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

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

Approved by:

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

August 2011

Major Subject: Genetics

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…

ACKNOWLEDGEMENTS

NOMENCLATURE

5meC	5-methylcytosine
ac	Acetylated
AI	Artificial insemination
ARTs	Assisted reproductive technologies
AS	Angelman syndrome
BWS	Beckwith-Wiedemann syndrome
CpG	Cytosine-Guanine (dinucleotides)
DMR	Differentially methylated region
DNMT	DNA methyltransferase
dpc	Days post-coitum
EGA	Embryonic genome activation
ET	Embryo transfer
H3K4	Histone 3 lysine residue 4
H3K9	Histone 3 lysine residue 9
H3K27	Histone 3 lysine residue 27
HAC	Histone acetyltransferase
HDAC	Histone de-acetylase
HMG	Histone modifying gene
HMT	Histone methyltransferase

HP1	Heterochromatic protein 1
hpf	Hours post-fertilization
ICC	Immunocytochemistry
ICR	Imprinting control region
IVF	*In vitro* fertilization
MBPs	Methyl CpG-binding proteins
MDBK	Madin-Darby bovine kidney (cells)
me2	Dimethylated
me3	Trimethylated
PGCs	Primordial germ cells
qPCR	quantitative real-time PCR
RNAi	RNA interference
RT	Reverse transcription
siRNAs	short interfering RNAs
TEs	Transposable elements
Xi	Inactivated X-chromosome

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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, Elsden *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, Mapletoft & 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.*

of these technologies in humans, and the birth of the first *in vitro* conceived child in

1978 (Steptoe & Edwards 1978). As a result of this success as well as a notable

decrease in human fertility over the last 50 years, *in vitro* technologies (particularly

superovulation, IVF, ICSI and ET) have since become routine treatments for infertile

couples (CDC's 2002 Annual ART Success Rates Report). However, the application of

these methods to bypass certain fertility issues has created other important medical

anomalies (Benoff & Hurley 2001, Khosla *et al.* 2001a, Khosla *et al.* 2001b, Maher *et al.*

2003, Fleming *et al.* 2004, Kelly & Trasler 2004, Niemitz & Feinberg 2004, Menezo *et al.*

2006, Paoloni-Giacobino 2007, Amor & Halliday 2008, Banwell & Thompson 2008,

Lawrence & Moley 2008, Grace & Sinclair 2009, Laprise 2009, Le Bouc *et al.* 2010).

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 Kcqnot1) 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.

EPIGENETICS

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

housekeeping genes, are hypomethylated and transcriptionally competent. A disruption of proper methylation patterns in the genome can lead to a wide range of moderate to severe disease states (Helwani *et al.* 1999, Jones & Laird 1999, Doherty *et al.* 2000, Jones & Takai 2001, Khosla *et al.* 2001a, Khosla *et al.* 2001b, Young *et al.* 2001, Shiraishi *et al.* 2002, El-Maarri *et al.* 2003, Das & Singal 2004).

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).
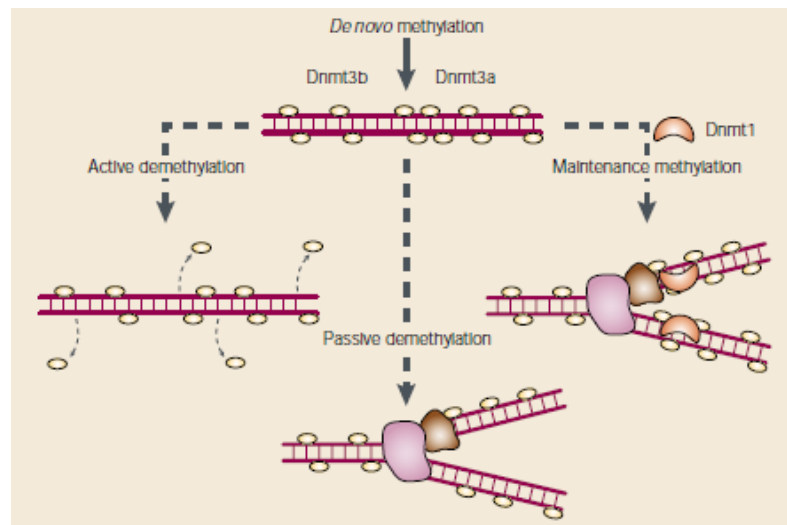
Figure 1.1 DNA methylation and demethylation in mammals. *Dnmt3a* and *Dnmt3b* introduce methyl groups to unmethylated DNA *de novo*. During DNA replication, *Dnmt1* recognizes methyl groups on hemimethylated DNA and copies the mark to the daughter strand. In the absence of *Dnmt1*, passive demethylation 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*

Table 1.1 Summary of the properties of the three catalytically active mammalian DNMTs (Geiman & Robertson 2002).

DNA methyltransferase	Proposed function	Localization	Interacting, chromatin-associated proteins	Proposed function of interaction
DNMT1	Maintenance methylation	Replication foci during S phase	HDAC1, 2	Deacetylate chromatin, transcriptional repression, facilitate DNA methylation?
			PCNA	Targeting of DNMT1 to replication foci
DNMT3A	*De novo* methylation	Heterochromatic foci in ES cells	HDAC1	Deacetylate chromatin, transcriptional repression, facilitate DNA methylation?
DNMT3B	*De novo* methylation	Heterochromatic foci in ES cells	HDAC1, 2	Deacetylate chromatin, transcriptional repression, facilitate DNA methylation?
			DNMT3L	Establishment of maternal methylation imprints

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

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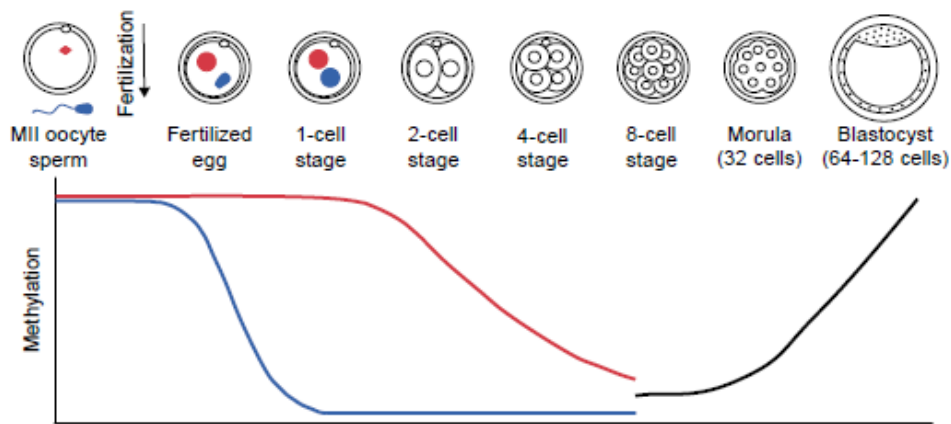
Figure 1.2 DNA methylation levels across bovine preimplantation embryonic development. The blue line represents active demethylation of the paternal genome, while the red represents passive demethylation of the maternal genome. After erasure, these marks are reestablished *de novo* when the embryonic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

wave of transcription (Kopecny 1989, Kopecny *et al.* 1989, Fulka *et al.* 2004, Wong *et al.* 2010). In mice however, EGA does not coincide with *de novo* DNA methylation. Transcription is initiated at the 2-cell stage, while the DNA remethylation event does not occur until after implantation (Monk *et al.* 1987, Kafri *et al.* 1992, Santos *et al.* 2002, Wang *et al.* 2004, Zeng & Schultz 2005).

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

Table 2.1 Oocytes and embryos collected for characterization of gene expression. Time of collection corresponds to the number of hours following *in vitro* fertilization.

Stage	Replicate	*n*	Time of collection
MII oocytes	1	17	0
MII oocytes	2	17	0
2-cells	1	15	30
2-cells	2	14	35
4-7-cells	1	17	48
4-7-cells	2	25	52
mid 8-cells	1	18	74
mid 8-cells	2	20	75
late 8-cells	1	22	94
late 8-cells	2	19	96
16-cells	1	21	124
16-cells	2	22	124
morulas	1	12	146
morulas	2	8	148
morulas	3	18	149
blastocysts	1	9	172
blastocysts	2	21	172
blastocysts	3	15	180

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.
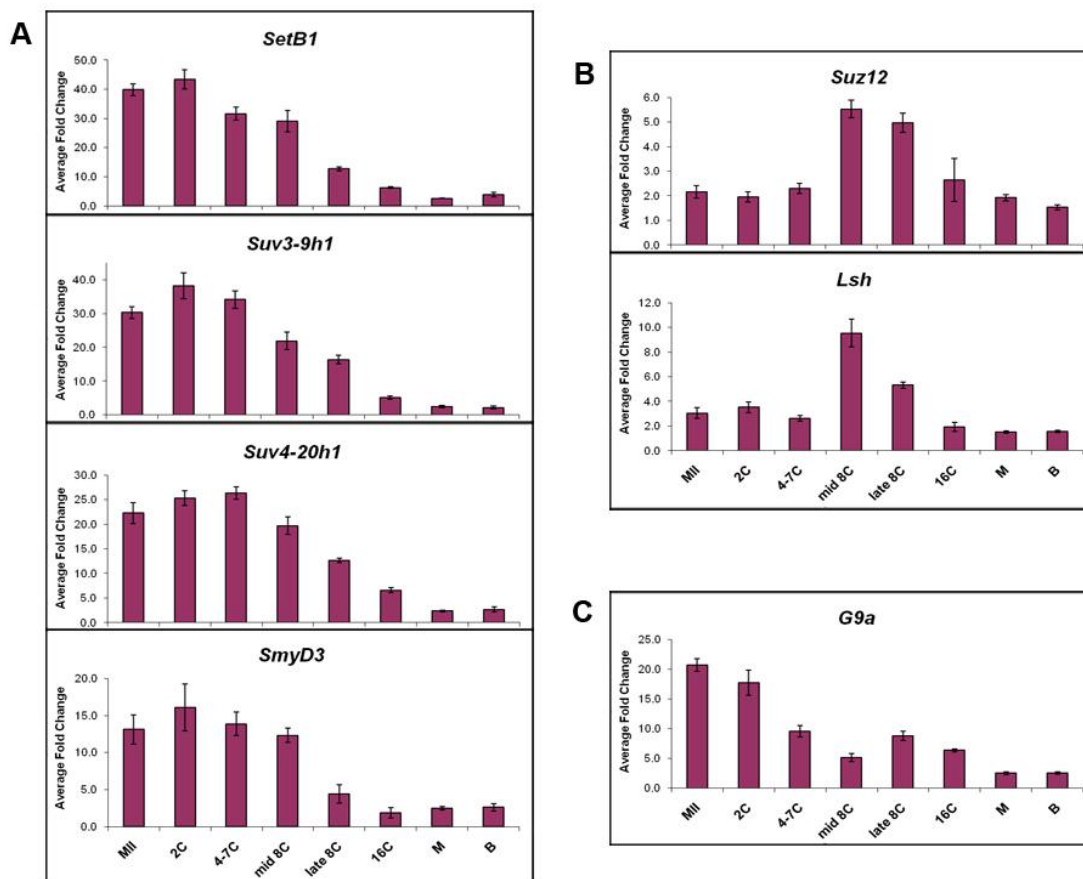
Figure 2.1 (A-C) Three characteristic expression patterns exhibited by several representative epigenetic modifiers in bovine preimplantation embryos. The *y-axis* represents the average fold change in expression seen across early embryonic stages, normalized to the geometric mean of three endogenous controls: *Gapdh*, *Ywhaz*, and *Sdha* (Goossens *et al.* 2005). Error bars represent S.E.M. for each stage examined.

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 3.1 siRNA sequences that were successful in reducing target gene expression when tested in MDBK cells. These siRNAs were further used for cytoplasmic microinjection into zygotes.

Gene Target	Name	bp Start	siRNA Target Sequence
Suz12	SUZ-316	316	5'-AAGGATGTAAGTTGTCCGATA-3'
Lsh	LSH-1575	1575	5'-AGCAGATACTGTTATCATTTA-3'
Lsh	LSH-447	447	5'-TCGGATATTGGTAAAGCATAT-3'
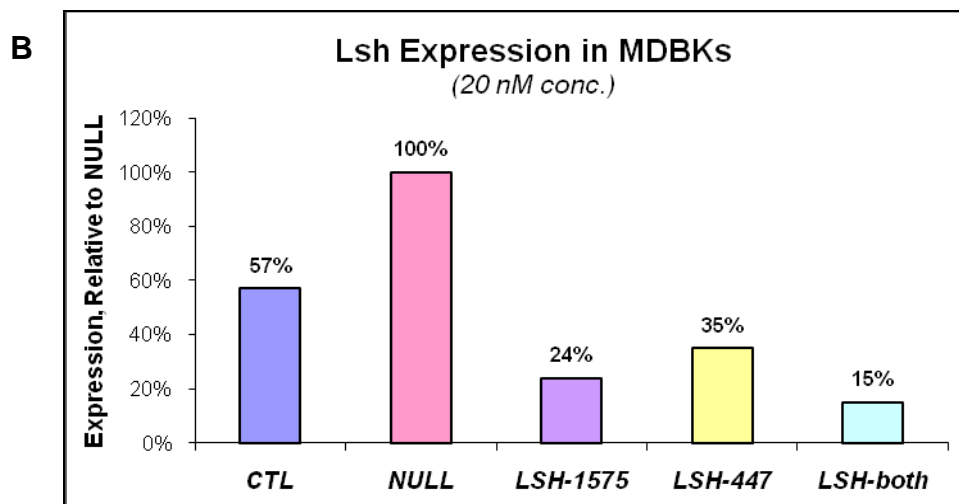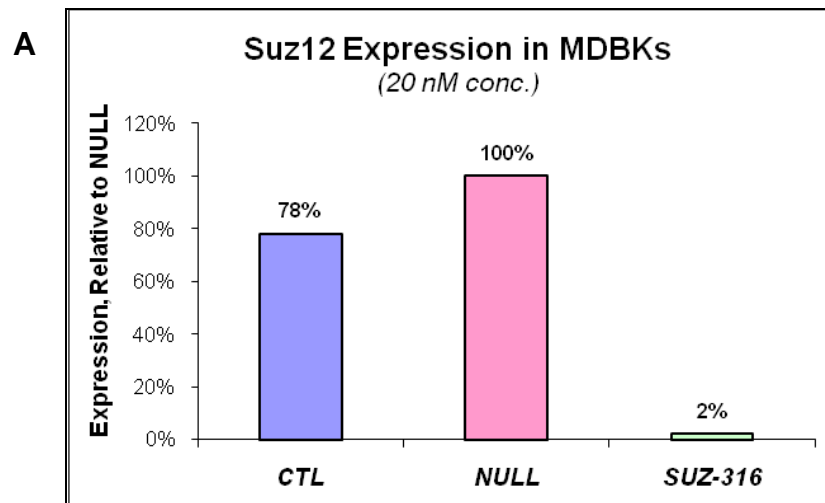
Figure 3.1 Targeted gene expression in MDBK cells, using 20 nM (low) siRNA concentration. (A) Bars represent *Suz12* expression following no treatment (CTL), transfection with a non-targeting siRNA control (NULL), or transfection with an *Suz12*-targeting siRNA (SUZ-316). (B) Bars represent *Lsh* expression 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_t method, adjusted according to individual PCR efficiencies

for each primer pair ($R^2 > 0.95$) and normalized to the geometric mean C_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.
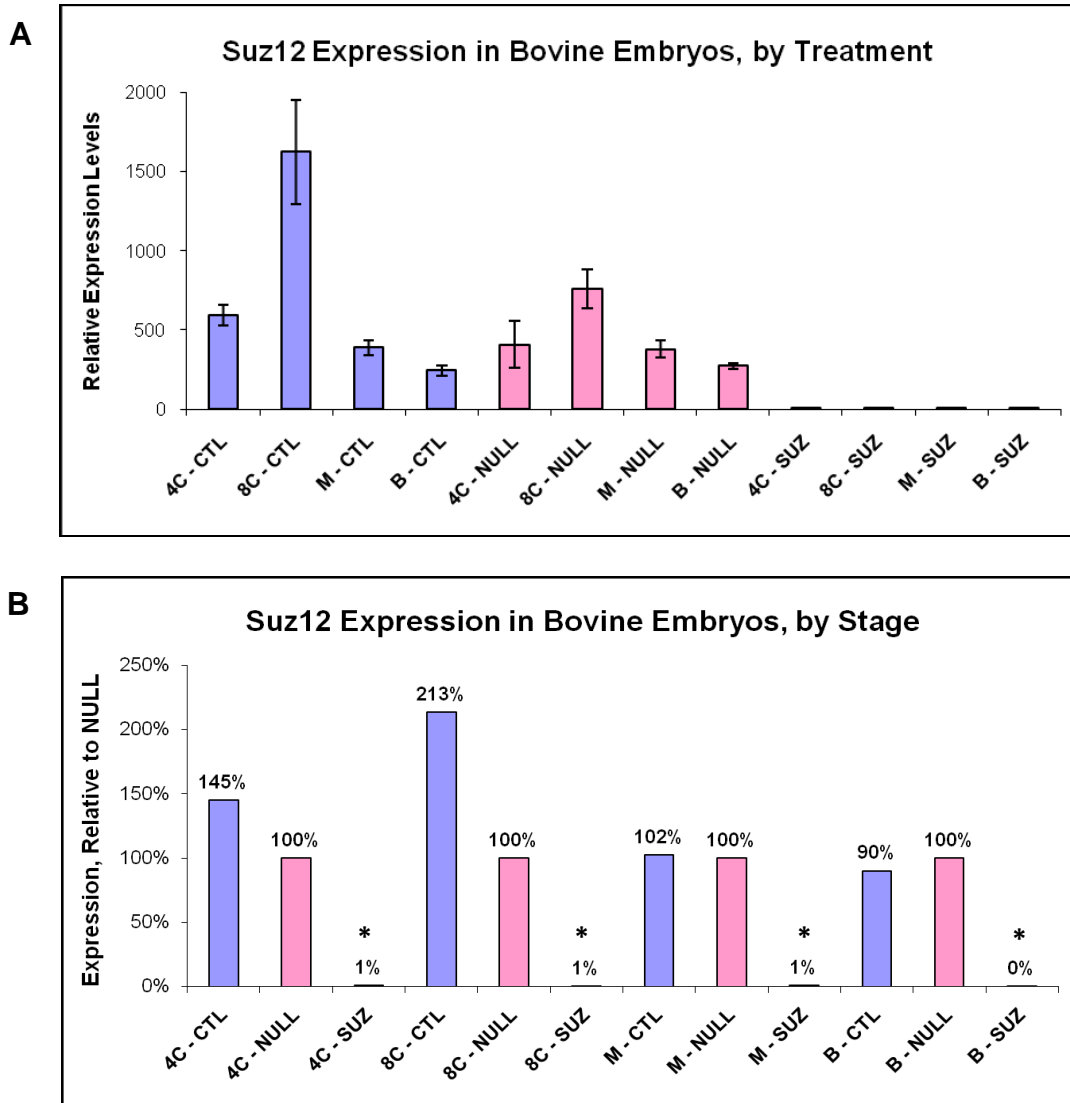
Figure 3.2 Expression levels of *Suz12* in bovine preimplantation embryos. (A) grouped by treatment, showing the average fold change (error bars represent S.E.M. for each treatment group and stage collected), and (B) by developmental stage with transcript levels in the NULL group set at 100% for each 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).

Treatment	n	Cleaved	*% Cleaved*	Blastocysts	*% Blastocysts*
CTL	508	394	77.6%[a]	96	18.9%[a]
NULL	580	384	66.2%[b]	34	5.9%[b]
SUZ	520	348	66.9%[b]	3	0.6%[c]

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 *Suz12* or *Lsh* into bovine zygotes. The experimental endpoints would determine whether a reduction in either *Suz12* or *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*

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. *Suz12* expression levels are shown in Figure 3.3, grouped either by treatment (A) to illustrate

A Suz12 Expression in Bovine Embryos, by Treatment

B Suz12 Expression in Bovine Embryos, by Stage
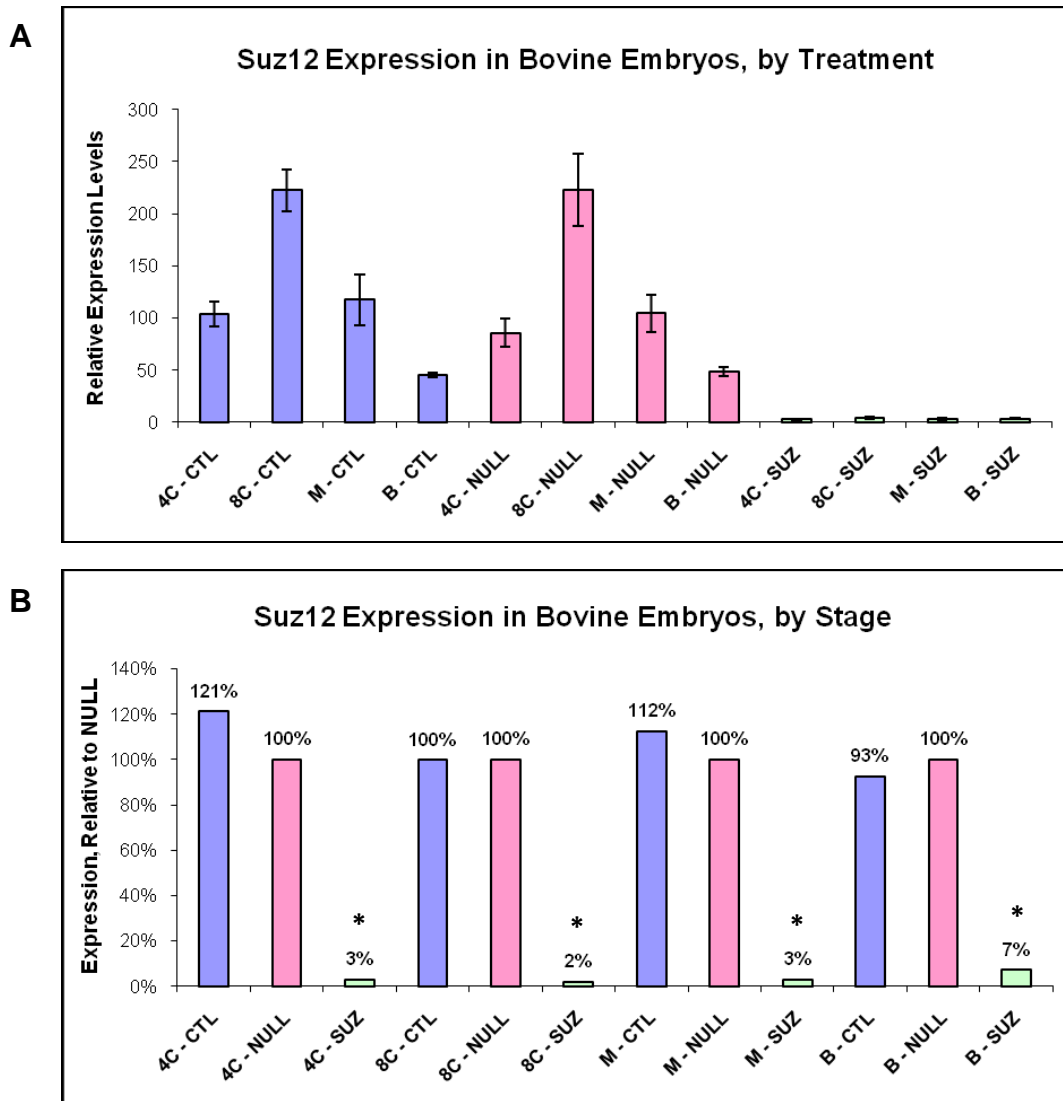
Figure 3.3 Relative expression levels of *Suz12* across embryo stages (4C, 4-cells; 8C, 8-cells; M, morulas; B, blastocysts), among 3 treatment groups (CTL, NULL or SUZ), determined by TaqMan qPCR. (A) Expression profiles of *Suz12* across development, grouped by treatment. Individual bars represent 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.

Treatment	n	Cleaved	% Cleaved	Blastocysts	% Blastocysts
CTL	1210	1009	83.4 ± 0.3%[a]	335	27.7 ± 0.3%[a]
NULL	1118	882	78.9 ± 0.3%[b]	229	20.5 ± 0.3%[b]
SUZ	946	726	76.7 ± 0.4%[b]	60	6.3 ± 0.2%[c]

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.

Upregulated	No change	Downregulated
none	Lsh	Suz12
	G9a	
	Suv4-20h1	
	Lsd1	
	Dnmt1	
	Dnmt3a	
	Dnmt3b	
	Oct4	
	Sox2	
	Cdx2	

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
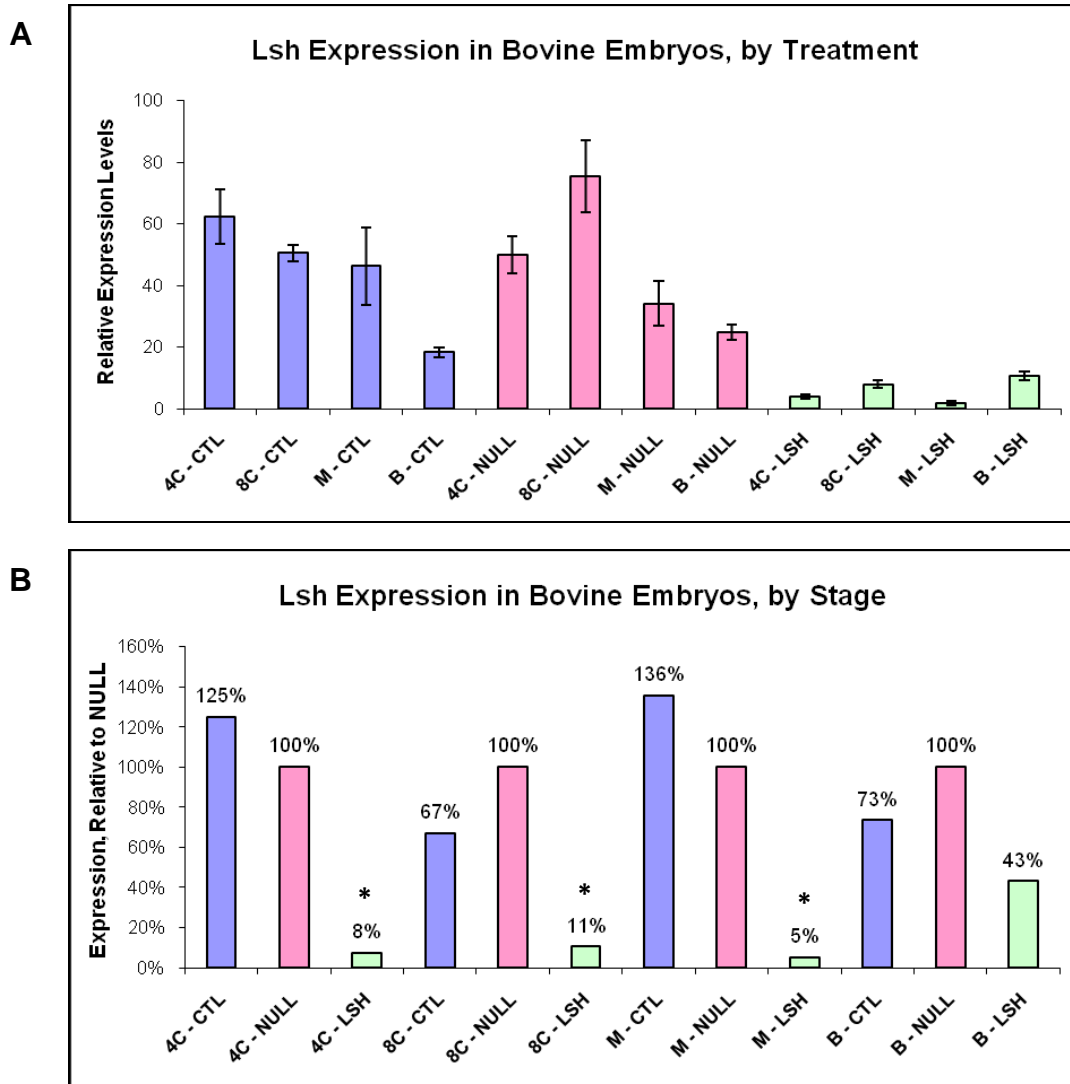
Figure 3.4 Relative expression levels of *Lsh* across embryo stages (4C, 8C, M and B), among 3 treatment groups (CTL, NULL or SUZ) determined by TaqMan qPCR. (A) Expression profiles of *Lsh* across development, grouped by treatment. Individual bars represent the treatment mean ± S.E.M. Asterisks refer 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 *Lsh* expression between the CTL and NULL treatment groups at any of the

stages examined ($p > 0.05$).

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

Modifiers

Targeted suppression of *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.

Table 3.5 Cleavage and development rates ± S.E.M. for bovine embryos in the *Lsh* targeted knockdown experiment. Chi-square comparisons were performed for all treatment groups; letters denote significantly different values ($p < 0.05$) between groups.

Treatment	n	Cleaved	% Cleaved	Blastocysts	% Blastocysts
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]

The effects of *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.

Upregulated	No change	Downregulated
	Suz12	*Lsh*
	G9a	
	Suv4-20h1	
	Lsd1	
	Dnmt1	
	Dnmt3a	
	Dnmt3b	
	Oct4	
	Sox2	
	Cdx2	

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.

	DNA	H3K27me3	merged	ratio H3K27/DNA
CTL				1.28 ± 0.08^A
NULL				1.32 ± 0.08^A
SUZ				0.52 ± 0.10^B
LSH				1.33 ± 0.10^A
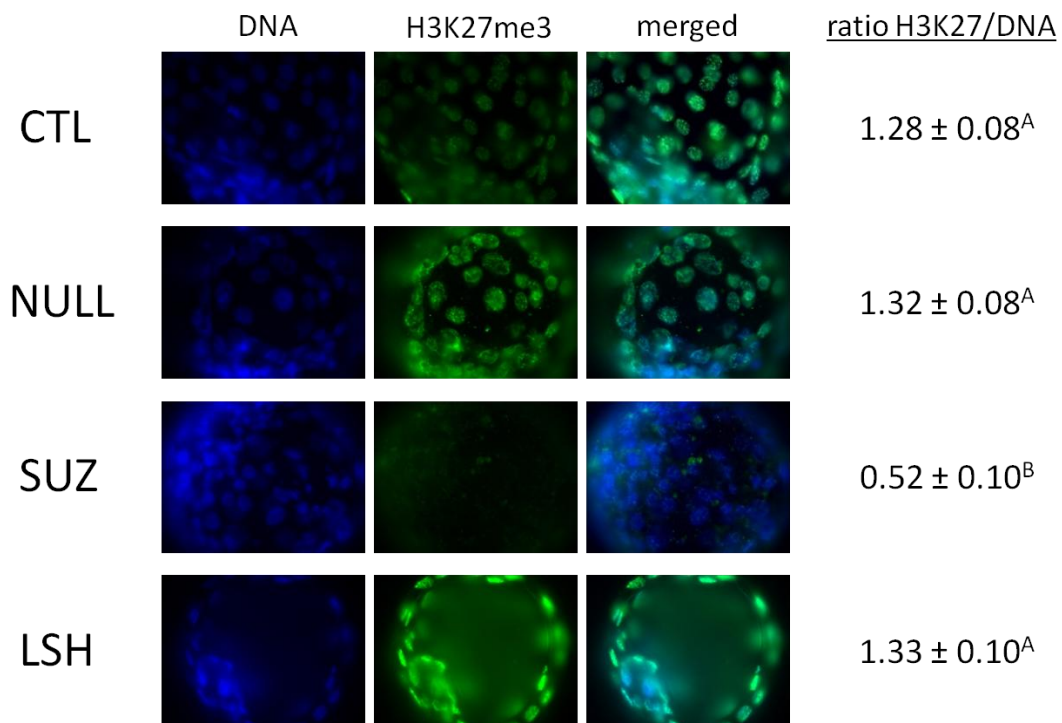
Figure 3.5 Immunostaining of DNA (blue) and H3K27 trimethylation (green) in blastocysts, illustrating differences between treatment groups. Ratio averages for each treatment are listed along with S.E.M. values. ^{A,B} Tukey Kramer adjusted $p < 0.05$.

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.

	DNA	H3K9me3	merged	ratio H3K9/DNA
CTL				$0.57 \pm 0.05^{B,C}$
NULL				0.89 ± 0.04^{A}
SUZ				$0.71 \pm 0.05^{A,B}$
LSH				$0.63 \pm 0.06^{B,C}$
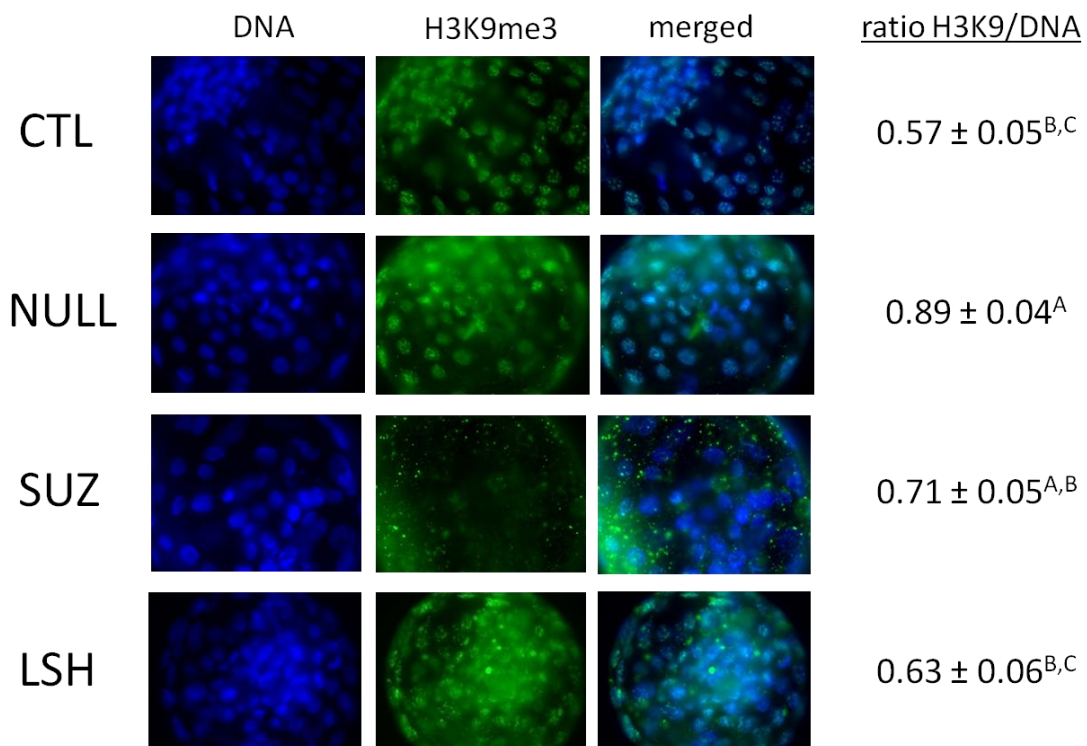
Figure 3.6 Immunostaining of DNA (blue) and H3K9 trimethylation (green) in blastocysts, illustrating differences between treatment groups. Ratio averages for each treatment are listed along with S.E.M. values. [A,B,C] Tukey Kramer adjusted $p < 0.05$.

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

	DNA	5meC	merged	ratio 5meC/DNA
CTL				1.04 ± 0.04^{B}
NULL				0.93 ± 0.04^{B}
SUZ				1.27 ± 0.04^{A}
LSH				1.23 ± 0.05^{A}

Figure 3.7 Immunostaining of DNA (red) and 5meC (green) in blastocysts, illustrating differences between treatment groups. Ratio averages for each treatment are listed with S.E.M. values. [A,B] Tukey Kramer adjusted $p < 0.05$.

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.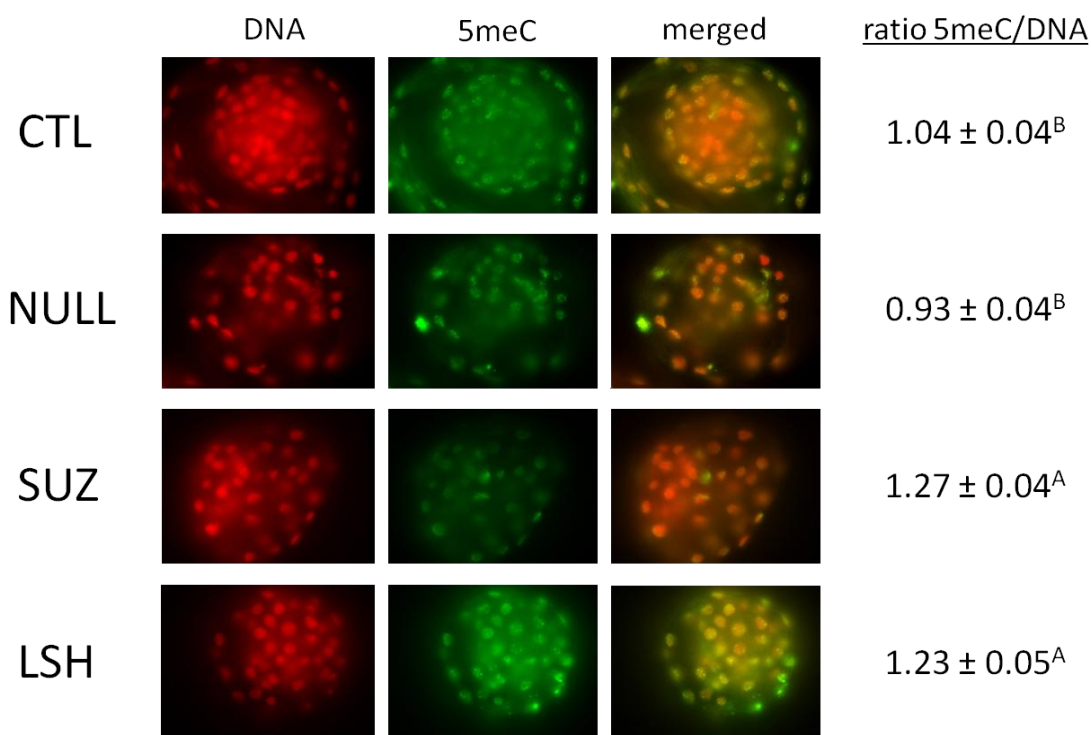* 2003b, Sarmento *et al.* 2004, de la Cruz *et al.* 2005, Santos *et al.* 2005, de la Cruz *et al.* 2007, Pasini *et al.* 2007, Puschendorf *et al.* 2008, Ross *et al.* 2008). In particular, characterization of these enzymes during the reprogramming events of preimplantation development would be helpful 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 approzimately 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% CO_2 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% CO_2 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% CO_2 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% CO_2, 5% O_2 and 90% N_2 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 (ie: 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 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.

A

	4-cell	8-cell	morula	blastocyst
Sample size (*n*)	15	20	10	10
Replicates per treatment	3	3	3	3*
Time of collection	44-46 hrs	70-72 hrs	146-148 hrs	172-174 hrs

B

	4-cell	8-cell	morula	blastocyst
Sample size (*n*)	15	20	10	10
Replicates per treatment	3	3	3	3
Time of collection	44-46 hrs	70-72 hrs	146-148 hrs	194-196 hrs

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_t method (Applied Biosystems, USA), and normalized to the

geometric mean C_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.

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

Along with the primers and probes, each 25 µL reaction consisted of: 2.5 µL 10X

Platinum Taq buffer (Invitrogen, USA), 4mM $MgCl_2$ (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 C_t 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.

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

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.

Labeling	Used to visualize	Filter	Exposure time (ms)
Hoescht	DNA (nuclei)	DAPI	200
Propidium iodide	DNA (nuclei)	CY3	300
Alexa 488	primary antibody	FITC	500

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 *in vitro*. These observations have led to more research aiming to uncover the mechanisms involved in reprogramming the early embryo and *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. *SetB1*, *SmyD3*, *Suv3-9h1* and *Suv4-20h1* exhibit transcript levels highest in the oocyte or 2-cell stage and dropping off by the morula and blastocyst stages. *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. *Suz12* and *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.

Suz12 and *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.

REFERENCES

2005 A Scientific Illustration of How Epigenetic Mechanisms Can Affect Health. Retrieved from http://commonfund.nih.gov/epigenomicsimg1.jpg.

Amor DJ & Halliday J 2008 A review of known imprinting syndromes and their association with assisted reproduction technologies. *Hum Reprod* **23** 2826-2834.

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC & Kouzarides T 2001 Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410** 120-124.

Banwell KM & Thompson JG 2008 *In vitro* maturation of mammalian oocytes: outcomes and consequences. *Semin Reprod Med* **26** 162-174.

Barnes F, Endebrock M, Looney C, Powell R, Westhusin M & Bondioli K 1993 Embryo cloning in cattle: the use of *in vitro* matured oocytes. *J Reprod Fertil* **97** 317-320.

Bartel DP 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116** 281-297.

Benoff S & Hurley IR 2001 Epigenetic and experimental modifications in early mammalian development: part I. Preface. *Hum Reprod Update* **7** 211-216.

Bernstein BE, Kamal M, Lindblad-Toh K, Bekiranov S, Bailey DK, Huebert DJ, McMahon S, Karlsson EK, Kulbokas EJ, 3rd, Gingeras TR, Schreiber SL & Lander ES 2005 Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* **120** 169-181.

Bestor TH 2000 The DNA methyltransferases of mammals. *Hum Mol Genet* **9** 2395-2402.

Bestor TH & Tycko B 1996 Creation of genomic methylation patterns. *Nat Genet* **12** 363-367.

Betteridge KJ 2003 A history of farm animal embryo transfer and some associated techniques. *Anim Reprod Sci* **79** 203-244.

Bird A 2002 DNA methylation patterns and epigenetic memory. *Genes Dev* **16** 6-21.

Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR *et al.* 2007 Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* **447** 799-816.

Bondioli KR, Westhusin ME & Looney CR 1990 Production of identical bovine offspring by nuclear transfer. *Theriogenology* **33** 165-174.

Botuyan MV, Lee J, Ward IM, Kim JE, Thompson JR, Chen J & Mer G 2006 Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* **127** 1361-1373.

Braude P, Bolton V & Moore S 1988 Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332** 459-461.

Caiafa P, Reale A, Allegra P, Rispoli M, D'Erme M & Strom R 1991a Histones and DNA methylation in mammalian chromatin. Differential inhibition by histone H1. *Biochim Biophys Acta* **1090** 38-42.

Caiafa P, Reale A, D'Erme M, Allegra P, Santoro R & Strom R 1991b Histones and DNA methylation in mammalian chromatin. II. Presence of non-inhibitory tightly-bound histones. *Biochim Biophys Acta* **1129** 43-48.

Campbell KH, McWhir J, Ritchie WA & Wilmut I 1996 Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380** 64-66.

Cao R, Wang H, He J, Erdjument-Bromage H, Tempst P & Zhang Y 2008 Role of hPHF1 in H3K27 methylation and Hox gene silencing. *Mol Cell Biol* **28** 1862-1872.

Cao R & Zhang Y 2004 SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell* **15** 57-67.

Carlson LL, Page AW & Bestor TH 1992 Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. *Genes Dev* **6** 2536-2541.

Chow JC & Brown CJ 2003 Forming facultative heterochromatin: silencing of an X chromosome in mammalian females. *Cell Mol Life Sci* **60** 2586-2603.

Chung YG, Ratnam S, Chaillet JR & Latham KE 2003 Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol Reprod* **69** 146-153.

Cuthill S 1994 Cellular epigenetics and the origin of cancer. *Bioessays* **16** 393-394.

Das PM & Singal R 2004 DNA methylation and cancer. *J Clin Oncol* **22** 4632-4642.

de la Cruz CC, Fang J, Plath K, Worringer KA, Nusinow DA, Zhang Y & Panning B 2005 Developmental regulation of Suz12 localization. *Chromosoma* **114** 183-192.

de la Cruz CC, Kirmizis A, Simon MD, Isono K, Koseki H & Panning B 2007 The polycomb group protein SUZ12 regulates histone H3 lysine 9 methylation and HP1 alpha distribution. *Chromosome Res* **15** 299-314.

De La Fuente R, Baumann C, Fan T, Schmidtmann A, Dobrinski I & Muegge K 2006 Lsh is required for meiotic chromosome synapsis and retrotransposon silencing in female germ cells. *Nat Cell Biol* **8** 1448-1454.

Dennis K, Fan T, Geiman T, Yan Q & Muegge K 2001 Lsh, a member of the SNF2 family, is required for genome-wide methylation. *Genes Dev* **15** 2940-2944.

Dodge JE, Kang YK, Beppu H, Lei H & Li E 2004 Histone H3-K9 methyltransferase ESET is essential for early development. *Mol Cell Biol* **24** 2478-2486.

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS & Schultz RM 2000 Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod* **62** 1526-1535.

Drahovsky D & Morris NR 1972 Mammalian DNA methylase: binding and methylation site selection. *Hoppe Seylers Z Physiol Chem* **353** 700-701.

Ehrlich M 2003 Expression of various genes is controlled by DNA methylation during mammalian development. *J Cell Biochem* **88** 899-910.

Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA & Gehrke C 1982 Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res* **10** 2709-2721.

Eichenlaub-Ritter U & Peschke M 2002 Expression in *in vivo* and *in vitro* growing and maturing oocytes: focus on regulation of expression at the translational level. *Hum Reprod Update* **8** 21-41.

El-Maarri O, Seoud M, Coullin P, Herbiniaux U, Oldenburg J, Rouleau G & Slim R 2003 Maternal alleles acquiring paternal methylation patterns in biparental complete hydatidiform moles. *Hum Mol Genet* **12** 1405-1413.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K & Tuschl T 2001a Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411** 494-498.

Elbashir SM, Lendeckel W & Tuschl T 2001b RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15** 188-200.

Elsden RP, Hasler JF & Seidel GE, Jr. 1976 Non-surgical recovery of bovine eggs. *Theriogenology* **6** 523-532.

Erhardt S, Su IH, Schneider R, Barton S, Bannister AJ, Perez-Burgos L, Jenuwein T, Kouzarides T, Tarakhovsky A & Surani MA 2003 Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development. *Development* **130** 4235-4248.

Esteve PO, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, Carey MF & Pradhan S 2006 Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev* **20** 3089-3103.

Fan T, Hagan JP, Kozlov SV, Stewart CL & Muegge K 2005 Lsh controls silencing of the imprinted Cdkn1c gene. *Development* **132** 635-644.

Fatemi M, Hermann A, Gowher H & Jeltsch A 2002 Dnmt3a and Dnmt1 functionally cooperate during *de novo* methylation of DNA. *Eur J Biochem* **269** 4981-4984.

Feil R & Khosla S 1999 Genomic imprinting in mammals: an interplay between chromatin and DNA methylation? *Trends Genet* **15** 431-435.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE & Mello CC 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391** 806-811.

Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ & Eckert JJ 2004 The embryo and its future. *Biol Reprod* **71** 1046-1054.

Fulka H, Mrazek M, Tepla O & Fulka J, Jr. 2004 DNA methylation pattern in human zygotes and developing embryos. *Reproduction* **128** 703-708.

Gama-Sosa MA, Midgett RM, Slagel VA, Githens S, Kuo KC, Gehrke CW & Ehrlich M 1983 Tissue-specific differences in DNA methylation in various mammals. *Biochim Biophys Acta* **740** 212-219.

Gardiner-Garden M & Frommer M 1987 CpG islands in vertebrate genomes. *J Mol Biol* **196** 261-282.

Geiman TM & Robertson KD 2002 Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? *J Cell Biochem* **87** 117-125.

Geiman TM, Tessarollo L, Anver MR, Kopp JB, Ward JM & Muegge K 2001 Lsh, a SNF2 family member, is required for normal murine development. *Biochim Biophys Acta* **1526** 211-220.

Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A & Peelman LJ 2005 Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Dev Biol* **5** 27.

Grace KS & Sinclair KD 2009 Assisted reproductive technology, epigenetics, and long-term health: a developmental time bomb still ticking. *Semin Reprod Med* **27** 409-416.

Gray KR, Bondioli KR & Betts CL 1991 The commercial application of embryo splitting in beef cattle. *Theriogenology* **35** 37-44.

Halliday J, Oke K, Breheny S, Algar E & D JA 2004 Beckwith-Wiedemann syndrome and IVF: a case-control study. *Am J Hum Genet* **75** 526-528.

Hashimoto H, Vertino PM & Cheng X 2010 Molecular coupling of DNA methylation and histone methylation. *Epigenomics* **2** 657-669.

Heape W 1891 Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. *Proc R Soc* **48** 457-458.

Helwani MN, Seoud M, Zahed L, Zaatari G, Khalil A & Slim R 1999 A familial case of recurrent hydatidiform molar pregnancies with biparental genomic contribution. *Hum Genet* **105** 112-115.

Hermann A, Gowher H & Jeltsch A 2004a Biochemistry and biology of mammalian DNA methyltransferases. *Cell Mol Life Sci* **61** 2571-2587.

Hermann A, Goyal R & Jeltsch A 2004b The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. *J Biol Chem* **279** 48350-48359.

Holmquist GP & Ashley T 2006 Chromosome organization and chromatin modification: influence on genome function and evolution. *Cytogenet Genome Res* **114** 96-125.

Huang J, Fan T, Yan Q, Zhu H, Fox S, Issaq HJ, Best L, Gangi L, Munroe D & Muegge K 2004 Lsh, an epigenetic guardian of repetitive elements. *Nucleic Acids Res* **32** 5019-5028.

Huen MS, Sy SM, van Deursen JM & Chen J 2008 Direct interaction between SET8 and proliferating cell nuclear antigen couples H4-K20 methylation with DNA replication. *J Biol Chem* **283** 11073-11077.

Huntriss J, Hinkins M, Oliver B, Harris SE, Beazley JC, Rutherford AJ, Gosden RG, Lanzendorf SE & Picton HM 2004 Expression of mRNAs for DNA methyltransferases and methyl-CpG-binding proteins in the human female germ line, preimplantation embryos, and embryonic stem cells. *Mol Reprod Dev* **67** 323-336.

Huntriss J & Picton HM 2008 Epigenetic consequences of assisted reproduction and infertility on the human preimplantation embryo. *Hum Fertil (Camb)* **11** 85-94.

Iida T, Nakahori Y, Tsutsumi O, Taketani Y & Nakagome Y 1994 The CpG island of the FMR-1 gene is methylated differently among embryonic tissues: implication for prenatal diagnosis. *Hum Reprod* **9** 1471-1473.

Iritani A & Niwa K 1977 Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *J Reprod Fertil* **50** 119-121.

Izzo A & Schneider R 2010 Chatting histone modifications in mammals. *Brief Funct Genomics* **9** 429-443.

Jia D, Jurkowska RZ, Zhang X, Jeltsch A & Cheng X 2007 Structure of Dnmt3a bound to Dnmt3L suggests a model for *de novo* DNA methylation. *Nature* **449** 248-251.

Johnson LA, G. CD & E. PC 1994 Recent advances of sex preselection of cattle: flow cytometric sorting of X- and Y-chromosome bearing sperm based on DNA to produce progeny. *Theriogenology* **41** 51-56.

Jones PA & Laird PW 1999 Cancer epigenetics comes of age. *Nat Genet* **21** 163-167.

Jones PA & Takai D 2001 The role of DNA methylation in mammalian epigenetics. *Science* **293** 1068-1070.

Kafri T, Ariel M, Brandeis M, Shemer R, Urven L, McCarrey J, Cedar H & Razin A 1992 Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev* **6** 705-714.

Kaslow DC & Migeon BR 1987 DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: evidence for multistep maintenance of mammalian X dosage compensation. *Proc Natl Acad Sci U S A* **84** 6210-6214.

Kautiainen TL & Jones PA 1985 DNA methylation in mammalian nuclei. *Biochemistry* **24** 5575-5581.

Kelly TL & Trasler JM 2004 Reproductive epigenetics. *Clin Genet* **65** 247-260.

Khosla S, Dean W, Brown D, Reik W & Feil R 2001a Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* **64** 918-926.

Khosla S, Dean W, Reik W & Feil R 2001b Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. *Hum Reprod Update* **7** 419-427.

Kim H, Heo K, Kim JH, Kim K, Choi J & An W 2009 Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. *J Biol Chem* **284** 19867-19877.

Klose RJ & Bird AP 2006 Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31** 89-97.

Ko YG, Nishino K, Hattori N, Arai Y, Tanaka S & Shiota K 2005 Stage-by-stage change in DNA methylation status of Dnmt1 locus during mouse early development. *J Biol Chem* **280** 9627-9634.

Koch CM, Andrews RM, Flicek P, Dillon SC, Karaoz U, Clelland GK, Wilcox S, Beare DM, Fowler JC, Couttet P, James KD, Lefebvre GC, Bruce AW, Dovey OM, Ellis PD, Dhami P, Langford CF, Weng Z, Birney E, Carter NP, Vetrie D & Dunham I 2007 The landscape of histone modifications across 1% of the human genome in five human cell lines. *Genome Res* **17** 691-707.

Kopecny V 1989 High-resolution autoradiographic studies of comparative nucleologenesis and genome reactivation during early embryogenesis in pig, man and cattle. *Reprod Nutr Dev* **29** 589-600.

Kopecny V, Flechon JE, Camous S & Fulka J, Jr. 1989 Nucleologenesis and the onset of transcription in the eight-cell bovine embryo: fine-structural autoradiographic study. *Mol Reprod Dev* **1** 79-90.

Kourmouli N, Jeppesen P, Mahadevhaiah S, Burgoyne P, Wu R, Gilbert DM, Bongiorni S, Prantera G, Fanti L, Pimpinelli S, Shi W, Fundele R & Singh PB 2004 Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. *J Cell Sci* **117** 2491-2501.

Laprise SL 2009 Implications of epigenetics and genomic imprinting in assisted reproductive technologies. *Mol Reprod Dev* **76** 1006-1018.

Law JA & Jacobsen SE 2010 Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* **11** 204-220.

Lawrence LT & Moley KH 2008 Epigenetics and assisted reproductive technologies: human imprinting syndromes. *Semin Reprod Med* **26** 143-152.

Le Bouc Y, Rossignol S, Azzi S, Steunou V, Netchine I & Gicquel C 2010 Epigenetics, genomic imprinting and assisted reproductive technology. *Ann Endocrinol (Paris)* **71** 237-238.

Li E, Beard C, Forster AC, Bestor TH & Jaenisch R 1993 DNA methylation, genomic imprinting, and mammalian development. *Cold Spring Harb Symp Quant Biol* **58** 297-305.

Li E, Bestor TH & Jaenisch R 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69** 915-926.

Lippman Z, Gendrel AV, Black M, Vaughn MW, Dedhia N, McCombie WR, Lavine K, Mittal V, May B, Kasschau KD, Carrington JC, Doerge RW, Colot V & Martienssen R 2004 Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430** 471-476.

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T & Boland MP 2003 Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod Domest Anim* **38** 259-267.

Luger K, Rechsteiner TJ, Flaus AJ, Waye MM & Richmond TJ 1997 Characterization of nucleosome core particles containing histone proteins made in bacteria. *J Mol Biol* **272** 301-311.

Maher ER, Afnan M & Barratt CL 2003 Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs? *Hum Reprod* **18** 2508-2511.

Mann MR & Bartolomei MS 2002 Epigenetic reprogramming in the mammalian embryo: struggle of the clones. *Genome Biol* **3** reviews1003.1-1003.4.

Mapletoft RJ & Hasler JF 2005 Assisted reproductive technologies in cattle: a review. *Rev Sci Tech* **24** 393-403.

Market-Velker BA, Fernandes AD & Mann MR 2010a Side-by-side comparison of five commercial media systems in a mouse model: suboptimal *in vitro* culture interferes with imprint maintenance. *Biol Reprod* **83** 938-950.

Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC & Mann MR 2010b Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet* **19** 36-51.

Memili E & First NL 2000 Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8** 87-96.

Menezo Y, Jr., Viville S & Veiga A 2006 Epigenetics and assisted reproductive technology. *Fertil Steril* **85** 269; author reply 269-270.

Mertineit C, Yoder JA, Taketo T, Laird DW, Trasler JM & Bestor TH 1998 Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development* **125** 889-897.

Monk M, Boubelik M & Lehnert S 1987 Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99** 371-382.

Morillon A, Karabetsou N, Nair A & Mellor J 2005 Dynamic lysine methylation on histone H3 defines the regulatory phase of gene transcription. *Mol Cell* **18** 723-734.

Muegge K 2005 Lsh, a guardian of heterochromatin at repeat elements. *Biochem Cell Biol* **83** 548-554.

Myant K & Stancheva I 2008 LSH cooperates with DNA methyltransferases to repress transcription. *Mol Cell Biol* **28** 215-226.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN & Bird A 1998 Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393** 386-389.

Napoli C, Lemieux C & Jorgensen R 1990 Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* **2** 279-289.

Newcomb R, Christie WB & Rowson LE 1978 Birth of calves after *in vivo* fertilisation of oocytes removed from follicles and matured *in vitro*. *Vet Rec* **102** 461-462.

Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P & Losson R 2001a Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol Cell* **7** 729-739.

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE & Kouzarides T 2001b Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412** 561-565.

Niemitz EL & Feinberg AP 2004 Epigenetics and assisted reproductive technology: a call for investigation. *Am J Hum Genet* **74** 599-609.

Okano M, Bell DW, Haber DA & Li E 1999 DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99** 247-257.

Okano M, Xie S & Li E 1998 Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* **19** 219-220.

Ooi SK, Qiu C, Bernstein E, Li K, Jia D, Yang Z, Erdjument-Bromage H, Tempst P, Lin SP, Allis CD, Cheng X & Bestor TH 2007 DNMT3L connects unmethylated lysine 4 of histone H3 to *de novo* methylation of DNA. *Nature* **448** 714-717.

Otani J, Nankumo T, Arita K, Inamoto S, Ariyoshi M & Shirakawa M 2009 Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. *EMBO Rep* **10** 1235-1241.

Owen CM & Segars JH, Jr. 2009 Imprinting disorders and assisted reproductive technology. *Semin Reprod Med* **27** 417-428.

Paoloni-Giacobino A 2007 Epigenetics in reproductive medicine. *Pediatr Res* **61** 51R-57R.

Pasini D, Bracken AP, Hansen JB, Capillo M & Helin K 2007 The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol Cell Biol* **27** 3769-3779.

Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E & Helin K 2004 Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J* **23** 4061-4071.

Patnaik D, Chin HG, Esteve PO, Benner J, Jacobsen SE & Pradhan S 2004 Substrate specificity and kinetic mechanism of mammalian G9a histone H3 methyltransferase. *J Biol Chem* **279** 53248-53258.

Pesavento JJ, Yang H, Kelleher NL & Mizzen CA 2008 Certain and progressive methylation of histone H4 at lysine 20 during the cell cycle. *Mol Cell Biol* **28** 468-486.

Peters AH, Mermoud JE, O'Carroll D, Pagani M, Schweizer D, Brockdorff N & Jenuwein T 2002 Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. *Nat Genet* **30** 77-80.

Picton H, Briggs D & Gosden R 1998 The molecular basis of oocyte growth and development. *Mol Cell Endocrinol* **145** 27-37.

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B & Zhang Y 2003 Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300** 131-135.

Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH & First NL 1987 Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol Reprod* **37** 859-866.

Puschendorf M, Terranova R, Boutsma E, Mao X, Isono K, Brykczynska U, Kolb C, Otte AP, Koseki H, Orkin SH, van Lohuizen M & Peters AH 2008 PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. *Nat Genet* **40** 411-420.

Ratnam S, Mertineit C, Ding F, Howell CY, Clarke HJ, Bestor TH, Chaillet JR & Trasler JM 2002 Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. *Dev Biol* **245** 304-314.

Reik W & Dean W 2001 DNA methylation and mammalian epigenetics. *Electrophoresis* **22** 2838-2843.

Reik W & Walter J 2001 Genomic imprinting: parental influence on the genome. *Nat Rev Genet* **2** 21-32.

Rein T, Kobayashi T, Malott M, Leffak M & DePamphilis ML 1999 DNA methylation at mammalian replication origins. *J Biol Chem* **274** 25792-25800.

Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, Shinkai Y & Allis CD 2003 Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* **12** 1591-1598.

Robertson AK, Geiman TM, Sankpal UT, Hager GL & Robertson KD 2004 Effects of chromatin structure on the enzymatic and DNA binding functions of DNA methyltransferases DNMT1 and Dnmt3a in vitro. *Biochem Biophys Res Commun* **322** 110-118.

Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B & First NL 1987 Nuclear transplantation in bovine embryos. *J Anim Sci* **64** 642-647.

Rodriguez KF & Farin CE 2004 Gene transcription and regulation of oocyte maturation. *Reprod Fertil Dev* **16** 55-67.

Ross PJ, Ragina NP, Rodriguez RM, Iager AE, Siripattarapravat K, Lopez-Corrales N & Cibelli JB 2008 Polycomb gene expression and histone H3 lysine 27 trimethylation changes during bovine preimplantation development. *Reproduction* **136** 777-785.

Rousseaux S, Faure AK, Caron C, Lestrat C, Govin J, Hennebicq S, Sele B & Khochbin S 2004 French [Organizing the sperm nucleus]. *Gynecol Obstet Fertil* **32** 785-791.

Sado T, Fenner MH, Tan SS, Tam P, Shioda T & Li E 2000 X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. *Dev Biol* **225** 294-303.

Santenard A, Ziegler-Birling C, Koch M, Tora L, Bannister AJ & Torres-Padilla ME 2010 Heterochromatin formation in the mouse embryo requires critical residues of the histone variant H3.3. *Nat Cell Biol* **12** 853-862.

Santos F, Hendrich B, Reik W & Dean W 2002 Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol* **241** 172-182.

Santos F, Peters AH, Otte AP, Reik W & Dean W 2005 Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Dev Biol* **280** 225-236.

Sarmento OF, Digilio LC, Wang Y, Perlin J, Herr JC, Allis CD & Coonrod SA 2004 Dynamic alterations of specific histone modifications during early murine development. *J Cell Sci* **117** 4449-4459.

Sasaki H, Allen ND & Surani MA 1993 DNA methylation and genomic imprinting in mammals. *EXS* **64** 469-486.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D & Jenuwein T 2004 A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* **18** 1251-1262.

Schultz RM & Worrad DM 1995 Role of chromatin structure in zygotic gene activation in the mammalian embryo. *Semin Cell Biol* **6** 201-208.

Shiraishi M, Oates AJ & Sekiya T 2002 An overview of the analysis of DNA methylation in mammalian genomes. *Biol Chem* **383** 893-906.

Shrivastava N & Srivastava A 2008 RNA interference: an emerging generation of biologicals. *Biotechnol J* **3** 339-353.

Sinclair KD 2008 Assisted reproductive technologies and pregnancy outcomes: mechanistic insights from animal studies. *Semin Reprod Med* **26** 153-161.

Sirinupong N, Brunzelle J, Doko E & Yang Z 2011 Structural insights into the autoinhibition and posttranslational activation of histone methyltransferase SmyD3. *J Mol Biol* **406** 149-159.

Smith C 1988a Applications of embryo transfer in animal breeding. *Theriogenology* **29** 203-212.

Smith C 1988b Genetic improvement of livestock using nucleus breeding units. *World Animal Review* **65** 2-10.

Steptoe PC & Edwards RG 1978 Birth after the reimplantation of a human embryo. *Lancet* **2** 366.

Summers MC & Biggers JD 2003 Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum Reprod Update* **9** 557-582.

Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, Fukuda M, Takeda N, Niida H, Kato H & Shinkai Y 2002 G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* **16** 1779-1791.

Takai D & Jones PA 2003 The CpG island searcher: a new WWW resource. *In Silico Biol* **3** 235-240.

Teitell M & Richardson B 2003 DNA methylation in the immune system. *Clin Immunol* **109** 2-5.

Thibier M 2003 More than half a million bovine embryos transferred in 2002: a report of the International Embryo Transfer Society Data Retrieval Committee. *IETS Newsletter* **21** 12-19.

Thompson SL, Konfortova G, Gregory RI, Reik W, Dean W & Feil R 2001 Environmental effects on genomic imprinting in mammals. *Toxicol Lett* **120** 143-150.

Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB & Schuebel KE 2004 Mammalian DNA methyltransferase 1: inspiration for new directions. *Cell Cycle* **3** 1024-1026.

Trasler JM, Alcivar AA, Hake LE, Bestor T & Hecht NB 1992 DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells. *Nucleic Acids Res* **20** 2541-2545.

Turek-Plewa J & Jagodzinski PP 2005 The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell Mol Biol Lett* **10** 631-647.

Tycko B & Morison IM 2002 Physiological functions of imprinted genes. *J Cell Physiol* **192** 245-258.

Vakoc CR, Sachdeva MM, Wang H & Blobel GA 2006 Profile of histone lysine methylation across transcribed mammalian chromatin. *Mol Cell Biol* **26** 9185-9195.

Wang J, Mager J, Chen Y, Schneider E, Cross JC, Nagy A & Magnuson T 2001 Imprinted X inactivation maintained by a mouse Polycomb group gene. *Nat Genet* **28** 371-375.

Wang KY & James Shen CK 2004 DNA methyltransferase Dnmt1 and mismatch repair. *Oncogene* **23** 7898-7902.

Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, Davis RW & Zernicka-Goetz M 2004 A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev Cell* **6** 133-144.

Wang SZ, Luo XG, Shen J, Zou JN, Lu YH & Xi T 2008 Knockdown of SMYD3 by RNA interference inhibits cervical carcinoma cell growth and invasion in vitro. *BMB Rep* **41** 294-299.

Watt F & Molloy PL 1988 Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev* **2** 1136-1143.

Willadsen SM 1986 Nuclear transplantation in sheep embryos. *Nature* **320** 63-65.

Willett EL, Black WG, Casida LE, Stone WH & Buckner PJ 1951 Successful transplantation of a fertilized bovine ovum. *Science* **113** 247.

Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KH 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385** 810-813.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM & Reijo Pera RA 2010 Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* **28** 1115-1121.

Woodcock DM, Simmons DL, Crowther PJ, Cooper IA, Trainor KJ & Morley AA 1986 Delayed DNA methylation is an integral feature of DNA replication in mammalian cells. *Exp Cell Res* **166** 103-112.

Xi S, Zhu H, Xu H, Schmidtmann A, Geiman TM & Muegge K 2007 Lsh controls Hox gene silencing during development. *Proc Natl Acad Sci U S A* **104** 14366-14371.

Xie S, Wang Z, Okano M, Nogami M, Li Y, He WW, Okumura K & Li E 1999 Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* **236** 87-95.

Yan Q, Cho E, Lockett S & Muegge K 2003a Association of Lsh, a regulator of DNA methylation, with pericentromeric heterochromatin is dependent on intact heterochromatin. *Mol Cell Biol* **23** 8416-8428.

Yan Q, Huang J, Fan T, Zhu H & Muegge K 2003b Lsh, a modulator of CpG methylation, is crucial for normal histone methylation. *EMBO J* **22** 5154-5162.

Yang H, Pesavento JJ, Starnes TW, Cryderman DE, Wallrath LL, Kelleher NL & Mizzen CA 2008 Preferential dimethylation of histone H4 lysine 20 by Suv4-20. *J Biol Chem* **283** 12085-12092.

Yoon HG, Chan DW, Reynolds AB, Qin J & Wong J 2003 N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. *Mol Cell* **12** 723-734.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I & Sinclair KD 2001 Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* **27** 153-154.

Young LE, Sinclair KD & Wilmut I 1998 Large offspring syndrome in cattle and sheep. *Rev Reprod* **3** 155-163.

Zeng F & Schultz RM 2005 RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. *Dev Biol* **283** 40-57.

Zhang Y, Cao R, Wang L & Jones RS 2004 Mechanism of Polycomb group gene silencing. *Cold Spring Harb Symp Quant Biol* **69** 309-317.

Zhu H, Geiman TM, Xi S, Jiang Q, Schmidtmann A, Chen T, Li E & Muegge K 2006 Lsh is involved in de novo methylation of DNA. *EMBO J* **25** 335-345.
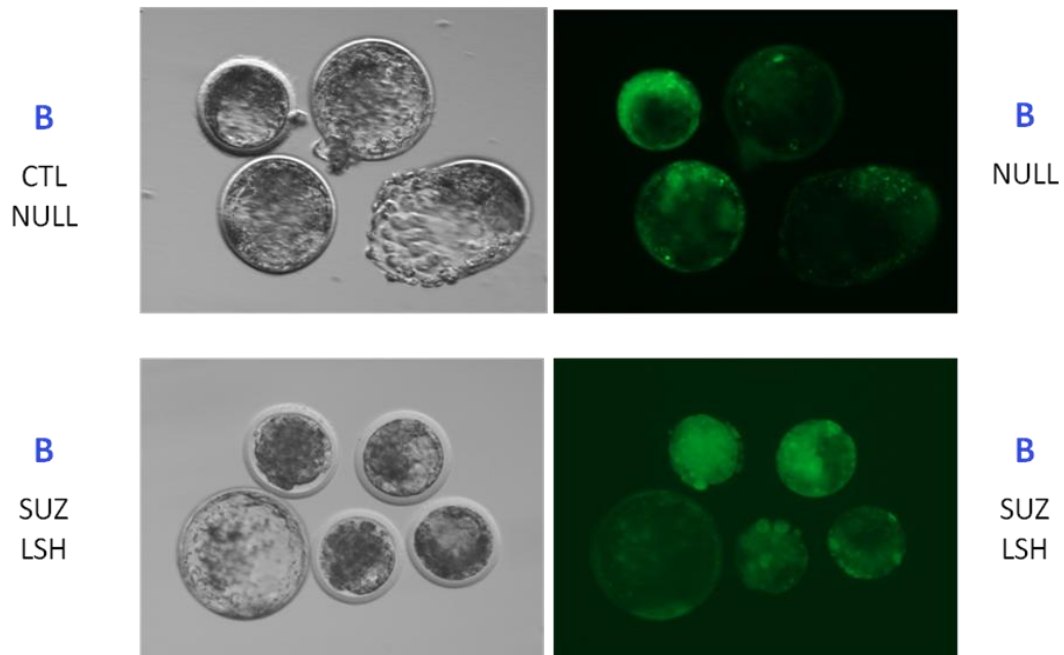
APPENDIX
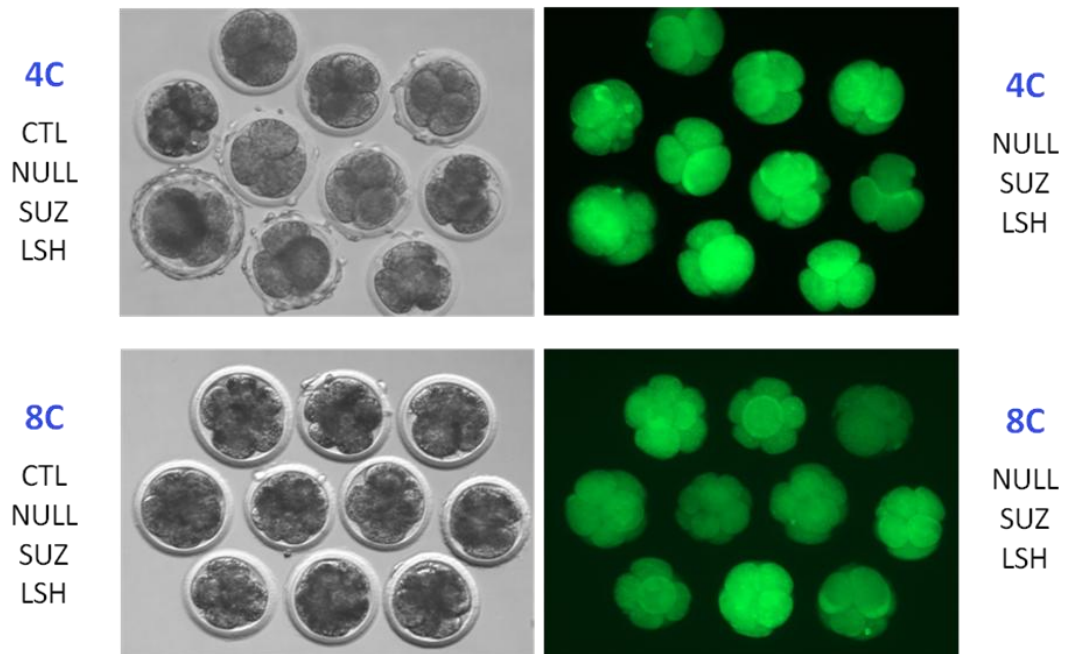
Figure A1 Pre-EGA representative morphological similarities exhibited by the SUZ and LSH embryos, as compared to the CTL and NULL embryos, at the 4-cell (4C) and 8-cell (8C) stages.
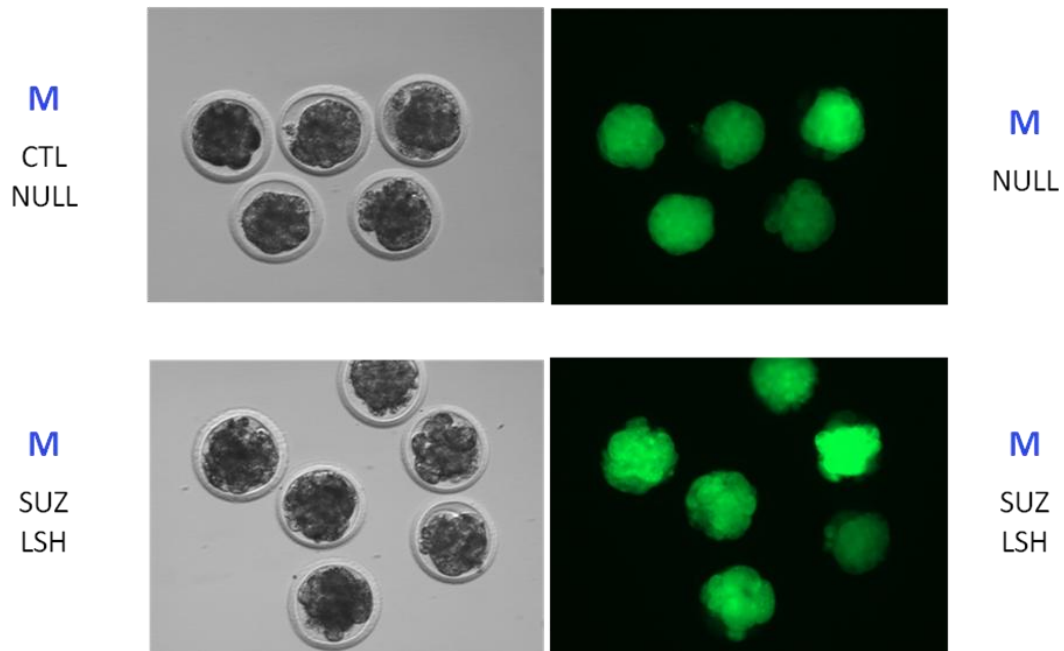
Figure A2 Post-EGA representative morphological differences exhibited by the SUZ and LSH embryos, as compared to the CTL and NULL embryos, at the morula (M) stage.

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.

VITA

Name: Gayle Linger Williamson

Address: Texas A&M Reproductive Sciences Lab
                   Department of Veterinary Physiology & Pharmacology
                   College of Veterinary Medicine
                   Mail Stop 4466 TAMU
                   College Station, TX 77843

Email Address: gdlwilliamson@gmail.com

Education: B.S., Genetics & Animal Science, Texas A&M University, 2006
                   M.S., Genetics, Texas A&M University, 2011

Publications: **Golding MC, Williamson GL, Stroud TK, Westhusin ME & Long CR** 2011 Examination of DNA methyltransferase expression in cloned embryos reveals an essential role for Dnmt1 in bovine development. *Mol Reprod Dev* **78** 306-317.