurs prior to differentiation, beginning at the 8-cell stage. However, in the mouse, remethylation does not begin until after differentiation, at the blastocyst stage. Also during the first few cleavage divisions, the maternal mRNA stores are gradually used up or degraded (Memili & First 2000). In order to continue

![Diagram of DNA methylation across bovine preimplantation embryonic development. The blue line represents active demethylation of the paternal genome, while the red represents passive demethylation of the maternal genome. After erasure, these marks are reestablished de novo when the embryonic genome is activated (Mann & Bartolomei 2002).](image)

Figure 1.2 DNA methylation levels across bovine preimplantation embryonic development. The blue line represents active demethylation of the paternal genome, while the red represents passive demethylation of the maternal genome. After erasure, these marks are reestablished de novo when the embryonic genome is activated (Mann & Bartolomei 2002).

development, the embryo must begin transcribing its own mRNA, an event termed embryonic genome activation (EGA). The EGA is also stage-dependent on species; it occurs at the 2-cell stage in mice and around the 4-8 or 8-16 cell stage in humans and bovids, respectively (Braude et al. 1988, Kopecny 1989, Kopecny et al. 1989, Memili & First 2000, Wang et al. 2004, Zeng & Schultz 2005, Wong et al. 2010). In human and bovine embryos, de novo DNA methylation is concurrent with EGA and the first major

The combination of changing chromatin configuration, changes in DNA methylation status and embryonic genome activation all occurring during a very short time period may suggest an epigenetic link connecting these events to each other. Because this is such a critical time in development of the epigenome, as evidenced by the fact that imprinting abnormalities have been associated with the prevalent use of ART procedures in human and livestock reproduction, we are investigating the roles of several epigenetic modifiers during preimplantation development. We hypothesize that a disruption in activity of these key enzymes likely leads to alteration in important downstream developmental processes.
CHAPTER II

EXPRESSION PROFILING OF HISTONE MODIFYING ENZYMES IN IN VITRO PRODUCED BOVINE PREIMPLANTATION EMBRYOS

INTRODUCTION

Although much information has been gleaned about DNA methylation characteristics during the reprogramming period in early embryonic development, changing chromatin configurations have been less explored. In cattle and humans, we know that the embryonic genome begins transcribing its own RNA at approximately the same stage as DNA remethylation, but it is unclear exactly which histone modifications are occurring at this time.

Our central goal was to determine which epigenetic modifiers (DNMTs and/or HMGs) could be playing a key role in early epigenetic reprogramming in the bovine preimplantation embryo. We hypothesized that expression profiles would either follow the pattern of maternal mRNA degradation between fertilization and the 8-cell stage or that the gene(s) would be expressed higher around the 8- to 16-cell stage, in association with embryonic genome activation and/or de novo DNA methylation. To address this hypothesis, we collected RNA from pooled in vitro-derived bovine embryos at specific time points (representing different cleavage stages) between fertilization and the blastocyst stage, reverse-transcribed it into cDNA, and profiled expression of several genes (SetB1, G9a, Suv3-9h1, Suv4-20h1, Suz12, Lsh, SmyD3 and Lsd1) via
qPCR. These particular genes were selected based on their known implications with histone modifications and/or DNA methylation, as described below.

*SetB1*

Set domain bifurcated 1 (*SetB1*) is an H3K9-specific methyltransferase, containing a putative methyl-binding domain (MBD) that potentially links H3K4 methylation to DNA methylation, or may do so through the interaction with the MBD-containing protein *MBD1* (Hashimoto *et al.* 2010). It has been demonstrated that *SetB1* only interacts with H3 in the absence of H3K4me3, as it fails to methylate H3K9 in the presence of substrates containing the H3K4me3 mark (Binda *et al.* 2010). In *Drosophila melanogaster*, trimethylation of H3K9 by *SetB1* mediates recruitment of *Dnmt2* and the *Drosophila* ortholog of mammalian *HP1* to target genes. In doing so, *SetB1* triggers DNA methylation and silencing of genes and certain retrotransposons (Gou *et al.* 2010). Although this has not been demonstrated in mammals, it provides a potentially interesting link between *SetB1* HMT activity and DNA methylation.

*G9a*

Euchromatic histone-lysine methyltransferase 2 (*Ehmt2*, or more commonly *G9a*) is known to methylate H3K9, aiding in gene silencing of euchromatic regions. While it is specific for mono- and dimethylation (Rice *et al.* 2003), some have suggested that *G9a* is indeed capable of trimethylation of H3K9 as well (Patnaik *et al.* 2004), although Suv3-
9h1 and Suv3-9h2 are primarily the enzymes responsible for this modification (Rice et al. 2003). Mice deficient in G9a die prematurely between embryonic days 9.5 and 12.5, displaying severe growth retardation due to deregulation of developmental genes (Tachibana et al. 2002). Interestingly, similar growth retardation was also observed in Dnmt1-null mice (Li et al. 1992), indicating a possible link between the activities of G9a and Dnmt1. Esteve et al. have reported a direct interaction between G9a and Dnmt1 based on their data in 2006. They demonstrated that these two proteins colocalize in the cell and at replication foci during S phase, and that G9a stimulates Dnmt1 activity, while Dnmt1 exhibits a similar, modest activity on G9a. They suggested that the Dnmt1-G9a binary complex is catalytically competent and can perform both histone and DNA methylation, so that H3K9 residues undergo histone methylation, concurrent with maintenance DNA methylation. In addition, the authors suggested that the presence of G9a in a Dnmt1 complex can activate Dnmt1 catalysis for additional de novo methylation (methylation spreading) as seen in silenced genes in cancer cells (Esteve et al. 2006). Clearly, G9a represents another HMG with close ties to DNA methylation, once again strengthening the argument for a link between histone modifications and the DNA methylation changes occurring during early development.

Suv3-9h1

In eukaryotes, suppressor of variegation 3-9 homolog 1 (Suv3-9h1) is required for formation of pericentric heterochromatin by catalyzing H3K9 trimethylation. Suv39h1-
mediated trimethylation of H3K9 leads to binding of HP1 and other HP proteins, which target the two Suv4-20h HMTs as well as Dnmts 3a & 3b in order to establish a transcriptionally repressed state (Puschendorf et al. 2008).

*Suv4-20h1*

Suppressor of variegation 4-20 homolog 1 (*Suv4-20h1*), which contains a SET domain, prefers catalyzation of H4K20 dimethylation but can also accomplish trimethylation of the same enzyme (Yang et al. 2008). Not much about the function of *Suv4-20h1* has been determined, but deficiency results in telomere elongation and derepression of telomere recombination (Benetti et al. 2007). Some have suggested that *Suv4-20h1*, in addition to *Suv3-9h*, is also active in establishing pericentric heterochromatin (Schotta et al. 2004).

*Suz12*

Suppressor of zeste 12 (*Suz12*) is a member of *PRC2* and *PRC3*, known to catalyze both di- and trimethylation (me2 and me3, respectively) of H3K27 during mouse embryogenesis. *Suz12* enhances the activity of *PRC2/3* by mediating the recruitment of the necessary histone-binding subunit RbAp48 to both of these complexes. In *Suz12* /-/- mice, data suggest the effects of *Suz12* loss arise around 8.5 days post-coitum (dpc), with near-complete fetal reabsorption by 10.5 dpc (Pasini et al. 2004). In *Drosophila*, *Su(z)12* has been implicated in the formation of both facultative and constitutive
heterochromatin (de la Cruz et al. 2007), and in mouse embryonic (ES) cells involves enrichment of Suz12 on the Xi during X-inactivation. In human cells, knockdown of SUZ12 via siRNA transfection resulted in global loss of H3K27me3 (de la Cruz et al. 2005). In addition to its involvement with H3K27 methylation, Suz12 has also been shown to be required for stable H3K9 trimethylation. In a separate experiment, knockdown of SUZ12 in human cells not only reduced the levels of H3K27me3, but also decreased levels of H3K9me3, thereby leading to the dissociation of HP1α from heterochromatin. The enrichment of H3K9me3 and HP1α on pericentric heterochromatin is required for centromere function & normal chromosome segregation (de la Cruz et al. 2007). Clearly, Suz12 plays an important role in histone methylation and heterochromatin formation during early development, and further study in the bovine model would shed helpful light on major epigenetic remodeling occurring during this time period.

Lsh

Lymphoid-specific helicase (Lsh) is known to be non-essential for murine embryonic development, but Lsh deficient mice die shortly after birth (Dennis et al. 2001) with dramatically reduced levels of DNA methylation, especially in normally-occurring heterochromatic regions, where transposable elements and other specific genes reside (Huang et al. 2004, Lippman et al. 2004, Fan et al. 2005). In mouse embryonic fibroblasts, Yan et al. reported that Lsh, in addition to Dnmt1 and Dnmt3B, is required
for DNA methylation of pericentric major satellite repeats (Yan et al. 2003b). Lsh has also been shown to co-immunoprecipitate with de novo methyltransferases Dnmt3a and Dnmt3b (Zhu et al. 2006). Most recently, researchers found that while transcriptional silencing by the LSH protein complex does not immediately result in DNA methylation, LSH directly recruits DNMT1, DNMT3B, and histone de-acetylases (HDACs) to the site, and the LSH-mediated increase in the local concentration of DNMTs on chromatin may lead to DNA methylation that further stabilizes a silenced chromatin state. These authors also showed that DNMT3B directly binds to the N-terminus of LSH, and that the interaction of DNMT1 with LSH in vitro and in vivo requires the presence of DNMT3B; DNMT1 is then believed to recruit HDAC1 and HDAC2 to the LSH-bound DNMT3B (Myant & Stancheva 2008). Clearly, Lsh is necessary for proper DNA methylation, especially during early development. However, due to differences between the timing of de novo DNA methylation activity in mice versus the more similar pattern seen between ruminants and humans, it would be helpful to further characterize Lsh activity during bovine preimplantation development.

SmyD3

SmyD3 is a member of the SmyD family class of chromatin regulators important in heart and skeletal muscle development (Sirinupong et al. 2011). Frequently overexpressed in different types of cancer cells, it contains a SET domain and is responsible for the accumulation of di- and trimethylation of H3K4 at induced estrogen
receptor genes (Kim et al. 2009). It has been suggested that SmyD3 plays a crucial role in HeLa cell proliferation and migration/invasion, as knockdown of this gene in HeLa cells inhibited these cellular functions (Wang et al. 2008). It is unclear what role SmyD3 plays in preimplantation development.

RESULTS

Following multiple rounds of IVF, we collected groups of bovine embryos at specific time points, representative of the following embryonic stages: 2-cells (day 1 post-IVF), 4-7-cells (day 2), early 8-cells (day 3), late 8-cells (day 4), 16-cells (day 5), morulas (day 6) and blastocysts (day 7). Groups of unfertilized MII oocytes were also collected on day 0, in order to have samples consisting of only maternally-derived mRNA, for comparison. Each replicate was produced from a different IVF round, and the sample size varied from one stage to another in order to compensate for decreasing levels of mRNA around the 8-16 cell stage (see Table 2.1).

RNA was isolated from each group and reverse-transcribed into cDNA. The cDNA was used (via SYBR Green qPCR) to determine a gene expression profile of the HMGs described above, across bovine preimplantation development. To account for both differences in cell number and varying mRNA content at each stage, gene expression was normalized to an average of three endogenous controls. We obtained 3 unique expression profiles for these epigenetic modifiers, displayed in Figure 2.1. SetB1, Suv3-9h1, Suv4-20h1 and SmyD3 were all moderately expressed in MII oocytes, higher
around the 2-4 cell stage, before levels tapered off by the morula & blastocyst stages. 

*G9a* represented a similar profile, but with levels increasing slightly at the late 8-cell stage. Interestingly, *Suz12* and *Lsh* mRNA levels were low during the earlier cleavage stages, increased noticeably around the 8- or 8-16 cell stages (respectively), then decreased again to levels similar to the 2-4 cell stages. Because we were primarily interested in obtaining the approximate expression patterns of these genes during this developmental window to use for further study, a statistical analysis of these data was not performed.
DISCUSSION

These data suggest that SetB1, Suv3-9h1, Suv4-20h1, and SmyD3 represent genes most active during the cleavage divisions, prior to and including the early stages of the EGA event. These transcripts are most likely maternally-derived, as their expression profiles exhibit strong similarity to levels of maternally-stored transcripts (levels decrease as the transcripts are used up or degraded prior to EGA) (Memili & First 2000). Transcript
levels of these genes following the 8-cell stage are likely embryonically-derived, but could also be residual maternal mRNA. Interestingly, because levels are still moderately high around the 8-cell stage, they could be actively involved in the EGA event and/or DNA remethylation. However, further examination of their actions would be necessary in order to support this hypothesis. Suz12 and Lsh represent a different and most unique expression profile. These mRNA were low in the earlier stages (maternal transcripts) and noticeably increased around the 8-cell stage (embryonic transcripts). This notable increase in expression coincides with both EGA and de novo DNA remethylation, thereby supporting the notion of their possible involvement in these key events. Further study of the enzymatic activities of Suz12 and Lsh (examining their impact on histone and DNA methylation) would be most informative in determining the significance of these genes during the EGA and DNA remethylation events. G9a represents a third profile, similar to that of both maternally-stored mRNA (decrease up to the 8-cell stage) and embryonic transcripts (increase in levels at or following the 8-cell stage). However, the increase is fairly small and slightly later than that of Suz12 and Lsh; further examination of the G9a profile during these stages, and additional characterization of its enzymatic activity affecting histone and DNA methylation is needed before an argument could be made that G9a plays a key role in EGA.

In this study, we aimed to collect each sample from a separate IVF round, so that any variation from one round to the next would be visible between samples. However,
using this method sometimes limited the number of embryos we could include in a sample, at each stage. If development was not as high as we expected for a particular round, we would then not have enough embryos for a minimum sample size, and therefore n would vary between samples. Also, this meant that we could not collect every stage from every IVF round, because if done this way, the sample sizes would not be sufficiently large enough for the amount of RNA needed for reverse transcription and qPCR analysis. With what we learned from this experiment, we decided that a better way to minimize variation while keeping n the same from sample to sample (with a minimum sample size set for each stage collected, to provide enough RNA) would be to collect all stages from each round of IVF. Because not enough embryos would be collected from each round to fulfill the required sample size, embryos at each stage would be pooled until the minimum n had been reached for the first sample, then the same for the second and third until all samples had been collected. Therefore, regarding all embryo samples collected for qPCR analysis following this experiment, we switched to using this approach.

Overall, this dataset provides an interesting window into the changing transcript levels of selected HMGs during bovine embryo preimplantation development in vitro, and thereby suggests at which time point(s) these genes could be active. However, a further look into protein levels and/or their enzymatic activities is needed before we can reach any sort of conclusion on their involvement with either the EGA event or DNA remethylation.
CHAPTER III
siRNA-MEDIATED KNOCKDOWN OF SELECTED GENES

INTRODUCTION

To address our goal of uncovering which epigenetic modifications are associated with early developmental reprogramming and EGA, we suppressed expression of a subset of the previously characterized HMGs and evaluated effects on development rates, gene function (specific histone modifications and/or DNA methylation) and gene expression of other known epigenetic modifiers. We hypothesized that genes with increased transcription around the 8-cell stage were likely involved in EGA, and that suppression of these transcripts would lead to a reduction in or complete block from further development.

To address this hypothesis, we selected the two most obvious candidates fitting the description of increased transcript levels around the 8-cell stage: Suz12 and Lsh. In short, the design of the experiment was to suppress the expression of these proteins individually via cytoplasmic microinjection of mRNA-targeting siRNAs at the zygote stage. Our endpoints for evaluation included development rates, the degree of gene knockdown via qPCR, any change in expression of non-targeted epigenetic modifiers and examination of any resulting changes in DNA or histone (H3K9 and H3K27) methylation via immunocytochemistry (ICC).
RESULTS

*Testing siRNA Effectiveness in a Bovine Cell Line*

In order to test which siRNAs were most successful in reducing target mRNA levels, we transfected MDBK cells at both low (20 nM) and high (50 nM) siRNA concentrations. Of the 2 Suz12-targeting siRNAs that were tested, 1 was found to have considerable knockdown efficiency at both low and high siRNA concentrations (low concentration shown in Figure 3.1). Of the 4 Lsh-targeting siRNAs we tested, 2 displayed notable knockdown efficiencies at both low and high siRNA concentrations; however, a further increase in efficiency was observed when these 2 siRNAs were transfected together (low concentration shown in Figure 3.1). The higher (50 nM) concentration of siRNAs did not result in an increased efficiency over the lower (20 nM) concentration; therefore, the 50 nM transfection data is not included here. Successful siRNA target sequences are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Name</th>
<th>bp Start</th>
<th>siRNA Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suz12</td>
<td>SUZ-316</td>
<td>316</td>
<td>5'-AAGGATGTAAGTTGTCCGATA-3'</td>
</tr>
<tr>
<td>Lsh</td>
<td>LSH-1575</td>
<td>1575</td>
<td>5'-AGCAGATACTGTTATCATTTA-3'</td>
</tr>
<tr>
<td>Lsh</td>
<td>LSH-447</td>
<td>447</td>
<td>5'-TCGGATATTGGTAAAGCATAT-3'</td>
</tr>
</tbody>
</table>
Figure 3.1 Targeted gene expression in MDBK cells, using 20 nM (low) siRNA concentration. (A) Bars represent Suz12 expression following no treatment (CTL), transfection with a non-targeting siRNA control (NULL), or transfection with an Suz12-targeting siRNA (SUZ-316). (B) Bars represent Lsh expression following no treatment (CTL), transfection with a non-targeting siRNA control (NULL), or transfection with Lsh-targeting siRNAs (LSH-1575, -447 or –both). The Lsh siRNAs were tested singly at 20 nM and in combination (20 nM each), in order to determine if a synergistic effect increased target reduction efficiency. Statistical comparisons were not performed.
Preliminary Data: Experimental Design Evaluation

We conducted a preliminary study in order to evaluate our experimental design, using embryos from a non-injected (CTL) group, or those injected with either non-targeting (NULL) or Suz12-targeting (SUZ) siRNAs. *In vitro* produced embryos were collected from each of these groups at the 4-cell, 8-cell, morula, and blastocyst stages. Cleavage and blastocyst rates were evaluated on days 2 and 7 following fertilization, respectively. RNA was isolated from 3 replicates of pooled embryos at each stage, except for the blastocysts, and reverse-transcribed into cDNA for use with qPCR. Relative gene expression levels from each sample were calculated in triplicate using the SYBR Green comparative C\textsubscript{T} method, adjusted according to individual PCR efficiencies for each primer pair (R\textsuperscript{2}> 0.95) and normalized to the geometric mean C\textsubscript{T} of of 3 endogenous controls (GAPDH, YWHAZ and SDHA), to account for differences in both cell number and amount of total mRNA present in each sample (Goossens et al. 2005).

In the SUZ embryos, we succeeded in suppressing Suz12 expression to 1% or less of levels seen in the NULL embryos, at each stage analyzed (Figure 3.2). As seen in Table 3.2, the microinjection procedure resulted in a decrease ($p < 0.0001$) in cleavage rates between injected (NULL and SUZ) and noninjected (CTL) groups, while no difference ($p = 0.8$) was observed between the NULL and SUZ groups. However, the blastocyst rates were unexpectedly low and significantly different ($p < 0.0001$) between all groups, with the SUZ group exhibiting less than 1% development to the blastocyst stage (only 3 SUZ blastocysts were obtained for qPCR analysis). Because all of the blastocyst rates were
lower than expected, we re-evaluated our methods and made several modifications before repeating the experiment.

Figure 3.2 Expression levels of Suz12 in bovine preimplantation embryos. (A) grouped by treatment, showing the average fold change (error bars represent S.E.M. for each treatment group and stage collected), and (B) by developmental stage with transcript levels in the NULL group set at 100% for each stage examined, to illustrate knockdown efficiency as percentage values. Asterisks reflect significantly different values, within each stage (Tukey Kramer adjusted p < 0.05).
Table 3.2 Development rates for bovine embryos in the preliminary study. Letters indicate significantly different values between treatment groups, as determined by Chi-square analysis, \((p < 0.05)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Cleaved</th>
<th>% Cleaved</th>
<th>Blastocysts</th>
<th>% Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>508</td>
<td>394</td>
<td>77.6%\textsuperscript{a}</td>
<td>96</td>
<td>18.9%\textsuperscript{a}</td>
</tr>
<tr>
<td>NULL</td>
<td>580</td>
<td>384</td>
<td>66.2%\textsuperscript{b}</td>
<td>34</td>
<td>5.9%\textsuperscript{b}</td>
</tr>
<tr>
<td>SUZ</td>
<td>520</td>
<td>348</td>
<td>66.9%\textsuperscript{b}</td>
<td>3</td>
<td>0.6%\textsuperscript{c}</td>
</tr>
</tbody>
</table>

**Targeted Gene Suppression in Bovine Embryos**

We tested the experimental hypothesis that epigenome modifiers are important during early embryonic development and specifically at the EGA by injecting the confirmed effective siRNAs targeting \textit{Suz12} or \textit{Lsh} into bovine zygotes. The experimental endpoints would determine whether a reduction in either \textit{Suz12} or \textit{Lsh} transcript levels prior to and during the reprogramming period would result in altered development rates, changes in non-targeted gene expression and alterations in histone and/or DNA methylation, thus highlighting the importance of these genes during bovine preimplantation development.

**Validation of Suz12 Suppression in Bovine Embryos**

\textit{Suz12} mRNA levels were suppressed at least 93\% at all embryonic stages analyzed, with the highest degree of suppression seen in the 4-cell, 8-cell and morula stages. \textit{Suz12} expression levels are shown in Figure 3.3, grouped either by treatment (A) to illustrate
Figure 3.3 Relative expression levels of Suz12 across embryo stages (4C, 4-cells; 8C, 8-cells; M, morulas; B, blastocysts), among 3 treatment groups (CTL, NULL or SUZ), determined by TaqMan qPCR. (A) Expression profiles of Suz12 across development, grouped by treatment. Individual bars represent the treatment mean ± S.E.M. Asterisks refer to significant differences in relative abundance of transcripts between treatment groups within each stage. (B) The same dataset grouped by stage, with expression levels shown as a percentage of the NULL treatment group mean at each stage, in order to illustrate the percent knockdown of the target gene. Asterisks reflect significantly different values, within each stage (Tukey Kramer adjusted p < 0.05).
expression levels across development or by stage (B) in order to illustrate the percentage knockdown at each stage, normalized to the NULL embryos. Overall, no significant difference was seen in Suz12 expression between the CTL and NULL treatment groups at any of the stages examined ($p > 0.05$).

**Effects of Suz12 Suppression on Development and Expression of Non-Targeted Epigenetic Modifiers**

Following siRNA-mediated suppression of Suz12 of the appropriate zygotes, examination of cleavage rates revealed a small, but significant decrease resulting from the microinjection procedure ($p < 0.01$), but no difference ($p = 0.24$) in cleavage rates was seen between the NULL and SUZ groups. However, Suz12 suppression clearly had effects on later development rates and embryo quality, beyond those resulting from the microinjection procedure. Cleavage and development rates are listed in Table 3.3, showing significantly different values between treatment groups. Although the NULL

**Table 3.3** Cleavage and development rates ± S.E.M. for bovine embryos in the Suz12 targeted knockdown experiment. Chi-square comparisons were performed for all treatment groups; letters denote significantly different values ($p < 0.05$) between groups for each experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Cleaved</th>
<th>% Cleaved</th>
<th>Blastocysts</th>
<th>% Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>1210</td>
<td>1009</td>
<td>83.4 ± 0.3%$^a$</td>
<td>335</td>
<td>27.7 ± 0.3%$^a$</td>
</tr>
<tr>
<td>NULL</td>
<td>1118</td>
<td>882</td>
<td>78.9 ± 0.3%$^b$</td>
<td>229</td>
<td>20.5 ± 0.3%$^b$</td>
</tr>
<tr>
<td>SUZ</td>
<td>946</td>
<td>726</td>
<td>76.7 ± 0.4%$^b$</td>
<td>60</td>
<td>6.3 ± 0.2%$^c$</td>
</tr>
</tbody>
</table>
embryos did exhibit a decrease in development compared to the CTL embryos ($p = 0.0001$), the blastocyst rate of the SUZ group was considerably lower ($p < 0.0001$) than both the NULL and CTL groups. Most of the SUZ morulas & blastocysts exhibited considerable fragmentation (grade 2 or lower), characteristic of poor quality or unhealthy embryos. In particular, the SUZ morulas that did go on to form blastocysts had fewer, larger compacting cells prior to blastocoele formation. Morphologically, the NULL and CTL embryos seemed to develop normally or with only minor fragmentation (grades 1-2), although the NULL embryos also exhibited somewhat lower blastocyst formation. Images of representative embryos from each stage and treatment can be seen in the Appendix.

To evaluate the effect of Suz12 suppression on several non-targeted genes involved in histone modification, DNA methylation or differentiation, we also used TaqMan-based qPCR to examine mRNA levels of these various genes. No significant differences were observed in any of the non-targeted genes ($p < 0.05$) when comparing the SUZ treatment to the NULL treatment, as illustrated in Table 3.4.
Table 3.4  Changes in mRNA levels of non-targeted epigenetic modifiers, resulting from Suz12 suppression in early bovine embryos at the 4-cell, 8-cell, morula and blastocyst stages. No significant change was detected in the expression of any of the non-targeted genes, comparing the NULL and SUZ treatments within each stage ($p < 0.05$). The significant decreases in Suz12 levels at each stage are illustrated in Figure 3.3.

<table>
<thead>
<tr>
<th>Upregulated</th>
<th>No change</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>Lsh</td>
<td>Suz12</td>
</tr>
<tr>
<td></td>
<td>G9a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Suv4-20h1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lsd1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dnmt1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dnmt3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dnmt3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oct4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sox2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cdx2</td>
<td></td>
</tr>
</tbody>
</table>

Validation of Lsh Suppression in Bovine Embryos

Similar results were observed when we injected Lsh-targeting siRNAs into bovine zygotes, although the Lsh-targeting siRNAs were not as effective at suppressing target mRNA levels as were those targeting Suz12. Despite this, we still achieved a minimum of 89% suppression in Lsh transcript levels at the 4-cell, 8-cell and morula stages. However, some Lsh suppression was lost by the blastocyst stage, as mRNA levels were only decreased by 57%. Lsh expression levels are illustrated in Figure 3.4, grouped either by treatment (A) to illustrate expression levels across development or by stage (B) in order to illustrate the percentage knockdown at each stage, normalized to the NULL embryos. Although the graphical representation of Lsh expression levels seems to show moderate differences between treatment groups at the blastocyst stage, these
Figure 3.4  Relative expression levels of Lsh across embryo stages (4C, 8C, M and B), among 3 treatment groups (CTL, NULL or SUZ) determined by TaqMan qPCR.  (A) Expression profiles of Lsh across development, grouped by treatment. Individual bars represent the treatment mean ± S.E.M. Asterisks refer to significant differences in relative abundance of transcripts between treatment groups within each stage.  (B) The same dataset grouped by stage, with expression levels shown as a percentage of the NULL treatment group mean at each stage, in order to illustrate the percent knockdown of the target gene. Asterisks reflect significantly different values, within each stage (Tukey Kramer adjusted p < 0.05).

differences were not significant (p = 0.60 and 0.44, when comparing the LSH group to the CTL and NULL groups, respectively). Overall, there was no significant difference
found in \textit{Lsh} expression between the CTL and NULL treatment groups at any of the stages examined ($p > 0.05$).

\textit{Effects of Lsh Suppression on Development and Expression of Non-Targeted Epigenetic Modifiers}

Targeted suppression of \textit{Lsh} resulted in lower cleavage rates in the injected groups as compared to the CTL embryos; again, there was no significant difference between the NULL and LSH injected groups. Analysis of blastocyst rates revealed that CTL embryos exhibited the highest development, followed by the NULL and finally, LSH groups. LSH morulas and blastocysts also exhibited fragmentation and the morulas had fewer, larger compacting cells. Cleavage and development rates are listed in Table 3.5, showing significantly different ($p < 0.05$) values between treatment groups. Images of representative embryos from each stage and treatment can be viewed in the Appendix.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Treatment} & \textbf{n} & \textbf{Cleaved} & \textbf{\% Cleaved} & \textbf{Blastocysts} & \textbf{\% Blastocysts} \\
\hline
CTL & 847 & 648 & 76.5 ± 0.4\%$^a$ & 173 & 20.4 ± 0.4\%$^a$ \\
NULL & 582 & 384 & 66.0 ± 0.4\%$^b$ & 89 & 15.3 ± 0.5\%$^b$ \\
LSH & 615 & 399 & 64.9 ± 0.6\%$^c$ & 30 & 4.9 ± 0.3\%$^c$ \\
\hline
\end{tabular}
\caption{Cleavage and development rates ± S.E.M. for bovine embryos in the \textit{Lsh} targeted knockdown experiment. Chi-square comparisons were performed for all treatment groups; letters denote significantly different values ($p < 0.05$) between groups.}
\end{table}

The effects of \textit{Lsh} suppression on the non-targeted genes involved in histone modification, DNA methylation or differentiation were determined as stated previously.
Overall, no changes ($p < 0.05$) in transcript levels of the non-targeted genes were observed when comparing the LSH treatment to the NULL treatment (illustrated in Table 3.6).

**Table 3.6** Changes in mRNA levels of non-targeted epigenetic modifiers, resulting from Lsh suppression in early bovine embryos at the 4-cell, 8-cell, morula and blastocyst stages. No significant change was detected in the expression of any of the non-targeted genes, comparing the NULL and LSH treatments within each stage ($p < 0.05$). The significant decreases in Lsh levels at each stage are illustrated in Figure 3.4.

<table>
<thead>
<tr>
<th>Upregulated</th>
<th>No change</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suz12</td>
<td>Lsh</td>
<td></td>
</tr>
<tr>
<td>G9a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suv4-20h1</td>
<td>Lsd1</td>
<td></td>
</tr>
<tr>
<td>Dnmt1</td>
<td>Dnmt3a</td>
<td></td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>Oct4</td>
<td></td>
</tr>
<tr>
<td>Sox2</td>
<td>Cdx2</td>
<td></td>
</tr>
</tbody>
</table>

**Immunocytochemical Analysis of Histone or DNA Methylation in Bovine Embryos**

We used antibodies specific to H3K27 trimethylation in order to verify any changes in this modification that may have resulted from suppression of either Suz12 or Lsh. By counterstaining the DNA of these same embryos with Hoescht and obtaining fluorescence intensity values for each staining, we obtained a ratio of H3K27me3 fluorescence divided by DNA fluorescence for each embryo. These values were averaged within treatment groups in order to obtain a mean ratio for each treatment.
We observed no difference between the CTL, NULL and LSH treatment groups, with ratios of 1.28, 1.32 and 1.33, respectively. However, we did observe a significant decrease in the fluorescence ratio of the SUZ group (0.52), compared with the other three treatments ($p < 0.05$), signifying a reduction in H3K27 trimethylation levels as a result of Suz12 suppression. Images of representative blastocysts from each treatment group with appropriate ratios H3K27me3:DNA are listed in Figure 3.5.

![Image](image.png)

**Figure 3.5** Immunostaining of DNA (blue) and H3K27 trimethylation (green) in blastocysts, illustrating differences between treatment groups. Ratio averages for each treatment are listed along with S.E.M. values. $^A,^B$ Tukey Kramer adjusted $p < 0.05$. 
For embryos stained specifically for H3K9 trimethylation, and using the same analysis of fluorescence values mentioned above, we obtained intensity ratios of H3K9me3 fluorescence divided by DNA fluorescence. Ratios for the CTL, NULL, SUZ and LSH treatment groups were 0.57, 0.89, 0.71 and 0.63, respectively. Images of representative blastocysts from each treatment group, along with the appropriate ratios for H3K9me3:DNA, are listed in Figure 3.6.

**Figure 3.6** Immunostaining of DNA (blue) and H3K9 trimethylation (green) in blastocysts, illustrating differences between treatment groups. Ratio averages for each treatment are listed along with S.E.M. values. A,B,C Tukey Kramer adjusted $p < 0.05$. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA</th>
<th>H3K9me3</th>
<th>Merged</th>
<th>Ratio H3K9/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td><img src="image1" alt="DNA" /></td>
<td><img src="image2" alt="H3K9me3" /></td>
<td><img src="image3" alt="Merged" /></td>
<td>0.57 ± 0.05&lt;sup&gt;B,C&lt;/sup&gt;</td>
</tr>
<tr>
<td>NULL</td>
<td><img src="image1" alt="DNA" /></td>
<td><img src="image2" alt="H3K9me3" /></td>
<td><img src="image3" alt="Merged" /></td>
<td>0.89 ± 0.04&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>SUZ</td>
<td><img src="image1" alt="DNA" /></td>
<td><img src="image2" alt="H3K9me3" /></td>
<td><img src="image3" alt="Merged" /></td>
<td>0.71 ± 0.05&lt;sup&gt;A,B&lt;/sup&gt;</td>
</tr>
<tr>
<td>LSH</td>
<td><img src="image1" alt="DNA" /></td>
<td><img src="image2" alt="H3K9me3" /></td>
<td><img src="image3" alt="Merged" /></td>
<td>0.63 ± 0.06&lt;sup&gt;B,C&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
For embryos stained for DNA methylation (5meC), and using the same analysis mentioned previously, we obtained intensity ratios of 5meC fluorescence divided by DNA fluorescence. Images of representative blastocysts from each treatment group, along with appropriate ratios for 5meC:DNA, are listed in Figure 3.7. No difference was seen in the ratios between the CTL & NULL treatments (1.04 and 0.93 respectively, \( p = 0.25 \)), or between the SUZ & LSH treatments (1.27 and 1.23 respectively, \( p = 0.96 \)). However, both the SUZ and LSH treatments resulted in significantly increased (\( p < 0.01 \))
levels of 5meC, indicating a general increase in DNA methylation when compared to the CTL and NULL treatments.

DISCUSSION

Histone modifying enzymes and the modifications they bring about represent an important and intensely investigated area of epigenetics due to its wide-reaching effects on development (Schultz & Worrad 1995, Dennis et al. 2001, Erhardt et al. 2003, Yan et al. 2003a, Yan et al. 2003b, Sarmento et al. 2004, de la Cruz et al. 2005, Santos et al. 2005, de la Cruz et al. 2007, Pasini et al. 2007, Puschendorf et al. 2008, Ross et al. 2008). In particular, characterization of these enzymes during the reprogramming events of preimplantation development would be helpful in determining which, if any, histone modifications lead to and/or control EGA in mammals. From these data, we can conclude that suppression of two of these enzymes, Suz12 and Lsh, in bovine embryos leads to lower development rates as well as alterations in both histone and DNA methylation at the blastocyst stage.

siRNA Validation

When deciding on types of siRNA oligos to use for the experiment, we chose Ambion’s Silencer® Select siRNAs over standard oligos, as these siRNAs are supposed to require lower siRNA concentrations than standard counterparts. However, in order to be sure we observed the highest possible knockdown with these siRNAs, we still tested them at
both low (20 nM) and high (50 nM) concentrations. After several rounds of testing, we obtained siRNAs that were suitable enough to use for microinjection, without needing the higher siRNA concentration.

**Preliminary Data**

In order to evaluate our experimental design, we conducted a preliminary experiment using the *Suz12* siRNAs. In this experiment, we observed the high degree of *Suz12* suppression we desired in the SUZ embryos, but were limited on any analyses to be performed on the blastocyst stage embryos, because so few SUZ treated embryos survived to the blastocyst stage. The extremely low rate of SUZ blastocysts could likely be attributed to the lack of Suz12 expression during the earlier stages, as nearly all of the SUZ morulas were severely fragmented in comparison with the NULL and CTL morulas. However, this still presented us with a problem, as we were greatly limited in the number of SUZ blastocysts feasibly obtained for analysis. In addition, the rate of CTL blastocyst formation was still much lower than we expected to see with our *in vitro* fertilization and culture system. Blastocyst rates of at least 25% for the CTL embryos would have been more appropriate for our system. After re-examining our techniques, we further minimized the time that the zygotes were out of the controlled CO$_2$/O$_2$ incubator for microinjection by working with smaller numbers at a time, and began holding them in a bicarbonate-buffered culture media in the incubator between handling periods, instead of the hepes-buffered holding media we had been using with
ambient air on warm plates. Furthermore, we learned that the bull whose semen we
were using was a proven producer of slower-developing embryos, typically not forming
many blastocysts until day 8. For all future embryos we evaluated, we collected
blastocysts and recorded development rates on day 8 instead of day 7.

In addition to the changes mentioned above, we also wanted the opportunity to be
able to look at additional non-targeted genes without needing to increase the number
of embryos collected in each sample. In order to accomplish this by multiplexing
reactions, we switched from using SYBR Green based qPCR to TaqMan based qPCR for
all future gene expression analyses.

*Gene Expression*

Depletion of *Suz12* transcript levels beginning at the zygote stage led to continued
suppression of the target gene throughout the 8-cell, morula and blastocyst stages,
although siRNA efficiency was slightly reduced in the blastocysts. Presumably, the
siRNA molecules were used up and/or degraded over time, allowing the embryo to
partially recover from *Suz12* suppression, leading to the slight decrease in knockdown
efficiency seen at the blastocyst stage. It is also possible that because we cannot
ensure the same volume is injected into every embryo, some embryos received more
siRNAs than others, and the ones that received the lowest siRNA treatment initially
were more capable of development to the blastocyst stage.
In addition to Suz12, we also wanted to see if suppression resulted in any off-target effects on other epigenetic modifiers. Studies have demonstrated an unexplainable effect of Suz12 depletion on transcript stability of Ezh2, another component of the PRC1 complex (Pasini et al. 2004). We selected several other epigenetic modifiers with known involvement in histone and/or DNA methylation to see if lowered Suz12 mRNA levels resulted in altered expression of these genes. No significant ($p > 0.05$) off-target changes in expression of these genes were observed. Although not reported in Table 3.4, we also looked to see if expression of HP1 was altered as a result of Suz12 suppression. Suz12 has been shown to be required for proper H3K9me3 and HP1 recruitment to initiate the spreading of heterochromatin (Bannister et al. 2001). While no changes from the NULL embryos were seen in HP1 expression, the primer/probe set we used did not meet our minimum PCR amplification requirements when the standard curves. Although we did not expect to see alteration in HP1 transcript levels, the analysis would need to be repeated with suitable primers in order to conclude any effects on HP1 expression.

Although not quite as efficient as the Suz12 knockdown, depletion of Lsh transcripts initiated at the zygote stage resulted in a moderate to high maintenance of Lsh suppression at the 4-cell, 8-cell and morula stages. However, the targeting siRNAs were not able to maintain suppression at a significant level ($p > 0.05$) through the blastocyst stage, and suppression was reduced to an efficiency of only 57%. Again, the lowered efficiency of the Lsh-targeting siRNAs observed at the blastocyst stage could
either be due to the siRNAs being used up or degraded over time, or because the embryos that survived to the blastocyst stage represent those that received less siRNAs during the microinjection procedure.

We observed no significant change in expression of any of the non-targeted genes in the LSH treatment group, compared with the NULL treatment group, at all stages examined. However, we did observe the following changes when comparing the LSH groups to the CTL groups: *Suv4-20h1* expression increased in the LSH blastocysts ($p = 0.04$) and *Dnmt3b* expression decreased in the LSH morulas ($p = 0.0009$), both compared to the CTL embryos at those stages. Dennis et al. reported no alteration of *Dnmt1, 3a* or *3b* protein levels, as well as *Dnmt1* activity when *Lsh* levels were depleted in mouse *Lsh* */-* tissues (Dennis et al. 2001). Others have suggested that *Lsh* directly participates in chromatin formation, as opposed to indirectly increasing transcription of other chromatin components (Muegge 2005). The effects of the LSH treatment we observed on *Suv4-20h1* and *Dnmt3b* expression cannot, for sure, be attributed to *Lsh* depletion because the microinjection treatment alone could have contributed to some of these changes. Further research would be necessary in order to draw any conclusions from these observations.

**Histone and DNA Methylation**

Although, *Suz12* suppression was not as efficient at the blastocyst stage, it was still sufficient enough to decrease methylation levels of H3K27 and H3K9, both expected
downstream effects (Cao & Zhang 2004, Pasini et al. 2004, de la Cruz et al. 2007, Pasini et al. 2007). However, we did observe an increase in DNA methylation levels of the SUZ embryos ($p < 0.01$). This result was quite unexpected, as we have no evidence of a downstream effect on DNA methylation, which results from alteration of H3K27 and H3K9 methylation. It has been speculated that DNA methylation does not intervene to silence genes that are actively transcribed, but only affects genes that have already been silenced by other means, such as histone methylation (Bird 2002). Under this idea, DNA methylation acts as a method of cell “memory” to reinforce the silencing of heterochromatin that was previously marked for methylation via histone modifications. It is possible that preventing necessary heterochromatic silencing (by preventing H3K27me3) might lead cells to use DNA methylation in order to try and prevent activation of these elements. It is known that different tissues, even within the same species, can employ PCG-mediated silencing and DNA methylation interchangeably (Iida et al. 1994, Sado et al. 2000, Wang et al. 2001, Bird 2002). If this were the case, then DNA methylation could act as the primary mark for silencing of heterochromatic regions, not simply as a method of reinforcing silencing initiated by histone methylation. However, this still does not explain how DNA methylation levels were increased in the SUZ-treated embryos, without any alteration in expression of the DNMTs examined. Therefore, how suppression of Suz12 can actually lead to increased DNA methylation cannot be explained within the scope of our experiment, and further examination/validation of this phenomenon is necessary.
Because of the reported requirement of Lsh for DNA methylation in the mouse (Dennis et al. 2001, Yan et al. 2003b), we anticipated seeing a decrease in 5meC staining intensity; however, the opposite of our expectation was observed. The intensity ratio of 5meC/DNA in the LSH embryos was significantly higher than both the CTL and NULL groups ($p < 0.01$), but explanation of this occurrence is beyond the scope of our experiment, as there was no significant increase in transcript abundance of $Dnmt1$, $3a$ or $3b$. As expected, we did not see any significant change ($p > 0.05$) in H3K27 trimethylation, as Lsh has not been demonstrated to have any effect on this modification. Unexpectedly, a slight decrease was seen when comparing the ratios of H3K9me3 in the LSH group to the NULL group ($p = 0.006$). It should be noted, however, that many of the embryo ratios for the LSH group fell within the range of ratios for the NULL group, and that by simple visual examination of the images, many of the LSH embryos looked no different from some of the NULL embryos.

Of further concern in this experiment was the extreme variation in signal ratios observed in H3K9me3 of the CTL embryos. Embryos that were “older” (stained and imaged a few months prior to the actual analysis) displayed a mean ratio similar to that of the NULL ($p = 0.91$), while the “newer” embryos (stained and imaged only days or weeks prior to the analysis) exhibited a mean ratio lower than any of the treatment groups, and one not significantly different from the IgG background controls ($p = 0.78$). Because there was significant variation in the controls from the beginning to the end of the experiment, the comparisons to the injected embryos cannot be trusted.
Development

The decrease in both blastocyst rate and quality of both the SUZ- and LSH-treated embryos serves as evidence of the importance of these genes during bovine preimplantation development. However, because some embryos did go on to form blastocysts, it is unclear if *Suz12* and *Lsh* are absolutely required for blastocyst formation. Near-complete suppression of *Suz12* severely limited development, but some embryos were still able to form blastocysts. Because we cannot standardize the volume of siRNA delivery into each zygote during the microinjection process, it is possible that these SUZ blastocysts survived only because they received less siRNAs than the embryos which arrested earlier in development. The partial “recovery” from *Suz12* suppression at the blastocyst stage would support this theory (97% or greater suppression in the earlier stages, reduced to 93%), as embryos which received a lower siRNA treatment at the zygote stage would probably exhibit reduced siRNA efficiency.

If we compare our results to those from studies generating *Suz12* knockout mice, we see some consistency in outcome, as *Suz12*−/− mice survive past implantation, but die *in utero* approximately 8.5 dpc, as a result of gastrulation defects arising around 7.5 dpc (Pasini *et al.* 2004). If these SUZ embryos were transferred into recipient cattle, we cannot say for sure if they would be expected to live full-term after *Suz12* suppression during the preimplantation reprogramming period. Severe alterations in histone modifications or DNA methylation could result in expression of genes or other elements
that are usually silenced in normal, untreated embryos (Yan et al. 2003b, Huang et al. 2004, Muegge 2005).

Although the Lsh-targeting siRNAs were not as efficient as those targeting Suz12, Lsh suppression at the earlier stages was sufficient enough to cause very low development rates and poorer quality embryos of those that did make it to the blastocyst stage. Again, because we cannot standardize the amount of siRNAs that get injected into each zygote, the LSH embryos that were able to form blastocysts could represent those which initially received a lower treatment volume, and therefore exhibit the reduced efficiency of Lsh suppression we observed at the blastocyst stage. Because we were not able to maintain a high level of Lsh suppression throughout all stages examined, it would be interesting to see whether or not similar development rates were observed if the experiment was repeated and a high degree of Lsh suppression maintained through the blastocyst stage.

It has been demonstrated that Lsh is required for proper DNA methylation, by recruiting DNMTs and HDACs to the site of potential methylation (Myant & Stancheva 2008). If we compare our observations to those from Lsh knockout mouse experiments, we might not expect any change in development rates, as Lsh -/- mice survive full term, but die shortly thereafter (Dennis et al. 2001, Geiman et al. 2001, Huang et al. 2004, Lippman et al. 2004, Fan et al. 2005). Although the mouse model differs from the bovine model, in that remethylation of the embryonic genome does not begin until around the time of implantation (as opposed to the 8-16 cell stage in
bovids), one would likely conclude that a knockout phenotype would be more severe than that of a short-term knockdown. Therefore, because $Lsh$-$/-$ mice do survive to term, we might expect the LSH embryos to also survive full-term, but be born with severe DNA hypomethylation defects, regardless of the differences in timing of DNA remethylation during early development. However, $Lsh$ probably has a much more important role in early bovine development than it does in early mouse development, based on our results here. In the mouse $Lsh$-$/-$ model, it was assumed that $Lsh$ depletion did not have a negative effect on implantation rates, because normal Mendelian frequencies were observed from 13 dpc until birth. Because DNA remethylation occurs post-implantation in the mouse (and $Lsh$ is believed to be required for proper DNA methylation), this observation would make sense. However, in the cow, DNA remethylation occurs prior to implantation, so if DNA methylation is somehow altered (i.e. by depletion of $Lsh$), this could cause reduced development to the blastocyst stage (just before implantation).

Also, because we cannot standardize the amount of siRNA material that is introduced into the cytoplasm during microinjection, it is likely that the embryos that were able to survive to the blastocyst stage represent those embryos which received less $Lsh$-targeting siRNA material initially. Due to the severe decrease in blastocyst formation observed with LSH-treated embryos, we would expect to see a further reduction in development rates with increased $Lsh$ suppression, possibly to the point of an early embryonic lethal phenotype.
Summary

Taken together, these results confirm the expected requirement of sufficient Suz12 expression for proper trimethylation of both H3K27 and H3K9, but suggest that Suz12 could somehow be involved in DNA methylation. As expected, reduction in Lsh transcripts did not have any observable effect on H3K27 trimethylation, but did have effects on H3K9 trimethylation (expected) and DNA methylation (unexpected). However, because Lsh suppression was not maintained through the blastocyst stage, the experiment would need to be repeated, preferably with siRNAs which could maintain a higher degree of Lsh suppression, over this period of time. Also, for both of these epigenetic modifiers, examining these same histone and DNA methylation marks one stage earlier (at the morula stage) might give us a clearer picture of gene activities, since gene transcript suppression was more efficient at this stage. Although we have demonstrated here the involvement of Suz12 and Lsh in both histone and DNA methylation, further examination of the activities of these two genes (including histone acetylation and methylation of other residues) would likely be informative in determining the specific roles of these two histone-modifying enzymes.
CHAPTER IV

METHODS

siRNA DESIGN AND TESTING

Two Silencer® Select siRNAs (Applied Biosystems, USA) were designed from published mRNA sequence for each gene, using Applied Biosystem’s GeneAssist™ Workflow Builder program and tested in an MDBK cell line. Concentrations of siRNAs were tested via transfection at 20 nM and 50 nM singly, and at 20 nM in combination, in order to find out the optimal efficiency of each siRNA and whether or not a synergistic effect was produced when transfecting both siRNAs together.

CELL CULTURE AND siRNA TRANSFECTION

MDBK cells were grown and maintained in culture in Modified Eagle’s Medium (Gibco, Invitrogen, USA), supplemented with 10% horse serum and 50 µg/mL gentamicin (Gibco, Invitrogen, USA), in a humidified atmosphere composed of 5% CO₂ in air at 37°C. Cells were washed, trypsinized, counted and plated at least 3 hours prior to transfection in order achieve approximately 50% confluency at time of transfection. siRNAs were transfected into the cells using a standard calcium phosphate kit (Invotrogen, USA); for each siRNA or combination of siRNAs being tested, three replicates were performed. Forty-eight hours following transfection, the cells were washed in 1X PBS and harvested in 600 µL of a guanidine thiocyanate-containing lysis
buffer (buffer RLT). The cell lysate was then vortexed and stored at -80°C until the RNA isolation step.

**OOCYTE AND EMBRYO PRODUCTION**

Multiple pools of mature bovine ova were obtained from TransOva Genetics (MN, USA), following standard *in vitro* maturation procedures. Briefly, viable cumulus-oocyte complexes (COCs) aspirated from slaughterhouse-derived ovaries of mixed-breed cows were matured *in vitro* in tissue-culture medium (TCM-199 Earle’s) supplemented with 10% fetal bovine serum (FBS), 0.02 I.U./mL bovine follicle-stimulating hormone (bFSH, Sioux Biochem, USA), 0.02 I.U./mL bovine luteinizing hormone (bLH, Sioux Biochem, USA), 12.5 mM sodium bicarbonate and 50 µg/mL gentamicin. Groups of approximately 50 COCs were matured for 23 hours (during overnight shipping) at 38.5°C in sealed tubes that had been equilibrated in a 5% CO₂ environment. Upon arrival, matured groups of COCs were washed through TL Hepes (Lonza, USA) and fertilized *in vitro* using frozen/thawed semen from a bull with proven fertility in a humidified atmosphere composed of 5% CO₂ in air at 38.5°C.

Approximately 19 hours following fertilization, presumptive zygotes were vortexed in 0.4% bovine hyaluronidase in TL Hepes to remove cumulus cells and cultured in a commercial culture media system, supplemented with bovine serum albumin (Probumin, Millipore, USA) and 50 µg/mL gentamicin (Gibco, Invitrogen, USA), under mineral oil in a humidified atmosphere composed of 5% CO₂, 5% O₂ and 90% N₂ at
38.5°C until time of collection. During specific time windows for each stage, groups of ova or embryos were collected, washed through 1X PBS, placed in buffer RLT and homogenized by vortexing for 1 minute, then frozen and stored at -80°C until all samples had been collected. Cleavage rates for each group were recorded on day 2, and blastocyst rates recorded on day 8 prior to collection; Chi-square analysis was used to determine significantly different (p < 0.05) development rates between groups.

**CYTOPLASMIC MICROINJECTION OF BOVINE ZYGOTES**

Following vortexing, presumptive zygotes were randomly sorted into three treatment groups: non-injected controls (CTL), nonsense siRNA injected controls (NULL) or targeted siRNA injected embryos (either SUZ or LSH). CTL embryos were moved into culture immediately, and the others moved into holding plates (with either hepes-buffered benchtop media on warm plates or culture media in the incubator) until injection. Embryos from the NULL, SUZ and LSH treatment groups were cytoplasmically microinjected with a mixture of 25 nM siRNAs (Ambion, USA) and 2 mg/mL fluorescein dextran (Invitrogen, USA) in TE buffer. The biologically inert dextran served as a marker to positively identify embryos that had been successfully microinjected. Approximate injection volume was estimated to be around 500-1000 pL, as determined by measuring the change in cytoplasmic volume (calculated using diameter measurements) before & after microinjection. Any embryos that were lysed as a result
of the microinjection procedure were removed prior to placing the embryos back into culture.

Separate groups of embryos were generated for each of the targeted knockdown experiments (i.e., the CTL and NULL embryos corresponding to the SUZ treatment group were not the same as the CTL and NULL embryos corresponding to the LSH treatment group). Embryos were collected at specific timepoints according to stage from multiple rounds of IVF, and pooled until three replicates were obtained for each stage evaluated. The number of embryos per sample \((n)\) varied from one stage to another in order to compensate for decreasing levels of mRNA around the EGA, and much higher levels by the blastocyst stage. However, \(n\) was the same for different treatment groups within the same stage. Sample size and time of collection for each stage are listed below in Table 4.1.

<table>
<thead>
<tr>
<th>A</th>
<th>4-cell</th>
<th>8-cell</th>
<th>morula</th>
<th>blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size ((n))</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Replicates per treatment</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3*</td>
</tr>
<tr>
<td>Time of collection</td>
<td>44-46 hrs</td>
<td>70-72 hrs</td>
<td>146-148 hrs</td>
<td>172-174 hrs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>4-cell</th>
<th>8-cell</th>
<th>morula</th>
<th>blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size ((n))</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Replicates per treatment</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Time of collection</td>
<td>44-46 hrs</td>
<td>70-72 hrs</td>
<td>146-148 hrs</td>
<td>194-196 hrs</td>
</tr>
</tbody>
</table>

Table 4.1 Embryos collected for the SYBR Green (A) or TaqMan (B) gene expression analyses of siRNA-mediated gene knockdown experiments. Time of collection corresponds to the number of hours following in vitro fertilization. The main difference was that we switched from collecting blastocysts on day 7 for the SYBR Green preliminary analysis to day 8 for the TaqMan analyses. *A total of only 3 SUZ blastocysts were generated in the preliminary experiment (SYBR Green qPCR), and were combined as a single replicate.
RNA ISOLATION (MDBK CELLS)

Total RNA was isolated from the MDBK cell samples using the standard version of the RNeasy kit protocol (Qiagen, USA). Frozen, homogenized cell lysate samples were defrosted on ice and vortexed again for 1 minute to ensure homogenization. All remaining steps were carried out at room temperature, and all wash/elution steps were performed via centrifugation.

An equal volume of 70% ethanol was added to the sample and gently mixed to promote selective binding of RNA to the RNeasy column membrane. The sample was then added to the column and centrifuged to allow the RNA to bind to the column. After washing with an ethanol-containing wash buffer (buffer RW1), the sample was treated on-column with RNase-free DNase I (Qiagen, USA) to remove any contaminating genomic DNA. The column-bound sample was then washed once more with buffer RW1 and twice with different ethanol-containing wash buffer (buffer RPE). Each sample was eluted in 40μL nuclease-free water. Reverse transcription was carried out immediately following the elution step in order to prevent degradation of the freshly isolated RNA.

RNA ISOLATION (OVA AND EMBRYOS)

Total RNA was isolated from oocyte & embryo samples using a slightly modified version of the RNeasy kit protocol (Qiagen, USA). Briefly, 80% ethanol was used instead of 70%, the second wash step in buffer RPE was replaced by washing in 80% ethanol and
the column was spun-dried for an additional 5 minutes just prior to the elution step. Because of the much lower amount of RNA present in these examples, each was eluted in only 20 µL nuclease-free water.

**REVERSE TRANSCRIPTION (RT)**

RT was performed using the qScript RT protocol (Quanta BioSciences, USA). For oocyte/embryo samples, 15 µL of each RNA sample was combined with 4 µL qScript reaction mix and 1 µL qScript reverse transcriptase (RTase) on ice and pipetted gently to mix. The remaining 5 µL of RNA from each sample was used to create a negative/“no RT” control for the qPCR step; to each of these samples, 1.33 µL of qScript reaction mix and 1.33 µL of nuclease-free water was added. For MDBK cell samples, the same volumes of qScript reaction mix and RTase were used, but only 500 µg of each RNA sample was used, diluted to 15 µL with nuclease-free water.

All samples were incubated at 25°C for 10 minutes for primer annealing followed by 35 minutes at 42°C. The RT reactions were stopped by heating the samples at 95°C for 5 min. Resulting cDNA (or RNA in the case of the “no RT” controls) was then diluted appropriately for qPCR amplification.

**SYBR GREEN qPCR**

All cDNA samples were run in triplicate, with a single “no RT” control for each. Per well, each 20 µL reaction consisted of 10 µL 2X Power SYBR Green PCR Master Mix (Applied
Biosystems, USA), 5 µL of a primer mix (3.33 µM forward & 3.33 µM reverse primers) and 5 µL of diluted template.

qPCR reactions were run in an ABI StepOne Plus Real-Time PCR System (Applied Biosystems, USA) in 48- or 96-well Optical Reaction Plates using the following conditions: 10 minutes at 95°C to allow for DNA denaturation and Taq Polymerase activation, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds, then 95°C for 15 seconds, and finally a gradual heating cycle from 60°C to 95°C in order to obtain a dissociation curve for the PCR products.

Relative gene expression from each sample was calculated in triplicate using the SYBR Green comparative C\text{t} method (Applied Biosystems, USA), and normalized to the geometric mean C\text{t} of 3 endogenous controls: Gapdh, Ywhaz and Sdha. This method accounts for differences in both cell number per embryo as well as total mRNA present in each sample, and corrects for changing gene expression levels of commonly used endogenous controls during this developmental period (Goossens et al. 2005). Each calculation was also adjusted to the individual PCR amplification efficiencies for each primer pair (R^2 > 0.95), estimated by constructing standard curves for each pair using serial dilutions of mixed oocyte and blastocyst cDNA (representative of both maternally and embryonically-derived mRNA).
TAQMAN qPCR

Primers used for TaqMan qPCR were multiplexed into 8 sets, ranging from 1 to 4 sets of primers and probes per set. Concentration of primers and probes in each set for a single 25 µl reaction are given in Table 4.2.

Table 4.2 Primer and probe concentrations used for TaqMan qPCR analysis.

<table>
<thead>
<tr>
<th>Set number</th>
<th>Target Gene</th>
<th>Primer pair concentration</th>
<th>Probe concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gapdh</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>1</td>
<td>Sdha</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>1</td>
<td>Ywhaz</td>
<td>0.8 µM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>1</td>
<td>Suv4-20h1</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>2</td>
<td>Dnmt3b</td>
<td>0.8 µM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>2</td>
<td>Lsh</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>3</td>
<td>Dnmt3a</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>3</td>
<td>Lsd1</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>4</td>
<td>G9a</td>
<td>0.8 µM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>4</td>
<td>Suz12</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>5</td>
<td>SetB1</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>6</td>
<td>Dnmt1</td>
<td>0.8 µM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>7</td>
<td>Oct4</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>8</td>
<td>Sox2</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>8</td>
<td>Cdx2</td>
<td>0.6 µM</td>
<td>0.1 µM</td>
</tr>
</tbody>
</table>

Along with the primers and probes, each 25 µL reaction consisted of: 2.5 µL 10X Platinum Taq buffer (Invitrogen, USA), 4mM MgCl₂ (Invitrogen, USA), 0.4mM dNTP mix (Invitrogen, USA), 1.25U Platinum Taq (Invitrogen, USA) and 2.5 µL of 1:10 diluted cDNA template. All cDNA samples were run in triplicate, with a single “no RT” control for each. In addition, 2 “no template” controls were run for each multiplexed primer and probe set.
qPCR reactions were run in an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, USA) in 96-well Optical Reaction Plates using the following conditions: 1 minute at 95°C to allow for DNA denaturation and Taq Polymerase activation, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. The reaction was then held at 4°C.

Relative gene expression from each sample was calculated in triplicate using the TaqMan comparative Ct method (Applied Biosystems, USA), and normalized to the geometric mean Ct of 3 endogenous controls: Gapdh, Ywhaz and Sdha. Each calculation was also adjusted to the individual PCR amplification efficiencies for each primer pair ($R^2 > 0.95$), estimated by constructing standard curves for each pair using serial dilutions of blastocyst cDNA.

**IMMUNOCYTOCHEMISTRY (ICC)**

Blastocysts were selected on day 8 of development from multiple pools of IVF-derived embryos, fixed by placing in cold methanol for a minimum of 1 minute, and stored in PBS-0.1% Tween 20 (PBS-Tw) at 4°C until further use. Embryos were permeabilized with 1% Triton X-100 in PBS (PBS-Tr) for 1 hour at room temperature while shaking and washed thoroughly through PBS-Tw over 15 minute intervals. Following the permeabilization and wash, embryos were blocked in fresh blocking buffer (10 mg/mL BSA, 2% v/v goat serum and 11.25 mg/mL glycine in 1X PBS) overnight in order to prevent any nonspecific antibody binding. Embryos that were to be labeled with 5meC
were washed at room temperature with 2M HCl for 30 minutes and subsequently neutralized with 100 mM Tris/HCl buffer (pH 8.5) for 10 minutes following permeabilization, but before washing and blocking overnight. Antibody labeling of histone modifications did not require an acid wash. Embryos were then labeled with the appropriate primary antibody, shaken at room temperature for one hour, washed through fresh blocking buffer again and labeled with the corresponding secondary antibody. Primary and secondary antibodies were all diluted to 0.5% v/v in PBS-Tw; see Table 4.3 for a list of all antibodies used.

Table 4.3 Antibodies used for ICC staining and analysis of bovine embryos.

<table>
<thead>
<tr>
<th>Type</th>
<th>Target</th>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>IgG</td>
<td>Normal rabbit IgG</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Primary</td>
<td>IgG</td>
<td>Normal mouse IgG</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>Primary</td>
<td>H3K9me3</td>
<td>Histone H3 trimethyl Lys9 Rabbit pAb</td>
<td>Active Motif</td>
</tr>
<tr>
<td>Primary</td>
<td>H3K27me3</td>
<td>Histone H3 trimethyl Lys27 Rabbit pAb</td>
<td>Active Motif</td>
</tr>
<tr>
<td>Primary</td>
<td>5meC</td>
<td>Monoclonal Antibody, 5-Methylcytidine Mouse</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>Secondary</td>
<td>rabbit IgG</td>
<td>Alexa 488 goat anti-rabbit IgG (H+L)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Secondary</td>
<td>mouse IgM</td>
<td>Alexa 488 goat anti-mouse IgM (H+L)</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Following antibody bonding, the embryos labeled for H3K9me3 and H3K27me3 were washed through PBS-Tw and counterstained with 0.5% v/v Hoescht in PBS-Tw; embryos labeled for 5meC were counterstained with 0.5% v/v propidium iodide in PBS-Tw instead of Hoescht. Following one more wash through PBS-Tw, each group of embryos was mounted separately (according to treatment & staining) on glass slides.
using Prolong Gold anti-fade reagent mounting media (Invitrogen), sealed and stored at 4°C protected from light until further examination. For each round of ICC, in addition to the specific staining (H3K9me3, H3K27me3 and 5meC) of all treatment groups (CTL, NULL, SUZ and LSH), a minimum of 2 CTL embryos were stained for IgG, in order to serve as a control for background intensity. Embryo treatment groups were visualized and z-scan images taken for analysis within 7 days of labeling/mounting, in order to prevent any fading of the fluorescently-tagged antibodies over time from affecting the actual intensities of each embryo.

**CONFOCAL LASER MICROSCOPY AND ANALYSIS**

All images were taken with a 63X water-immersion objective lens. To represent fluorescence intensity of the entire embryo, 6-7 optical sections were taken for each embryo, evenly divided between the bottom and top of the embryo. Thickness of the Z-scans among embryos varied from 6 to 10 µm, and depended on the size of each embryo and other uncontrollable mounting variables between each slide. The area photographed and characterized as “representative” of a single blastocyst was determined by the location and visibility (80-100%) of the inner cell mass (ICM) of each embryo. The average intensity of these measurements for an individual blastocyst was considered illustrative of the intensity of the entire embryo. Exposure times for each filter are listed in Table 4.4. Each filter setting was determined according to minimum and maximum fluorescent values of IgG controls and replicate controls across all group
replicates and treatments; thus, filter settings were set and remained the same with each replicate and experimental treatment.

Table 4.4 Exposure times used to image embryos for each channel/filter.

<table>
<thead>
<tr>
<th>Labeling</th>
<th>Used to visualize</th>
<th>Filter</th>
<th>Exposure time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoescht</td>
<td>DNA (nuclei)</td>
<td>DAPI</td>
<td>200</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>DNA (nuclei)</td>
<td>CY3</td>
<td>300</td>
</tr>
<tr>
<td>Alexa 488</td>
<td>primary antibody</td>
<td>FITC</td>
<td>500</td>
</tr>
</tbody>
</table>

Average fluorescence intensity ratios were calculated for each embryo by dividing the intensity of the specific target (H3K27me3, H3K9me3 or 5meC) by the intensity of DNA staining (Hoescht or propidium iodide). Ratios for each embryo were then averaged to obtain a mean ratio for each treatment group and staining combination. Using these means, a one-way ANOVA model was constructed in order to determine significant differences between treatment groups for each staining ($p < 0.05$).
CHAPTER V

SUMMARY

The increased application of ARTs in both humans and animals has led to the increased incidence of imprinting and other epigenetic abnormalities in animals or people conceived \textit{in vitro}. These observations have led to more research aiming to uncover the mechanisms involved in reprogramming the early embryo and \textit{in vitro} perturbations during this critical time period. However, the area of epigenetics still remains somewhat unexplored in the mammalian preimplantation embryo, especially as it relates to histone tail modifications, the enzymes that catalyze these changes and downstream effects of the modifications.

We characterized gene expression via qPCR of several known histone modifying enzymes during bovine preimplantation development. \textit{SetB1, SmyD3, Suv3-9h1} and \textit{Suv4-20h1} exhibit transcript levels highest in the oocyte or 2-cell stage and dropping off by the morula and blastocyst stages. \textit{G9a} is expressed moderately during the earlier cleavage stages pre-EGA, but also possibly active during EGA, because an increase in expression is observed around the 8-cell stage. \textit{Suz12} and \textit{Lsh} reflect genes with low expression pre-EGA and a notable increase at the 8-16 cell stage. These transcript levels are then reduced to basal levels again by the morula and blastocyst stages.

\textit{Suz12} and \textit{Lsh} were selected as obvious candidates for further exploration of gene function during this developmental window, since their increases in expression
coincided with the EGA event as well as reprogramming of DNA methylation. After testing and confirming siRNAs targeted at each of these genes in MDBK cells, we employed cytoplasmic microinjection to introduce these molecules into bovine embryos at the zygote stage. Embryos were collected at the 4-cell, 8-cell, morula and blastocyst stages for analysis of genes expression via qPCR. Development rates were also evaluated to the blastocyst stage and blastocysts from each treatment group were collected and used for ICC analysis of either specific histone (H3K9me3, H3K27me3) or DNA methylation. Near-complete (>90%) suppression of Suz12 resulted in decreased development rates ($p < 0.01$) and lower morphological quality of embryos that did make it to the blastocyst stage, when compared to the CTL and NULL embryos. In addition, Suz12 suppression led to expected decreases in both H3K9 ($p = 0.07$) and H3K27 trimethylation ($p < 0.0001$), and an unexpected increase in DNA methylation ($p < 0.0001$). Moderate to high (55-95%) suppression of Lsh similarly resulted in lower development rates ($p < 0.0001$), with poorer quality embryos that formed blastocysts. Lsh suppression also led expectedly to no change in H3K27 trimethylation levels ($p > 0.05$), but unexpectedly to changes in both H3K9 trimethylation (decreased, $p = 0.006$) and DNA methylation (increased, $p < 0.0001$). Because these results both confirm some and disagree with other previously published activities for these two genes, further characterization of their activities would be necessary in order to determine their potential role(s) in the reprogramming events occurring during preimplantation development.
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Figure A1 Pre-EGA representative morphological similarities exhibited by the SUZ and LSH embryos, as compared to the CTL and NULL embryos, at the 4-cell (4C) and 8-cell (8C) stages.
**Figure A2** Post-EGA representative morphological differences exhibited by the SUZ and LSH embryos, as compared to the CTL and NULL embryos, at the morula (M) stage.

**Figure A3** Post-EGA representative morphological differences exhibited by the SUZ and LSH embryos, as compared to the CTL and NULL embryos, at the blastocyst (B) stage.
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