

ABSTRACT

Title of Document: EVALUATION OF LEF1 TRANSCRIPTION
FACTOR IN MAMMALIAN
PREIMPLANTATION EMBRYOS

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The first lineage differentiation of cells during pre-implantation embryo development is critical for continued embryonic growth. Though several transcription factors (TFs) have been identified that are involved in this transition, a specific mechanism of regulation has yet to be determined. Previous studies in our laboratory have shown the TF Lef1 is involved in differentiation of mouse embryonic stem cells into trophoblastic stem cells through the Wnt signaling pathway. Lef1 is known to be involved in lineage determination of adult skin stem cells. As we observed that two isoforms of Lef1 were expressed at different stages of pre-implantation development, we hypothesized that Lef1 was interacting with the other well-established lineage differentiation TFs, Nanog, Oct4, and Cdx2, in these early embryos. At the blastocyst stage, no significant changes in mRNA expression were seen when siRNAs, specifically designed to knockdown Lef1 expression, were injected in early embryos; however, knockdown of Lef1 did not interfere with blastocyst formation.

EVALUATION OF LEF1 TRANSCRIPTION FACTOR IN MAMMALIAN
PREIMPLANTION EMBRYOS

By

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Table of Contents

Acknowledgements.....	ii
Table of Contents.....	iii
List of Tables.....	iv
List of Figures.....	v
List of Abbreviations.....	vi
Chapter 1: Literature Review.....	1
Early Embryo Development.....	1
Transcription Factors.....	4
Oct4.....	7
Nanog.....	9
Cdx2.....	12
Wnt Pathway.....	13
Lef1.....	15
Knock down of Lef1 using siRNA.....	16
Chapter 2: Methods.....	20
Embryo culture.....	20
Cell culture.....	21
Murine and bovine siRNA sequences.....	21
Transfection of cells with Lef1 siRNA.....	22
Microinjection of Lef1 siRNA into embryos.....	23
RT-PCR and qRT-PCR.....	25
Statistical Analysis.....	26
Chapter 3: Evaluation of Lef1 in mammalian pre-implantation embryos.....	28
Introduction.....	28
Rationale.....	29
Results.....	30
Expression of early differentiation transcription factors in bovine embryos.....	30
Validation of Lef1 siRNA in bovine fetal fibroblasts.....	31
Microinjection of siRNA into bovine embryos.....	33
Validation of Lef1 siRNA in mouse ES cells.....	34
Microinjection of siRNA into mouse embryos.....	35
Chapter 4: Discussion.....	38
Chapter 5: Conclusions.....	44
Summary.....	44
Future Studies.....	44
Appendices.....	69
Bibliography.....	81

List of Tables

Table 1: Expression of early differentiation transcription factors at different stages in bovine embryos.....	46
Table 2: Bovine embryo cleavage rates after injection of Lef1 and missense siRNA	52
Table 3: Murine embryo cleavage rates after injection of siRNA.....	60
Appendix Table 1: Numbers of bovine embryos collected to determine gene expression at different early development stages	69
Appendix Table 2: Numbers of bovine 4-8 cell embryos collected after siRNA injection.....	70
Appendix Table 3: Numbers of murine 4-8 cell embryos collected after siRNA injection.....	71
Appendix Table 4: Numbers of murine blastocysts collected after siRNA injection.	72
Appendix Table 5: Bovine RT-PCR primer sequences	73
Appendix Table 6: Bovine qRT-PCR primer sequences	74
Appendix Table 7: Mouse qRT-PCR primer sequences.....	75

List of Figures

Figure 1: Pre-implantation mammalian embryo development	3
Figure 2: Known transcription factors involved in early embryo development	6
Figure 3: Expression of Lef1 in early bovine embryos and cell lines	47
Figure 4: Validation of bovine Lef1 siRNA in bovine fetal fibroblasts	48
Figure 5: Bovine embryos after injection of siRNA-TRD.....	50
Figure 6: Bovine embryos after siRNA injections.....	53
Figure 7: Bovine 4- to 8- cell staged bovine embryos after injection with Lef1 siRNA	55
Figure 8: Summary of qRT-PCR data in the bovine.....	57
Figure 9: Validation of murine specific Lef1 siRNA in mouse ES cells.....	58
Figure 10: Mouse 4-8 cell embryos after Lef1 siRNA injection	61
Figure 11: Murine blastocyst morphology after siRNA injection	63
Figure 12: mRNA expression of key transcription factors in mouse blastocysts after siRNA injection	64
Figure 13: Expression of Tcf/Lef family members in mouse blastocysts after Lef1 siRNA injection	66
Figure 14: Summary of qRT-PCR data in the mouse.....	68
Appendix Figure 1: Bovine Lef1 gene.....	76
Appendix Figure 2: Alignment of bovine, human, and mouse Lef1 genes	77
Appendix Figure 3: Injection of siRNA into bovine embryo	79
Appendix Figure 4: Direct incubation pilot study	80

List of Abbreviations

TF(s)	Transcription Factor(s)
ICM	Inner Cell Mass
TE	Trophectoderm
ES cells	Embryonic stem cells
TS cells	Trophoblast stem cells
Cdx2	Caudal-type homeobox transcription factor 2
Lef1	Lymphoid enhancer-binding factor 1
Tcf1, Tcf3, Tcf4	T-cell (Transcription) factor 1, 3, 4
LIF	Leukemia Inhibitory Factor
siRNA	small interfering RNA
RT-PCR	Reverse Transcription Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
TRD	Neutral Conjugated Texas Red Dextran

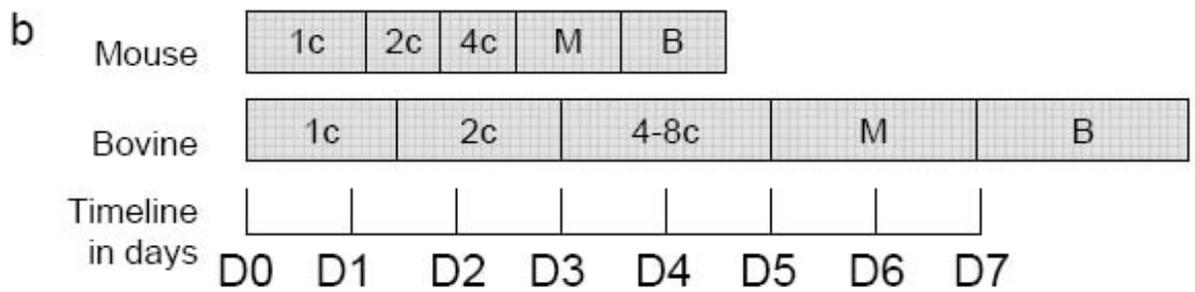
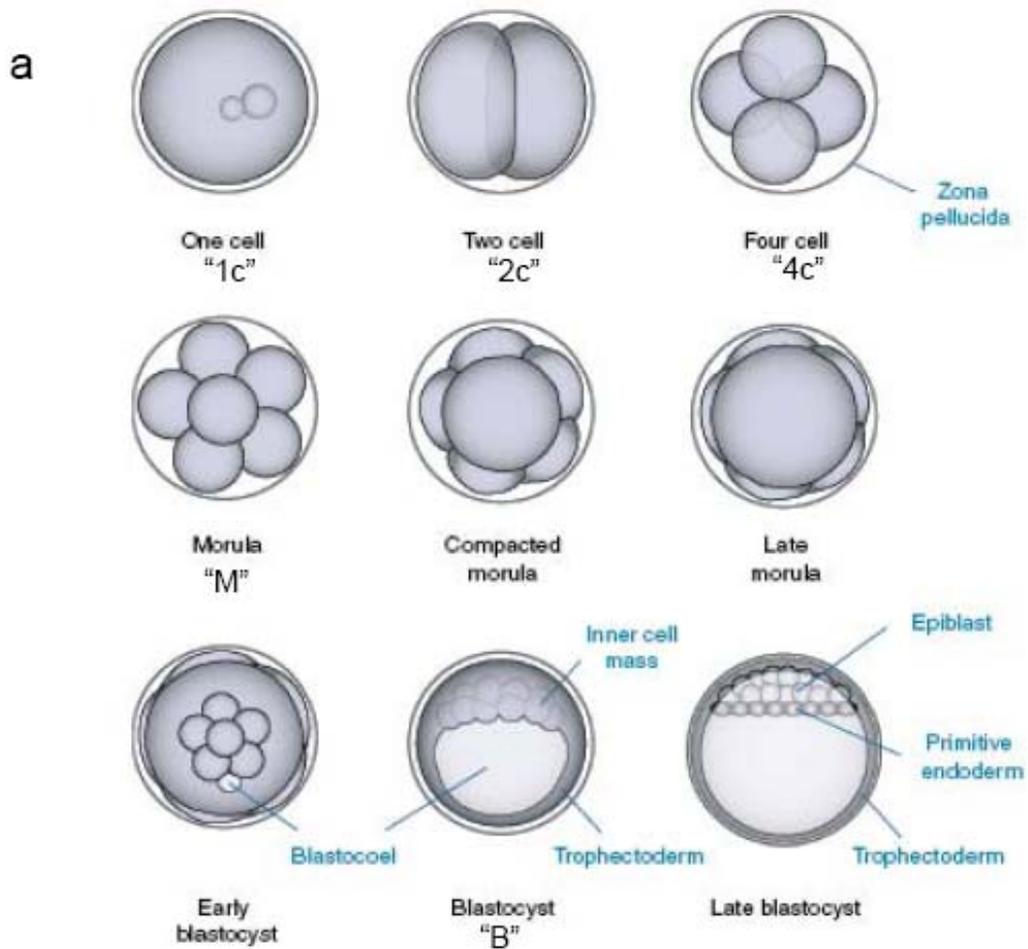
Chapter 1: Literature Review

Early Embryo Development

Early in mammalian development, the cells of the embryo are able to become any one of the many different cell types found in the body. However, just before blastocyst formation, in a process known as compaction, the cells of the embryo compress causing the blastomeres to flatten and form tight junctions between the cells. It is at this stage when the cell population begins to differentiate into two different cell types signifying the formation of a blastocyst (Figure 1). The single layer of multipotent cells surrounding the perimeter of the embryo have an epithelial-like morphology and are referred to as trophoblast (TE) cells (Ralston and Rossant, 2005). These trophoblast cells later become the fetal portion of the placenta. Fluid enters into the embryo forming an open cavity, which is referred to as the blastocoel. The other cell type found at this time in the early embryo is a pluripotent collection of inner cells, known as the inner cell mass (ICM); these cells are the predecessors for all cell types of the embryo proper. At the late blastocyst stage a second differentiation occurs in which the ICM further separates into the epiblast and primitive endoderm. Multipotent primitive endoderm cells then migrate to cover the blastocoelic surface. These cells later become the visceral and parietal yolk sac, structures that provide nutrients and patterning information for the developing embryo. The other emerging cell type at this time is the epiblast. Epiblastic cells can be found between the TE and the primitive endoderm; and it is these cells that form the three germ layers of the embryo; endoderm, mesoderm, and ectoderm. It is

imperative that these two differentiation processes are seamless as both cell lineages are required for normal embryonic and fetal development.

Figure 1: Pre-implantation mammalian embryo development



(Ralston and Rossant, 2005)

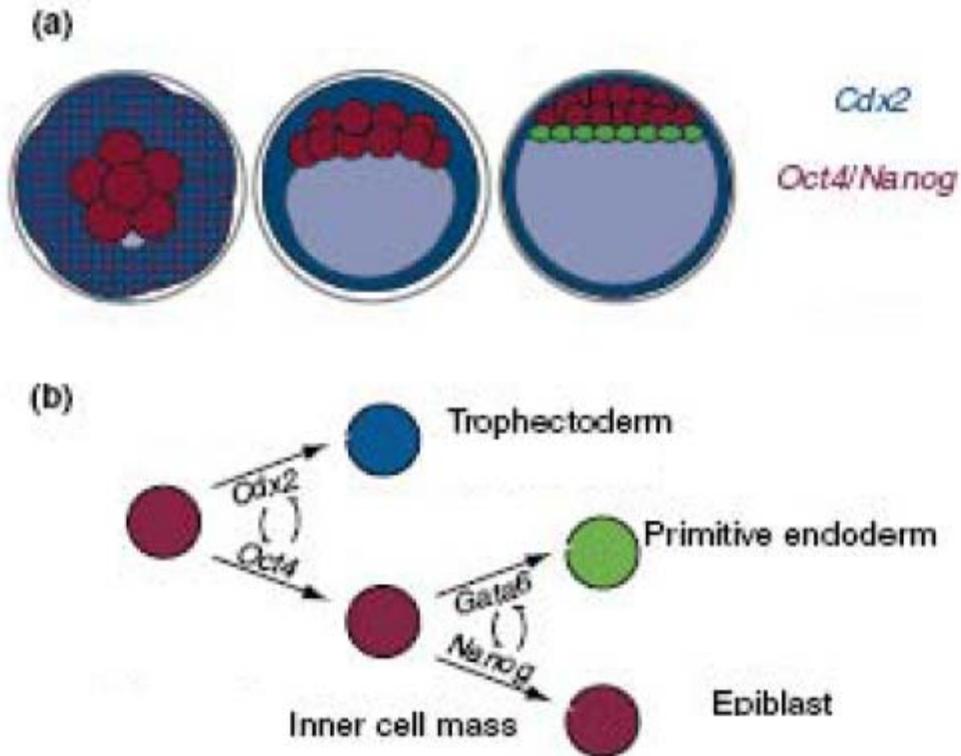
Once these cell types are established and the blastocyst hatches from the protective zona pellucida, implantation occurs in mammals. The trophoblastic cells mature to form the placenta; an organ that aids in gas and nutrient exchange as well as in waste elimination. In some mammalian species, implantation occurs relatively quickly. In mice, once the embryo hatches from the zona pellucida, implantation occurs. However in other mammals, particularly ruminants, there is a delayed implantation during which the extensive structures of the placenta and uterus develop (Guillomot, 1995). Unlike humans and mice where the embryonic cells invade the uterine lining, ruminants form structures on both the maternal and embryonic surfaces that interlock to allow exchange of nutrients (Guillomot, 1995; Lee and DeMayo, 2004). In cows and ewes, where the tube-like uterus must support the growth of a relatively large fetus, there is an extended elongation period during which the embryo stretches to approximately 150 mm long before attaching. Therefore, the ruminant trophoblastic cells must be able to proliferate and remodel the cellular shape without fully differentiating (Wintenberger-Torrés and Fléchon, 1974). In ruminants and other species in which implantation is delayed, the integrity of the trophoblast cells is critical; the embryo must rely on these cells for nutrients (Wintenberger-Torrés and Fléchon, 1974). Without the precise balance of TE and ICM in the first differentiation step, implantation would fail.

Transcription Factors

Initial work with mouse carcinoma cell lines showed that altering the expression of certain factors could change the lineage identity of cells. This led some

researchers to believe that there are proteins or other factors involved in maintaining pluripotency. These factors, known as transcription factors, are directly associated with DNA and either enhance or repress transcription of genes. Then in the 1980s, embryonic stem cells (ES) were derived from the ICM of mouse blastocysts. These cells provided a better system in which to study early developmental transcription factors (Evans and Kaufman, 1981; Martin, 1981). Current research to determine which transcription factors are involved in the establishment and ability to sustain pluripotency has largely been based on work with mouse embryos or embryonic stem cell lines because those cell lines have been well characterized and fully validated. It has been difficult to determine the molecular components and pathways utilized in early differentiation because they are part of a complex network of proteins that are involved in self-regulation and regulation of other factors and pathways. Several transcription factors such as Oct4, Nanog, and Cdx2 have been recognized as key players involved in this complex regulation in ES cells and early embryos (Figure 2).

Figure 2: Known transcription factors involved in early embryo development



(Ralston and Rossant, 2005)

Oct4

Oct4, also known as Oct3/4 or Pou5f1, is a POU-domain (Pit-Oct-Unc) transcription factor encoded by the Pou5f1 gene. Common among all POU family members, Oct4 recognizes the sequence ATGCAAAT and turns on genes that contain an octamer motif in the promoter. Early studies showed Oct4 being expressed in germ cells and early embryos, which made Oct4 a leading candidate for establishing or maintaining pluripotency of cells (Nichols et al., 1998; Schöler et al., 1990; Schöler et al., 1989). Initially, it was considered a master regulator for the maintenance of pluripotency (Pesce and Scholer, 2001). Since then many researchers have studied Oct4 to determine its role in early embryos and stem cells. In mice, Oct4 can initially be found in all cells of the early embryo, but after compaction it is isolated to ICM; the site where pluripotent cells originate (Pan et al., 2006; Pan et al., 2002; Pesce and Scholer, 2001; Ralston and Rossant, 2005). In bovine, goat, and porcine blastocysts, however, Oct4 can be found in both the ICM and TE cells (Degrelle et al., 2005; He et al., 2006; Kirchhof et al., 2000). Oct4 is expressed in both cell types of bovine blastocysts until approximately day 10, but by day 12 Oct4 is downregulated in both the ICM and TE cells (van Eijk et al., 1999). It is important to point out that though Oct4 is highly conserved in mammalian species, the pattern of expression is not the same in murine and bovine early embryos.

In murine embryos missing the Oct4 gene, implantation does not occur and the inner cells of the blastocysts look more like trophoctoderm cells (Nichols et al., 1998; Niwa et al., 2000). Also, no ICM markers are expressed, and stem cells can not

be cultured from the embryos (Rossant, 2001). These findings identified Oct4 as a necessary factor for proper early development. Because the ICM of embryos missing Oct4 had a trophoctoderm-like morphology, this led to the idea that the trophoctoderm lineage is a default pathway for early embryonic cells and a pluripotent factor like Oct4 would be needed to overcome a TE fate.

Studies were conducted that tried to define the specific mechanism by which Oct4 maintained pluripotency. Although Oct4 is predominately found in pluripotent and germ-line cells, constitutive expression of Oct4 in ES cells was not enough to prevent the cells from differentiating (Niwa et al., 2000; Pesce and Scholer, 2001). This finding seemed to dispel the thought of Oct4 as the sole factor involved in maintaining cell pluripotency. Furthermore, overexpression of Oct4 in mouse stem cell lines led to differentiation into extraembryonic endoderm (Niwa et al., 2000). When the murine Oct4 promoter was attached to an enhanced green fluorescent protein and transfected into early bovine and porcine embryos, all preimplantation stages of both species expressed GFP (Kirchhof et al., 2000). This study indicated that the Oct4 promoter regulation was conserved across several mammalian species. The variation of Oct4 expression seen in these species was more likely an adaptation because of differences in peri-implantation timing, rather than alteration of the transcription factor function.

Subsequent studies using reporter assays and small interfering RNA (siRNA) showed that Oct4 worked in concert with Cdx2 to establish specific lineages (Hay et

al., 2004; Niwa et al., 2005). When Cdx2 was induced, Oct4 was no longer able to function correctly to maintain pluripotency (Niwa et al., 2005). When Oct4 expression was knocked down using siRNA there was an increase in Cdx2 expression (Hay et al., 2004). Because Cdx2 and Oct4 do not appear to be directly competing to bind DNA, it was proposed that either the two proteins interacted through an autoregulatory element or they were in close proximity to one another when bound to DNA (Niwa et al., 2005). These studies bring to light that not only is the presence or absence of these factors important, but the concentration at which they are present must be considered. Niwa suggested there was a threshold of Oct4 that was needed for cells to maintain pluripotent characteristics, but if an excess of Oct4 accumulated the cells would differentiate (Niwa et al., 2000; Niwa et al., 2005). To maintain a steady expression of Oct4, it is likely that this transcription factor acts as its own repressor to decrease production when too much protein is being made (Pan et al., 2006).

Nanog

One important regulator of Oct4 may be the Nanog protein. Nanog, coined from legendary land of Tír na nÓg meaning *land of forever young*, was first characterized in 2003 and was given its name because it was found to be involved in establishing and maintaining pluripotency in embryonic cells (Chambers et al., 2003; Mitsui et al., 2003). This homeobox protein was found using digital differential display and though the sequence was similar to other homeobox family members, the amino acid sequence was noticeably different making Nanog very unique (Matsui and

Okamura, 2005; Mitsui et al., 2003). Nanog is not expressed until the morula stage in mouse embryos, and is generally found in the ICM of blastocysts, in early germ cells, and embryonic stem (ES) cells (Cavaleri and Scholer, 2003; Chambers et al., 2003; Matsui and Okamura, 2005; Mitsui et al., 2003). In bovine spherical blastocysts, Nanog was restricted to the ICM tissues, but as the embryo elongated Nanog could be found in both embryonic and extra-embryonic tissues (Degrelle et al., 2005).

When first discovered, Nanog was considered a repressor of trophectoderm lineage by inhibiting genes involved in signaling the cell to differentiate (Chambers et al., 2003; Mitsui et al., 2003). However, later it was found that Nanog contains two strong trans-activation domains at the C-terminus end dispelling the notion that Nanog acts only as a repressor (Pan et al., 2006; Pan et al., 2002). In mice, it has been shown that both Nanog and Oct4 work together to maintain the undifferentiated cellular population of the ICM (Ralston and Rossant, 2005). Then as lineage determination progresses, Nanog is involved in maintaining pluripotency of the inner cells of the ICM. These cells are the precursors for the formation of the epiblast, and cells not expressing Nanog differentiate into primitive endoderm (Chambers et al., 2003).

To accurately understand the mechanisms by which Nanog is affecting cells, ablation and overexpression experiments were completed. In mutant mouse ES cell lines in which the Nanog gene was removed, the cells still expressed ES cell specific markers. These cells were also able to grow on a layer of STO feeder cells, but the

growth was very slow (Mitsui et al., 2003). When the Nanog gene was removed from mouse embryos, the Nanog mutant was morphologically indistinguishable from normal embryos at 3.5 dpc, but Nanog deficient embryos did not survive (Mitsui et al., 2003). The ICM from these Nanog deficient embryos was dissected out and plated onto culture dishes. The cultured cells presented a primitive endoderm-like morphology (Cavaleri and Scholer, 2003; Mitsui et al., 2003; Ralston and Rossant, 2005). When Nanog was overexpressed in mouse ES cells, there was no longer a need to supplement the medium with the cytokine LIF (Cavaleri and Scholer, 2003; Chambers et al., 2003; Mitsui et al., 2003). Previously, mouse ES cells required the addition of LIF to maintain pluripotent characteristics. Though Nanog may not be explicitly needed for initial formation of blastocysts, it does appear to be necessary for the establishment of the epiblast and, therefore, the three germ layers of the fetus.

Both Oct4 and Nanog have been shown to be important factors involved in establishing and maintaining pluripotency in their own right, but it is clear neither works alone. There is an Oct4 binding site 181 base pairs upstream of the Nanog promoter which suggests Nanog may be a target of Oct4 (Pan et al., 2006). This finding helped explain why overexpression of Oct4 led to an endoderm lineage rather than maintenance of pluripotency. Mouse ES cells that overexpressed Oct4 or cells with reduced Nanog expression looked very similar (Pan et al., 2006). When Nanog was expressed below baseline levels, Oct4 activated the Nanog promoter to increase expression, but when Nanog levels were high Oct4 worked as a repressor (Pan et al., 2006). These studies further indicated that many transcription factors associated with

pluripotency are not regulated in an on/off manner but rather through a more complex network. The expression of these transcription factors is stimulated or repressed based on the concentration of other factors present. Both Nanog and Oct4 appear to be part of a these feedback loops that are tightly regulated to maintain pluripotency of cells.

Cdx2

Nanog and Oct4 have been established as markers of pluripotency and of the ICM, but what about TE cells? Caudal-type homeobox transcription factor 2, or Cdx2, is one of a few transcription factors found specifically in the outer trophectodermal (TE) cells and is, therefore, commonly used as a trophectoderm specific marker. Cdx2 mRNA is first expressed at the eight-cell stage; and by the early morula, Cdx2 can be seen in the nucleus and cytoplasm of the outer presumptive trophectoderm cells (Beck et al., 1995). In bovine embryos, Cdx2 was restricted to the TE at the ovoid and filamentous blastocyst stages (Degrelle et al., 2005).

Cdx2 has been implicated in lineage restriction of the pluripotency factors, Oct4 and Nanog (Strumpf et al., 2005). It was suggested by Pesce and Scholer in 2001, that the TE lineage is a default cellular pathway rather than one that is triggered by specific events or factors because TE cells form when Oct4 is removed (Pesce and Scholer, 2001). However, in embryos in which Cdx2 has been removed the trophectodermal cells display morphology inconsistent with that of normal cells.

Without Cdx2, TE integrity and function are lost and the future stem cell population is distorted (Chawengsaksophak et al., 1997; Strumpf et al., 2005). In the Cdx2 mutants, Oct4 and Nanog were expressed at normal level in the perimeter cells of the embryo (Strumpf et al., 2005). These findings enforced the idea that Cdx2 may be needed to override the signal that maintains pluripotency in ICM cells and push the outer embryonic cells into another lineage. Cdx2 may specify the TE fate of the outer cells by blocking or decreasing the expression of Oct4 and Nanog. Another group showed that adding Cdx2 to ES cells was sufficient to push the cells into a trophectoderm-like morphology. However, if Oct4 expression had already begun to decrease, Cdx2 was not required (Niwa et al., 2005). At this time, the mechanism by which Nanog and Oct4 are segregated to the inner cells while Cdx2 is exclusively expressed in the outer cells is unclear. Presumably there are other upstream factors being activated through context-dependent receptor pathways that can recognize the surrounding environment, which starts a cascade of events orchestrating these events.

Wnt Pathway

The Wnt signaling pathway is an established network of proteins that has recently been shown to be involved in mouse ES cell differentiation (Ogawaa et al., 2006; Pilon et al., 2006; Reya and Clevers, 2005). Also, several Wnt ligands, including Wnt3a, are expressed in early mouse preimplantation embryos (Lloyd et al., 2003). Receptors for the Wnt pathway send signals down the canonical pathway for cell fate determination and to the noncanonical pathway for tissue polarity control and cellular movement (Katoh and Katoh, 2007). The canonical Wnt pathway was the

first to be characterized, and it acts through a specific set of proteins that are highly conserved across many different species. This pathway has been linked with cellular behavior, proliferation, and even apoptosis through the regulation of target genes (Moon et al., 2001). Both the canonical and noncanonical Wnt signaling pathways are transduced through Frizzled family receptors, but only the canonical pathway utilizes the protein β -catenin. Without a Wnt signal, β -catenin is phosphorylated in the cytoplasm of cells and degraded. With β -catenin degraded, the transcription factors Tcf and Groucho act together to stop the expression of Wnt specific target genes. After the Wnt signal binds to its cell surface receptor, β -catenin is dephosphorylated allowing it to accrue in the cytoplasm and enter into the nucleus. In the nucleus β -catenin has access to the genome and replaces Groucho to form a complex with Lef/Tcf, allowing these factors to work as transcriptional activators (Gordon and Nusse, 2006; Reya and Clevers, 2005). It is possible that cell surface receptors stimulate the Wnt pathway and its target genes in response to neighboring cell signals to either maintain pluripotency or allow differentiation in mouse ES cells and early embryos. Since the Tcf/Lef1 family is a component of the canonical Wnt pathway, it is likely that one or more of these family members is involved. In mouse ES cells it was recently shown that Tcf3, transcription factor 3, interacts with the Nanog promoter through a Groucho binding domain to decrease Nanog expression. This interaction appears to be regulating the expression of Nanog and allowing lineage commitment of the cells (Pereira et al., 2006). Another member of the Tcf/Lef1 family, Lef1, has been associated with adult stem cells, and it also contains a Groucho binding domain.

Lef1

Lymphoid enhancer-binding factor 1 (Lef1) was first identified from lymphoid-specific cDNA clones in 1991, and was found to contain a sequence-specific DNA-binding domain known as an HMG (High Mobility Group) box (Travis et al., 1991). HMG boxes have the unique ability to bind in the minor groove of DNA. Once bound an amino acid side chain is inserted between two base pairs causing the DNA to bend. This allows other proteins to aggregate together to either activate or repress surrounding genes. Early studies showed that Lef1 was expressed during murine embryogenesis (Oosterwegel et al., 1993), but it wasn't until 1996, with the aid of yeast two-hybrid screens, that Lef1 was recognized as part of the Wnt pathway.

Lef1 mRNA is alternatively spliced, and different isoforms are expressed in early development. In mouse 8-cell embryos the full-length Lef1 is expressed, but after compaction of the morula and development into the blastocyst another form of Lef1 lacking the sixth exon, (Lef1^{Δ6}), is expressed (He, unpublished). Though the β-catenin binding site and the HMG DNA domain are still present, the Groucho binding domain is missing in this isoform.

In mouse ES cells Lef1^{Δ6} was the dominant isoform expressed, but overexpression did not seem to affect Nanog (He, unpublished). However, mouse ES cells induced to express increased levels of Lef1^{Δ6}, by removal of LIF, responded to the cytokine Wnt3a with an increase of Cdx2 expression. When these cells were grown in TS (trophoblast stem) cell medium they differentiated into trophoblast-like cells (He,

unpublished). It may be that the different Lef1 isoforms allow Lef1 and other family members to be involved in both Nanog regulation and TE differentiation in a context dependent manner. Lef1 has been found to be involved in differentiation of multipotent skin stem cells (Merrill et al., 2001). These studies point to Lef1 as a player in the lineage specification of cells.

Knock down of Lef1 using siRNA

Removing Lef1 from early embryos and mouse ES cells should shed some light onto the molecular context that Lef1 plays in early development. Previously a Lef1 “knockout” would be created that removed the gene of interest utilizing homologous recombination. This approach, the former gold standard for disrupting the mouse genome, is lengthy and expensive, especially in bovids where the gestation period is longer and embryonic stem cells have not been established. Also, many genes involved in early differentiation are difficult to knockout because they are needed to create a viable embryo (Nichols et al., 1998; Strumpf et al., 2005). Instead, RNA interference (RNAi) can be used in early embryos as mRNA degradation and, therefore, protein expression can be specifically targeted at a particular stage.

RNAi is mediated through post-transcriptional sequence specific gene silencing. Double-stranded RNA with a homologous sequence to the gene that is to be silenced, is first cleaved into short strands of 21-22 base pairs long utilizing an enzyme known as Dicer (Bernstein et al., 2001; Elbashir et al., 2001b; Hamilton and Baulcombe, 1999; Hammond, 2005). These short double-stranded RNA pieces are

known as small interfering RNAs (siRNA). Both strands of the siRNA combine with a conglomerate of proteins known as an RNA-induced silencing complex (RISC) (Rana, 2007). The antisense portion of the siRNA guides the RISC to its complementary RNA sequence in the cytoplasm where the mRNA is cleaved, preventing the protein from being made. Before RNAi was introduced in 1998 (Fire et al., 1998), single-stranded antisense and sense RNA were used, which did not provide consistent gene silencing.

Not only did RNAi provide an easier more specific mode of gene silencing, but the machinery was also found to be conserved across many other species including mammals (Elbashir et al., 2001a; Kennerdell and Carthew, 1998; Svoboda et al., 2000; Wargelius et al., 1999; Wianny and Zernicka-Goetz, 2000). The first attempts at mammalian gene silencing using double stranded RNA (dsRNA) were completed in mouse oocytes in 2000 (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). Two independent groups were able to knock down genes by microinjection of dsRNA into oocytes, and resulting embryos were comparable to knockout mouse embryos. dsRNA and siRNA were also used to knock down specific mRNA expression in mammalian cell lines. In some cell types, siRNA can be added directly to the culture media of cells and the molecules can cross cellular membranes unaided, however, in most cases lipid complexes such as Lipofectamine™ must be used to promote in transfection.

Because embryos are surrounded by a zona pellucida, different approaches are needed to transfer siRNA molecules into the cytoplasm. Currently three main techniques have been used to transfect siRNA into embryos: electroporation, microinjection, and direct incubation. Electroporation uses electricity to create small holes in the cellular membranes allowing the small RNA molecules to enter the cell (Calegari et al., 2002; Tompers and Labosky, 2004). Microinjection uses micromanipulation tools to puncture the zona pellucida of the embryo and inject siRNA into specific cells. Microinjection is a common technique used in embryo manipulation because it is a controlled method of getting specific amounts of materials into an embryo (Jaffe and Terasaki, 2004). The third technique uses a higher concentration of siRNA added directly into the embryo culture media. At a higher concentration, this complex is able to penetrate into a cell or embryo without the use of electroporation or direct injection (Kawamura et al., 2003; Kigami et al., 2003; Sandy et al., 2005). Since handling of embryos can affect developmental progress, the direct incubation of embryos in siRNA offers a way to transfect without an added manipulation step. Another approach gaining popularity is to transfect the embryo with short hairpin RNA which when transduced produces the siRNA needed for silencing of gene expression.

The use of RNAi in mammalian embryos opens up an efficient way to study transcription factors that have been identified as important players in differentiation. Gestational losses due to malformed TE are a major contributor to inefficiencies observed in advanced reproductive technologies. Knowledge about how the

trophectoderm emerges may lead to a better understanding of how early embryo manipulation alters the potential for the embryo, especially in its ability to implant into the uterus. Also, understanding what triggers cells to differentiate during early embryonic development could help derive and maintain embryonic stem cells in other animals.

Chapter 2: Methods

Embryo culture

Bovine embryos were purchased from a commercial company (Bomed, Madison, WI). Embryos used for determining expression pattern of key transcription factors were shipped as fertilized zygotes and were harvested at 2-cell, 4-8 cell, morula, and blastocyst stages. The number of embryos collected from each stage can be found in Appendix Table 1.

Bovine oocytes used for siRNA microinjections were also shipped from Bomed. Frozen-thawed Jersey bull semen was spun at 7,000 x G in 10 mL PBS containing magnesium and calcium supplemented with 10 mg/mL BSA and penicillin-streptomycin. Sperm were incubated for 15 minutes in Tyrode's Albumin-Lactate-Pyruvate (TALP) containing 4.5 U of heparin and then diluted in 11 mL of TALP-FM solution. 500 μ L of diluted sperm and 10-25 oocytes were placed in 4-well IVF culture plates (Falcon) at a final concentration of around 2×10^6 sperm cells/mL for approximately 20 hours. All presumptive zygotes were cultured *in vitro* in sequential G1/G2 medium at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂.

Murine embryos were purchased from Embryotech Laboratories Inc. (Wilmington, MA) as cryogenically frozen 1-cell embryos. Straws of embryos were thawed according to manufacture's protocol. After injections, embryos were cultured *in vitro* in KSOM at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂.

Cell culture

Bovine fetal fibroblasts were obtained from the USDA (Beltsville, MD) and cultured in a basic feeder medium containing DMEM (Dulbecco's Modified Eagle's Medium; Gibco, Carlsbad, CA) supplemented with 10% FBS, and 2 mM L-glutamine.

R1 mouse ES cells (ATCC SCRC-1011) were grown in ES medium on 0.1% gelatin-coated plates in the absence of feeder cells. The ES medium was comprised of DMEM (Gibco, Carlsbad, CA) supplemented with 15% FBS (Fetal bovine serum), 2 mM L-glutamine, 1x non-essential amino acids, 0.1 mM β -mercaptoethanol, and 1000 U/ml LIF (Leukemia Inhibitory Factor) (Chemicon, Billerica, MA) to prevent differentiation.

Murine and bovine siRNA sequences

Stealth small interfering RNA (siRNA) oligos (Invitrogen, Carlsbad, CA) were designed using the BLOCK-iT RNAi Designer from Invitrogen and Tuschl guidelines (Elbashir et al., 2002). The annealed duplexes were prepared as recommended by the manufacturer. The murine sense strand was determined based on the Lef1 gene starting from the 136 base of Lef1 open reading frame (ORF) (NM_010703) and was as follows: 5'- GGCGACUUAGCCGACAUCAAGUCAU-3' and the antisense strand was 5'-AUGACUUGAUGUCGGCUAAGUCGCC -3'. The bovine Lef1 siRNA oligos were designed against the predicted Lef1 sequence starting

from the 139 base of Lef1 ORF. The sense strand used to target the bovine Lef1 gene was 5'-GAAGGUGACUUAGCCGACAUCAAGU-3' and the antisense strand was 5'-ACUUGAUGUCGGCUAAGUCACCUUC-3'.

Transfection of cells with Lef1 siRNA

To validate the effect of Lef1 siRNA on knockdown of Lef1 mRNA, both mouse ES cells and bovine fibroblasts were transiently transfected with StealthTM Lef1 siRNA or a scrambled, StealthTM missense siRNA. All cell transfections were one of three treatment groups: Lef1 siRNA used to knock down expression of Lef1, missense siRNA used as a transfection control to identify off-target effects from transfection procedure, or a control treatment in which no siRNA was added to the cells.

The bovine fetal fibroblasts were plated in 24-well plates (2×10^5 /well) in triplicate for each treatment. This experimental design was replicated on three different days. After cells were 70% confluent 5 pmol of missense or Lef1 stealth RNAi (80 nM) and 2 μ L Lipofectamine 2000 were randomly added to each designated treatment well. siRNA complexes were prepared in Opti-MEM (Invitrogen, Carlsbad, CA) according to manufacturer's guidelines and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to enhance siRNA uptake. The third treatment type, designated as control, was not transfected with siRNA or Lipofectamine; instead equivalent amounts of Opti-MEM, the media used to dilute

the siRNA, were added to the wells. After 24 hours, cells were collected in lysis buffer and flash frozen in liquid nitrogen for a later recovery of RNA.

Mouse ES cells were transfected in duplicate for each treatment, and the experimental design was replicated on three different days. ES cells were trypsinized and plated into 24-well gelatin-coated culture plates in 400 μ L of ES medium. To each well, 100 μ L of one of the three treatments was added. For RNAi transfection, ES cells (1×10^5 /well) were transfected with complexes consisting of 5 pmol Lef1 or missense stealth siRNA (80 nM) and 2 μ L Lipofectamine 2000 mixed in Opti-MEM (Invitrogen, Carlsbad, CA). Only Opti-MEM was added to cells designated as control. Cells were cultured for 24 hours. After this period, the cells were collected in lysis buffer and flash frozen in liquid nitrogen for a later recovery of RNA.

Microinjection of Lef1 siRNA into embryos

Both murine and bovine zygotes were randomly sorted into one of three groups: control in which the oocytes were not injected, missense siRNA in which the oocytes were injected with a scrambled missense siRNA sequence to identify detrimental effects from the siRNA injection procedure, or Lef1 siRNA to knock down expression of Lef1. Embryo manipulation plates were prepared using Optilux (BD Biosciences, Franklin Lakes, NJ) culture dishes with a 150 μ L drop of the handling media EmCare (ICPbio, Auckland, New Zealand) overlaid with mineral oil (Sigma, St.Louis, MO). Small groups of 10 embryos were placed into the injection drop and microinjections were completed on an inverted microscope (Leica DMIRB).

For bovine embryo injections, 40 μM of siRNA was mixed with 10 mg/mL of 40,000 MW neutral Texas red-conjugated dextran (TRD) diluted in saline (Invitrogen, Carlsbad, CA) in a 1:1 ratio giving a final concentration of 20 μM of siRNA. For murine embryos 20 μM of siRNA and TRD were mixed in a 3:1 ratio for final concentration of 15 μM . 5 μL of siRNA-TRD mixture, missense siRNA or Lef1 siRNA, was loaded into a Femtotips II injection needle purchased from Eppendorf (Eppendorf, Hamburg, Germany) and attached to a FemtoJet (Eppendorf, Hamburg, Germany). A volume of approximately 10-20 pmols of dsRNA was injected into the cytoplasm of the embryos. After injections, bovine embryos were placed into G1 culture media and mouse embryos into KSOM.

Bovine and murine 4-8 cell embryos from each treatment were placed in a lysis buffer and immediately flash frozen in liquid nitrogen. Number of bovine and murine embryos collected can be found in Appendix Table 2 and Appendix Table 3 respectively. Remaining bovine embryos were observed seven days after insemination to determine blastocyst rates and morphology. Four days after being thawed, murine embryos were checked for morphology and blastocyst rates. All blastocysts of a treatment group were collected in lysis buffer and flash frozen in liquid nitrogen. The number of murine blastocysts collected from each treatment group can be found in Appendix Table 4.

RT-PCR and qRT-PCR

Total RNA was extracted from embryos using an Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA). The RNA was then quantified at the Center for Advanced Research in Biotechnology (CARB, Rockville, MD) using an Agilent 2100 bioanalyzer. An aliquot of RNA (8 ng) was reverse transcribed into cDNAs using SuperScript III (Invitrogen, Carlsbad, CA).

Total RNA from murine and bovine cells was extracted using an Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). RNA from these cells was quantified using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA). For both cell types, an aliquot of RNA (60 ng) was reverse transcribed into cDNA by means of SuperScript III.

Equivalent amounts of cDNA were used as templates for Reverse transcription PCR (RT-PCR), and Platinum Taq (Invitrogen, Carlsbad, CA) was used as the DNA polymerase. Primer sequences used are shown in Appendix Table 5. The PCR thermocycling conditions were the following: an initial denaturation step at 95°C for 3 min followed by 35 cycles of: 95°C for 30 sec, primer specific annealing temperature for 30 sec, and 72°C for 45 sec extension, with a final extension at 72°C for 10 min.

Quantitative real-time PCR (qRT-PCR) was performed using the iCycler apparatus (BioRad Inc., Hercules, CA) and mRNA presence was detected with the fluorochrome SYBR Green (IQ SYBR Green Supermix, BioRad Inc.). All qRT-PCR reactions were performed in a 20 μ L reaction volume using 500 nM of each specific primer. Primer sequences used for bovine qRT-PCR reactions are shown in Appendix Table 6, and murine sequences are shown in Appendix Table 7. Equivalent amounts of cDNA were used as templates for qRT-PCR. Reactions were performed in triplicate for each sample. The PCR program consisted of an initial denaturation step at 95°C for 3 minutes to activate the polymerase, followed by 40 cycles of: denaturation at 95°C for 30 seconds, specific annealing of each primer pair for 30 seconds, and extension for 30 seconds at 72°C at which time fluorescence was measured.

Statistical Analysis

Transcription factor expression was recorded as present or absent in groups of bovine embryos analyzed at each stage of development. Results were analyzed using a generalized linear mixed model in SAS version 9.0 (SAS institute, Cary, NC) with a binomial distribution.

Percentage of embryo cleavage and development to blastocysts after injection of siRNA were recorded. Frequencies of cleavage between treatments were analyzed using a χ^2 test with a Bonferoni adjustment.

Ct values from quantitative real-time PCR were normalized to β -Actin and the control treatment, standardized to 100, using the $\Delta\Delta^{ct}$ method. Statistical analysis was performed with SAS version 9.0 (SAS institute, Cary, NC). Data are presented as means \pm standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) with variance component estimated by treatment due to nonhomogeneous variances in samples. Treatments within a graph denoted with different letters were significantly different ($P < 0.05$).

Chapter 3: Evaluation of Lef1 in mammalian pre-implantation embryos

Introduction

In mammals the network of transcription factors involved in cell fate and lineage differentiation of preimplantation embryos is critical for normal embryo development and implantation. The differentiation of cells into either the inner cell mass (ICM) or trophoctoderm (TE) at the end of the morula stage is the first segregation of cells into different fates. The cells of the ICM are the progenitor cells of the three germ layers that eventually become the embryo proper, whereas the TE cells become placental tissues. Knowing that proper differentiation of cells into the ICM and TE is critical for embryo development, the question to ask is which transcription factors are influencing this push into different cell types at this stage. In addition to the well characterized Oct4, Nanog, and Cdx2, our laboratory has found another transcription factor, known as Lef1, which has different isoforms expressed in early development. In preliminary work conducted in our laboratory using mouse ES cells and early embryos, we found a full-length Lef1 isoform being expressed in adult tissues and mouse embryonic fibroblasts (MEF), but it was nearly undetectable in the more pluripotent cells of morulae and blastocysts. On the other hand, Lef1^{Δ6}, which lacks the sixth exon, was seen at the morula and blastocyst stages in mouse embryos and down-regulated in MEF and adult tissues. The expression of different isoforms in early development implies Lef1 could be involved in this network of transcription factors determining cell fate.

Rationale

Other studies in our laboratory, using mouse ES cells, indicate that Lef1 may be involved in early TE lineage determination. In this study, we wanted to determine if Lef1 was involved in cell fate determination of preimplantation embryos. Since failure of proper TE development is one of the major causes of early gestational loss in cattle following embryo transfer (ET) of manipulated embryos, we chose to look at the role of Lef1 in bovine embryos. In addition to bovine embryos, mouse embryos were used as a basis to understand Lef1 function since mouse strains have been well characterized and offer a relatively uniform population of embryos with high blastocysts rates. Furthermore, as we were interested in the period in which murine and bovine development begins to diverge, we needed to assess expression in both bovine and murine embryos in order to gain a better understanding of the function and expression patterns of Lef1 during this critical phase.

First we wanted to determine the normal expression pattern of Lef1 and other key transcription factors at different stages in bovine pre-implantation embryo development. Because many other studies have been conducted to determine expression of pluripotent markers in murine embryos, we can compare the expression pattern of transcription factors in these two mammalian species. Second, we wanted to determine the role that Lef1 plays in early embryo development. To accomplish this, we have chosen to use siRNA against the Lef1 gene. This approach allows for a targeted knockdown of mRNA during early development to determine the role of Lef1.

Results

Expression of early differentiation transcription factors in bovine embryos

Pools of bovine embryos collected at different stages were assayed to determine if Oct4, Nanog, Cdx2, and Lef1 mRNAs were present (Table 1). Our results show that Oct4 was present in all preimplantation embryo stages collected. All three replicates indicated Oct4 was expressed throughout preimplantation embryo development from oocytes through blastocysts. In oocytes, Nanog and Cdx2 mRNAs were not present in any of the embryo pools tested, but both isoforms of Lef1 were expressed. Similar results were found in the 2-cell embryos collected. In one of the three pools of 2-cell embryos, however, the full-length Lef1 was not present, as indicated by the 66% “+/-.” By the 4- to 8-cell stage some embryos were positive for Nanog and Cdx2, but most did not express Nanog or Cdx2 at this stage. The amount of full-length Lef1 isoform appeared to decrease by this time, whereas most of the 4-8 cell embryos were still positive for the $\Delta 6$ isoform. Pools of morulae collected indicated a mixed population of embryos. Most of the morulae were still not expressing Nanog, and the Lef1 ^{$\Delta 6$} isoform appeared to be the dominant form of Lef1 at this stage. Cdx2 expression shifted and was present in most morulae tested. By the blastocyst stage there was an inconsistent expression of Nanog, Cdx2, and both Lef1 isoforms; one pooled population of embryos was positive, while another was not. Looking specifically at Lef1 (Figure 3), the Lef1 ^{$\Delta 6$} isoform was dominant in the early embryo where the cells were more pluripotent. Both isoforms were present in 2-cell embryos, but it was clear that the $\Delta 6$ form was expressed at higher levels. At 4- to 8-

cell, morulae, and blastocyst stages only the $\Delta 6$ isoform was present, and it appeared that as the embryo develops, expression of this isoform was decreasing. In bovine cell lines and oocytes, both isoforms were present. In the cell lines there was no apparent difference in expression between the isoforms, but oocytes clearly had more Lef1 ^{$\Delta 6$} present.

Validation of Lef1 siRNA in bovine fetal fibroblasts

To ensure that the siRNA specifically designed to decrease expression of bovine Lef1 was functioning properly, Lef1 siRNA oligonucleotides and non-specific missense siRNA oligonucleotides were transfected into bovine fetal fibroblasts. Results from quantitative real-time PCR indicated that the Lef1 siRNA decreased Lef1 expression in these cells (Figure 4). Lef1 siRNA-transfected cells were statistically different from both control cells in which no siRNA was added to the cells (P=0.0035) and from the missense siRNA transfected cells (P=0.0010). The missense transfected cells and control cells were not statistically different (P=0.08).

The expression of Cdx2 in these cells was also significantly decreased when the Lef1 siRNA was used. In fact, there was a significant difference between all of the treatments analyzed. The cells treated with Lef1 siRNA had a lower Cdx2 expression than both the missense treated cells (P=0.0308) and untreated control cells (P<0.0001). Also, there was a significant difference between the control and

missense siRNA transfected cells ($P=0.0002$) which may be indicating an off-target effect on Cdx2 in the missense siRNA treatments.

Microinjection of siRNA into bovine embryos

Once the Lef1 siRNA was validated, we had to determine the best way to deliver the siRNA into embryos. Because the development of *in vitro* derived embryos depends on the environment and how the embryos are handled, a non-invasive direct incubation of the embryos with high concentrations of siRNA was attempted first (Kawamura et al., 2003). Unfortunately, the embryos did not survive a 22-hour incubation with the siRNA (Appendix Figure 4). The increased concentration of EDTA in the commercially designed siRNA buffer probably had detrimental effects on the embryos. Therefore, an alternative approach using microinjection was used. Lef1 or missense siRNA were injected into the cytoplasm of 1-cell bovine embryos. A FemtoJet was used to provide a more consistent volume of injection. The siRNA was mixed with Neutral Conjugated Texas Red Dextran (TRD) to visualize the amount being ejected from the microinjection needle. After a successful injection of the siRNA-TRD, TRD was seen in the cytoplasm (Figure 5). TRD could also be seen in the perivitelline space of the embryos, which was caused by leakage from the needle during the injection process. Embryo survivability, as determined by cleavage after injection, was observed the next day (44-48 hours post fertilization). An average of $41.2 \pm 2.3\%$ of the embryos had cleaved (Table 2). Although the survivability rate of the injected embryos was low ($41.2 \pm 2.3\%$), the control group also only had 42.4% of embryo cleaved by this time (Table 2). No morphological differences were seen between the three treatment groups of bovine embryos (Figure 6). At this time all dead embryos were removed. The following day when the embryos were at the 4- to 8-cell stage, groups of approximately 20 embryos

were pooled together and placed in lysis buffer for extraction of total RNA. The remaining embryos were kept in culture until day 7 after fertilization when blastocysts should have been formed. Unfortunately, no bovine blastocysts were present in any of the three treatment groups so no bovine blastocysts could be collected for analysis.

Quantitative real-time PCR was used to determine the expression of Lef1 and Oct4 mRNA in early bovine embryos after the injection of siRNA. Both TFs were shown to be expressed in bovine 4-8 cell embryos (Figure 7). As expected, Lef1 was significantly decreased in bovine embryos; expression was decreased by about 90%. There was no significant difference in Oct4 mRNA expression in any of the three treatment groups in the 4-8 cell embryos. Levels of Nanog and Cdx2 mRNAs were not analyzed in the bovine 4-8 cell embryos because RT-PCR results indicated neither factor was expressed at that stage in development. A summary of the qRT-PCR data from both bovine fetal fibroblasts cells and injected bovine embryos can be found in Figure 8.

Validation of Lef1 siRNA in mouse ES cells

Although no bovine blastocysts developed, we were able to use mouse embryos to examine at the role of Lef1 in early blastocyst differentiation. Before the murine specific Lef1 siRNA could be injected into embryos, the expected knockdown of Lef1 needed to be validated in mouse cell lines. The murine Lef1 siRNA and

missense siRNA were transiently transfected into mouse ES cells. Once again the Lef1 siRNA appeared to be decreasing the expression of Lef1 mRNA in cells by roughly 60%, and there was no significant difference between the control treatment and those cells treated with missense siRNA (Figure 9).

In mouse ES cells the addition of Lef1 siRNA did not affect the expression of Cdx2; all treatments were not statistically different (Figure 9). Both Nanog and Oct4 mRNA expression were increased by about 40% in the mouse cell lines, but only the expression of Nanog was significantly different ($P=0.0178$) from the control group. However, it must be pointed out that although the control and siRNA groups were different there was no difference in Nanog expression between the missense and Lef1 siRNA transfected cells. The expression of Oct4 increased after Lef1 siRNA transfection, but it was not statistically different from the missense or control groups.

Microinjection of siRNA into mouse embryos

After the validation of the mouse specific Lef1 siRNA in mouse ES cells, the Lef1 and missense siRNA were injected into the cytoplasm of thawed 1-cell murine embryos. The same microinjection techniques were used in both bovine and murine embryos. When embryo survivability after injection was observed the next day, cleavage had occurred in 58.6% of the embryos injected with missense siRNA and 75.4% in embryos injected with the Lef1 siRNA (Table 3). In the control embryos, 95.9% had divided into at least 2-cells 24-hours after being thawed (Table 3). There

was a significant difference ($P < 0.01$) in frequency of cleavage between all injected treatments. The following day, when the embryos had cleaved into 4-8 cells, groups of approximately 20 embryos were pooled together and placed in lysis buffer for extraction of total RNA. The remaining embryos were kept in culture until day 3.5 after thawing when blastocysts should have been formed. In all three groups about half of the remaining embryos had developed into blastocysts.

Quantitative real-time PCR was used to determine the expression of Lef1, Cdx2, and Oct4 mRNA in early mouse embryos after the injection of siRNA. Either missense siRNA or Lef1 siRNA was injected into embryos. Levels of Lef1 mRNA could not be detected in most of the missense siRNA samples using qRT-PCR. Therefore, only the control and Lef1 siRNA treatments were shown (Figure 10). Results revealed that Lef1 expression was significantly ($P = 0.0345$) decreased in embryos injected with Lef1 siRNA when compared to the non-injected control embryos. Cdx2 expression was decreased by about 70% ($P = 0.0050$) from the control group and by 15% ($P = 0.0224$) from the missense group following injection with the Lef1 siRNA. Oct4 was decreased by approximately 50% from the control ($P = 0.0241$), but only about 20% from the missense siRNA treatment making it statistically not significant ($P = 0.1794$). Other missense and Lef1 siRNA groups were not statistically different.

The murine blastocysts that developed from each of the three groups had no major morphological differences; all had a defined inner cell mass (ICM) and

trophectoderm (TE) layer (Figure 11). However, in the embryos injected with Lef1 siRNA, cells of the ICM did not appear as compact as those of the missense and control groups. To better understand the consequence of decreasing Lef1 expression in a molecular-context, RNA was collected from the blastocysts and qRT-PCR was used to analyze changes in mRNA expression (Figure 12). Results showed Lef1 expression was significantly decreased from the control ($P=0.0038$) after injection of the Lef1 siRNA, but it was not statistically different from the missense injected siRNA, although there was a trend ($P=0.0693$). There was also a significant decrease in Nanog ($P=0.0358$) and Oct4 ($P=0.0088$) mRNA expression when compared to the control treatment, but there was no significant difference (Nanog $P=0.5161$; Oct4 $P=0.3502$) when compared with the missense siRNA injected embryos. There was no significant difference in Cdx2 expression between any of the treatment groups.

To ensure Lef1 depletion was not being compensated by one of the other three Tcf/Lef1 family members, the expression of these transcription factors was also tested. The results indicated there was no significant difference between the control and siRNA or missense injected embryos in any of the other Tcf family members tested (Figure 13). A summary of the qRT-PCR data from both mouse ES cells and injected murine embryos can be found in Figure 14.

Chapter 4: Discussion

Our studies show that different isoforms of Lef1 are being expressed in early bovine and murine embryos, and that we can knockdown mRNA for this transcription factor using siRNA. However, the injection of Lef1 siRNA into bovine and murine embryos did not provide any conclusive results as to the role that Lef1 plays in early embryo development.

In the first experiments, we determined the expression of Oct4, Nanog, Cdx2, and Lef1 in bovine embryos to obtain an indication of when each transcription factor was active in early development. Results showed Oct4 was present in bovine unfertilized oocytes and embryos through the blastocyst stage. This pattern of expression was very similar to what has been seen in mouse early embryos; Oct4 was also found throughout early murine development (Boiani et al., 2002; Nichols et al., 1998; Schöler et al., 1990). Since Oct4 was present in 2-cell embryos it must be one of the stored maternal mRNAs found in oocytes because the embryonic genome is not yet active at this stage of development. Our findings support data from the mouse that indicate Oct4 is needed early in development for normal blastocyst formation.

Both Nanog and Cdx2 are expressed in murine embryos at the late morula and blastocyst stages as the cells of the embryo are beginning to diverge into different lineages (Beck et al., 1995; Chambers et al., 2003). A similar pattern of expression is also seen in early bovine pre-implantation embryos. Both Nanog and Cdx2 are not

expressed in oocytes and at the 2-cell stage, but by the 4-8 cell stage some embryos were positive for Nanog and Cdx2 mRNA expression. Perhaps these findings were due to mRNA expression in some of the more advanced 8-cell embryos. In sets of pooled morulae and blastocysts at least half of the pools express Nanog and Cdx2.

The expression of Lef1 in early mouse and bovine embryos was dramatically different. In bovine oocytes and 2-cell embryos, where the maternal stores of mRNA are present, both isoforms are expressed. By the maternal zygotic transition (MZT) only the $\Delta 6$ isoform is present in the bovine embryos. As the embryo develops, expression of this isoform appears to decrease with each cellular division. This pattern is not seen in mouse early embryos, where there is a switch from full-length Lef1 expression at the 8-cell stage to Lef1 ^{$\Delta 6$} . In murine morulae and blastocysts both isoforms are expressed, although the $\Delta 6$ isoform is dominant (He, unpublished). These results seem to indicate Lef1, particularly the $\Delta 6$ isoform, is another transcription factor involved in the lineage specification of cells in early embryos; altered isoform expression coincides with lineage divergence of cells.

When we looked at the normal expression of Oct4, Nanog, Cdx2, and Lef1 in preimplantation bovine embryos, there was variation in the pools of embryos collected. Because the oocytes were obtained from slaughterhouse animals that have very diverse genetic and management backgrounds it is not surprising that we see such variability. Other studies conducted in our laboratory with the same type of bovine embryos have also shown a great deal of variance in transcription factor

expression from embryo to embryo. This study points to embryo quality as a contributing factor for variability in transcription factor expression. Also, there is a transition around the 8-cell stage in bovine embryos. This transition is known as the maternal zygotic transition or MZT (Vigneault et al., 2004), and may be another reason why we saw variation of mRNA expression in embryos collected at this stage.

In the second set of experiments completed, we used cell lines to validate how effectively Lef1 siRNA knocked down expression of total Lef1 mRNA. The results indicated that the respective Lef1 siRNAs were effectively decreasing Lef1 mRNA expression in both bovine fetal fibroblasts and mouse ES cells. However, we also observed a decrease in Cdx2 expression in the bovine fetal fibroblasts transfected with the missense siRNA. Since the missense siRNA is a scrambled sequence of RNA with no known mRNA target, the decrease in Cdx2 expression was unexpected. When the bovine and murine missense siRNA sequences were compared against all known mRNA sequences in the bovine and murine genome there were no significant matches to any particular genes. The decrease in Cdx2 after missense siRNA transfection may be due to off-target effects that are affecting the expression of Cdx2. These off-target effects may be from partial complementation of the missense siRNA to Cdx2 or another target upstream of Cdx2 that is strong enough to cause degradation the mRNA by the RISC complex or decreased transcription. Regardless, the expression of Cdx2 in cells transfected with Lef1 siRNA was significantly different from both the control and missense transfected cells. From this experiment it appears that decreasing total Lef1 mRNA in bovine fetal fibroblast also decreases

Cdx2 expression, providing further evidence for a connection between Cdx2 and Lef1.

In our final experiments, Lef1 and missense siRNA were injected into bovine and murine embryos to determine the role of Lef1 in early embryo development. In the 4-8 cell embryos collected, there was a decrease in Lef1 mRNA after the embryos were injected with the Lef1 siRNA, indicating microinjection is an effective method of siRNA delivery into embryos. Unfortunately, in the bovine embryos no blastocysts developed, even in the non-injected control group of embryos. Since the lack of blastocyst development cannot be attributed to a decrease in Lef1 expression, it is probably due to the quality of embryos collected. As with the embryos used in the expression study, these embryos were from slaughterhouse animals with variable genetic and management backgrounds. Even though the fetal fibroblasts indicated a possible link between Cdx2 and Lef1 in the bovine, we cannot examine this relationship in early embryos because no bovine blastocysts development. At this time, we cannot make any claims about the role that Lef1 plays in early bovine embryos.

To overcome the issue with embryo quality, mouse embryos were used. These commercially purchased embryos have a 90% blastocyst development rate so any effect seen should not be due to poor embryo quality. In the 4-8 cell embryos, Lef1 expression in embryos injected with missense siRNA were unable to be analyzed accurately, because several data points were undetectable by qRT-PCR for

unknown reasons. Even though the missense treatment was not used, we still determined that Lef1 expression appeared to decrease in murine embryos injected with Lef1 siRNA. In the other two genes examined at this stage, the missense siRNA appeared to have significantly decreased expression when compared to the control embryos. This should not be because of off-target effects since none were seen in mouse ES cells. Instead, micromanipulation of the embryos was probably the cause of the observed significant difference between the control and missense siRNA treatments. To better understand why we are seeing this difference, another control treatment of injected siRNA buffer and TRD would be needed to help determine if the decrease in expression at this stage was caused by the microinjection process rather than a poorly designed missense siRNA. As in the bovine fetal fibroblasts, we also see a decrease at the 4-8 cells stage in Cdx2 expression after the embryos were injected with the Lef1 siRNA when compared to both the control and the missense injected embryos. However, this effect does not continue in the murine blastocysts sampled. Previous work in our laboratory has shown a connection between Lef1 and Cdx2 expression in mouse ES cells, but only when Wnt3a, an agonist of the Wnt pathway, was also added to the cells (He, unpublished). Even though it appears that a decrease in Lef1 mRNA affected Cdx2 expression, further studies that also look at the role of Wnt3a need to be conducted before we can determine how these factors are interacting.

One might expect that if Lef1 is involved in TE differentiation, a more pluripotent population of cells expressing higher levels of Nanog and Oct4 would be

seen following Lef1 mRNA knock-down in blastocysts. This, however, is not what we see in the murine embryos. There was no change in Nanog and Oct4 expression in embryos injected with Lef1 siRNA when compared to the missense injected embryos. In addition, we did not see a difference in Cdx2 expression in any of the treatments. Since the missense treatment did not affect mRNA levels in mESC, it is likely that the injection process was altering mRNA levels. Therefore, we assumed that the missense siRNA treatment was the better control for comparison of changes in mRNA expression. We would need to add another treatment group of embryos that are injected with TRD and siRNA buffer, but no siRNA, to ensure there are no off-target effects in embryos from the missense siRNA.

Other Tcf/Lef1 family members were also analyzed to determine if another closely related family member was upregulated to compensate for the decreased Lef1 in the early embryos. There were no significant changes in expression of the family members in any of the treatment groups. It is possible that even though blastocysts form, the alteration of Lef1 expression could affect the ability of the embryo to implant into the uterus. It would be interesting to test whether blastocysts from each different treatment group could actually implant.

Chapter 5: Conclusions

Summary

Although a definitive role of Lef1 in early embryo development was not identified in this study, we were able to determine the expression pattern of Lef1 and other transcription factors during bovine preimplantation development. Furthermore, we have demonstrated that embryos can survive microinjection of siRNA, although it appears that the injection process does have some detrimental effects on the embryos. To make more definitive conclusions, additional replicates of larger embryo pools would need to be tested. This should decrease the large variation seen with some of the transcription factors tested. Also, the use of qRT-PCR to detect minute quantities of transcription factors from such a small number of cells may be near or below the limitations of the system. Until there is a consistent method that is sensitive enough to detect changes in such small amounts of mRNA, larger pools of embryos will be required.

Future Studies

Our studies show a decrease in Cdx2 expression when Lef1 mRNA is knocked down in the bovine fetal fibroblasts. Results from the mouse 4-8 cell embryos also suggest there is a relationship between Cdx2 and Lef1. Recent work in our laboratory has shown that addition of the Wnt agonist Wnt3a in embryonic stem cells permits an increase of Lef1 mRNA to act synergistically to increase expression

of Cdx2 (He, unpublished). These findings suggest that Wnt, Lef1, and Cdx2 are all interconnected. It would be interesting to look at the change in Cdx2 and Lef1 expression following stimulation of the Wnt pathway by Wnt3a in early murine embryos. Results could reveal if Cdx2 and/or Lef1 are targets of the Wnt pathway in these early stages of embryo development. Assuming that there is a relationship between the factors in murine embryos, experiments using siRNA against Lef1 and Cdx2 as well as antagonists of the Wnt pathway could be completed to determine mRNA expression with qRT-PCR and protein location and interactions using immunocytochemistry and western blots.

As we learned from this study, there is considerable variability in embryos produced using slaughterhouse-derived oocytes. If we were to conduct additional experiments with bovine embryos, results should be more conclusive if larger numbers of embryos were analyzed and the embryos were obtained from cattle with similar genetic and management backgrounds such as the Wye Angus herd.

Table 1: Expression of early differentiation transcription factors at different stages in bovine embryos

Summary of data from RT-PCR amplification of each transcription factor. Three pools (n=20) of embryos at each stage were analyzed except at the blastocyst stage where only 2 pools (n=9 or 10) were obtained. A minus “-” indicates mRNA was not detected, a plus “+” indicates mRNA expression was seen, and a plus-minus “+/-” indicates a different results were obtained from one group of embryos to the next. Numbers below denote the percentage of embryo pools that were positive for transcription factor expression.

	Oocytes	2-cells	4-8 cells	Morula	Blastocysts
Oct4	+ 100%	+ 100%	+ 100%	+ 100%	+ 100%
Nanog	- 0%	- 0%	+/- 33%	+/- 33%	+/- 50%
Cdx2	- 0%	- 0%	+/- 33%	+/- 66%	+/- 50%
Full-length Lef1	+ 100%	+/- 66%	- 0%	+/- 33%	+/- 50%
Lef1 ^{Δ6}	+ 100%	+ 100%	+/- 66%	+/- 66%	+/- 50%

Figure 3: Expression of Lef1 in early bovine embryos and cell lines

Lef1 mRNA expression patterns in bovine pre-implantation embryos, bovine cumulus cell lines, and bovine fetal fibroblasts. A white band indicates Lef1 mRNA is being expressed at the embryo stage identified. Upper bands are representative of full-length Lef1 expression and the lower bands indicate presence of the Lef1^{Δ6} isoform.

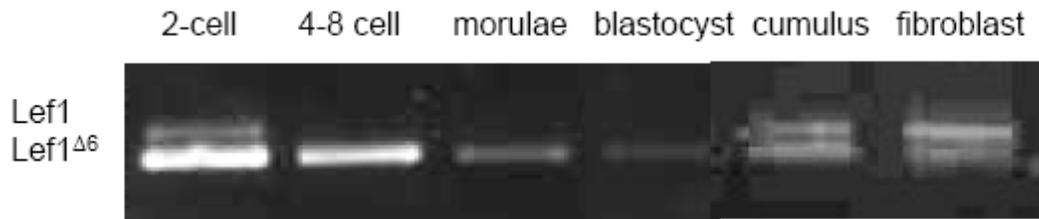
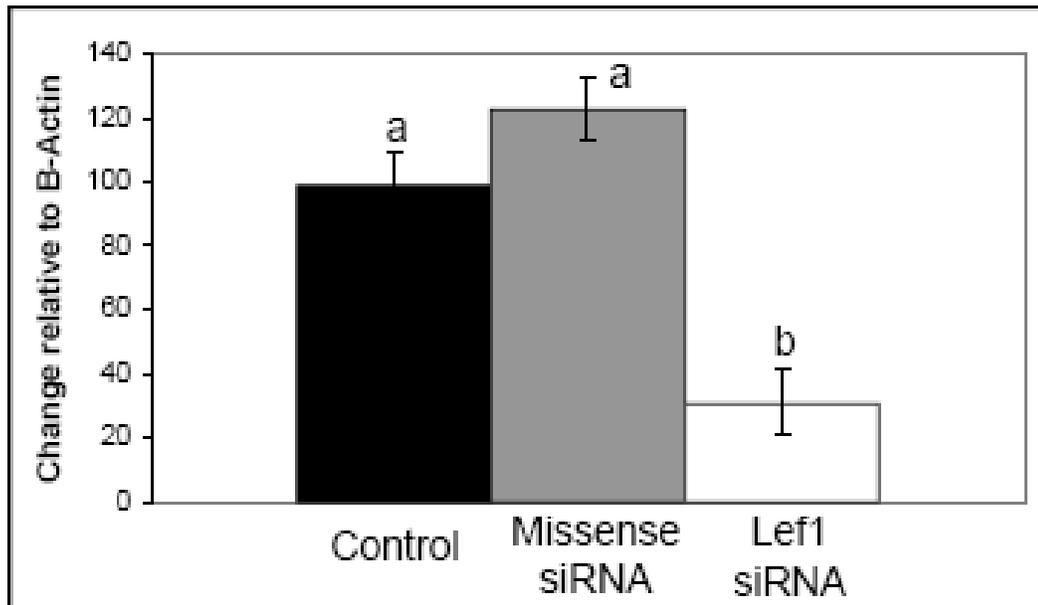


Figure 4: Validation of bovine Lef1 siRNA in bovine fetal fibroblasts

Bovine fetal fibroblasts were transfected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control, another group of cells were not transfected (black). Quantitative real-time PCR was used to determine expression of Lef1 and Cdx2 in the fibroblasts. Presented is the mean \pm SEM of the change in Lef1 or Cdx2 relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin as well as an average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

Lef1



Cdx2

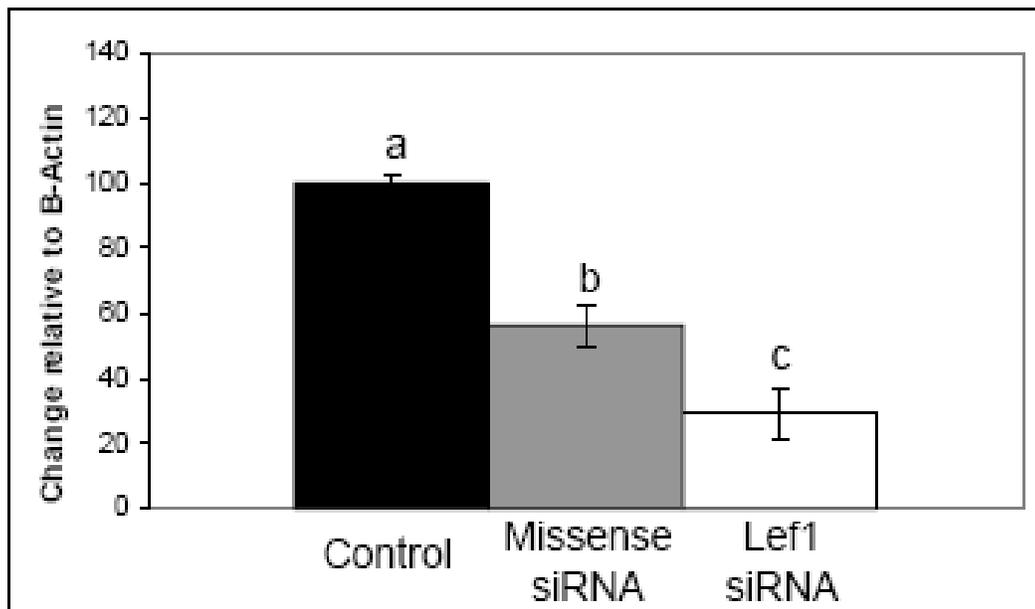


Figure 5: Bovine embryos after injection of siRNA-TRD

Bovine 1-cell embryos were injected with approximately 10-20 pmol of Lef1 or missense siRNA, each were mixed with Texas Red Dextran (TRD). The presence of TRD acts as a fluorescent marker indicating successful injection. Below are a group of these embryos after injection. Top panel (a) is a group of the bovine embryos viewed under a light-microscope at 20x magnification. The middle panel (b) is the same group of embryos shown with fluorescence emission. The bottom panel (c) represents an overlap of the two images.

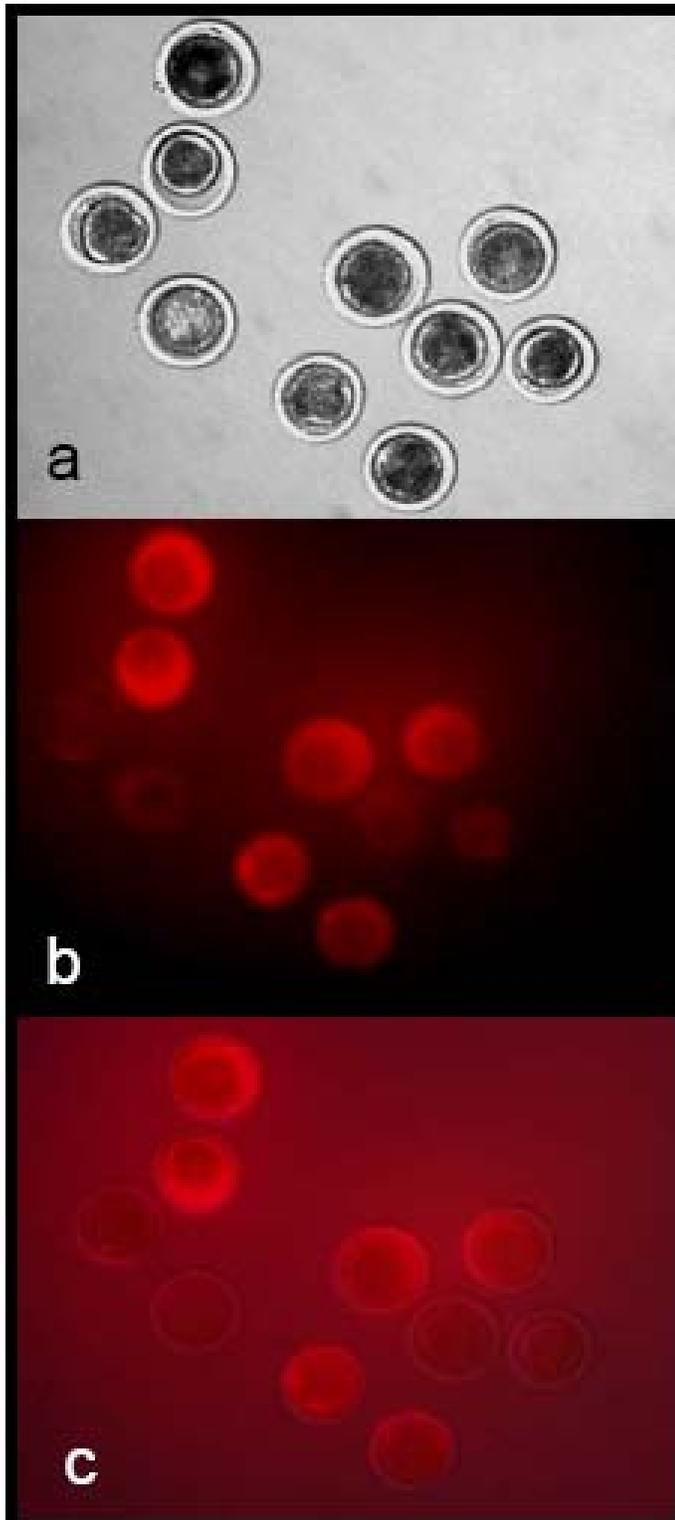


Table 2: Bovine embryo cleavage rates after injection of Lef1 and missense siRNA

Total number of embryos used for each treatment group, as well as, the number cleaved after 44-48 hours post fertilization (hpf). The last column is percentages of embryos that were cleaved. Treatments not significantly different were denoted by the same superscript letter.

Treatment	Total	Cleaved after 44-48 hpf	Percent cleaved
Control	271	115	42.4% ^a
Missense siRNA	272	106	38.9% ^a
Lef1 siRNA	271	118	43.5% ^a

Figure 6: Bovine embryos after siRNA injections

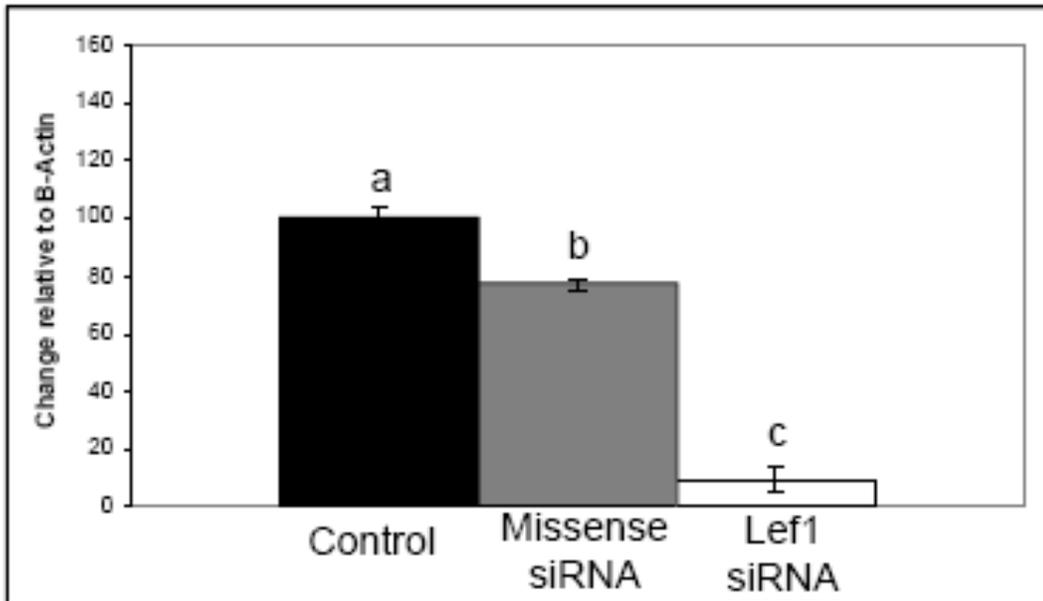
Bovine embryos 24 hours after siRNA injection. The top group of embryos (a) represent the control treatment of embryos that were not injected. In the middle (b) is a representative group of embryos that were injected with missense siRNA. On the bottom (c) is a typical group of embryos that were injected with Lef1 specific siRNA.



Figure 7: Bovine 4- to 8- cell staged bovine embryos after injection with Lef1 siRNA

Bovine 1-cell embryos were injected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control another group of cells were given no treatment (black). Embryos were collected at the 4-8 cell stage and total RNA was isolated. Quantitative real-time PCR was used to determine expression of Lef1 and Oct4 in the embryos. Presented results are the mean \pm SEM of the change in Lef1 and Oct4 relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin and an average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

Lef1



Oct4

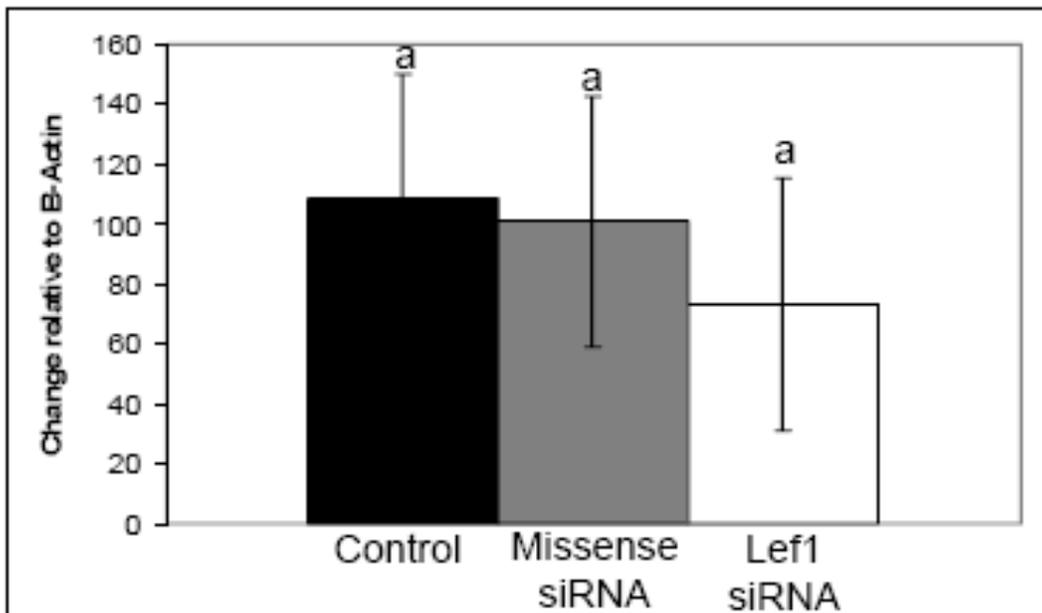


Figure 8: Summary of qRT-PCR data in the bovine

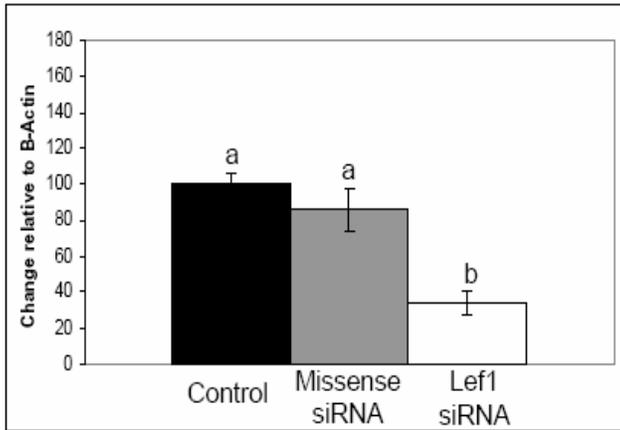
The table below is a summary of the transcription factors analyzed after transfection or injection of Lef1 siRNA in the different bovine cells collected. A “↓” indicates mRNA expression is significantly decreased after addition of the Lef1 siRNA when compared to the control specified above. A “↔” indicates no statistical difference in mRNA expression when compared to the control specified above. The “N/A” indicates that particular transcription factor was not analyzed because the mRNA was not expressed in the cells collected.

Bovine	Fetal fibroblasts		4-8 cell embryos
	<i>Change Relative to Control</i>	<i>Change Relative to Missense Control</i>	<i>Change Relative to Missense Control</i>
Lef1	↓	↓	↓
Cdx2	↓	↓	N/A
Nanog	N/A	N/A	N/A
Oct4	N/A	N/A	↔

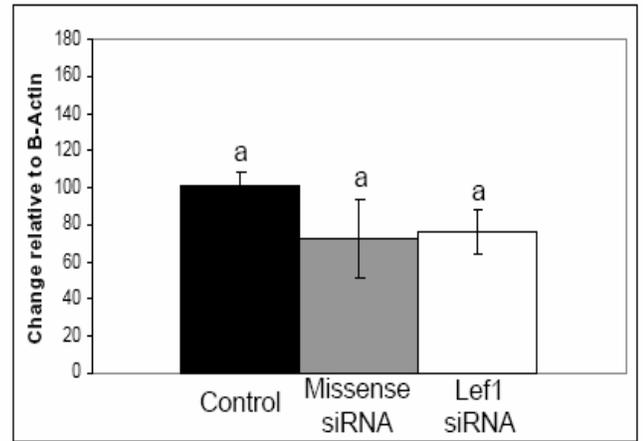
Figure 9: Validation of murine specific Lef1 siRNA in mouse ES cells

Mouse ES cells were transfected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control another group of cells were not transfected with either siRNA (black). Cells were collected 24-hours after transfection and total RNA was isolated. Quantitative real-time PCR was used to determine expression of Lef1, Cdx2, Nanog and Oct4 in these cells. Presented results are the mean \pm SEM of the change in Lef1, Cdx2, Nanog, and Oct4 expression relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin and an average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

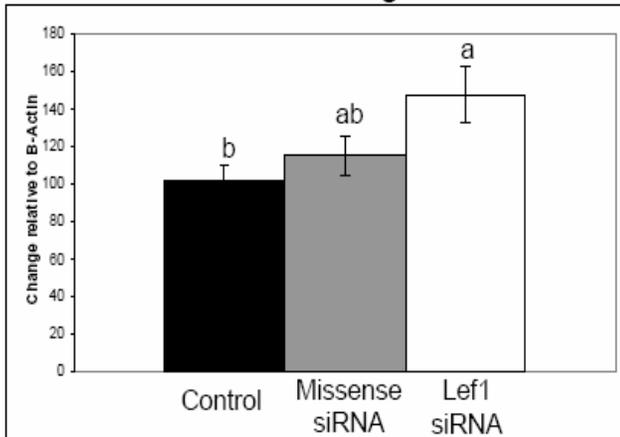
Lef1



Cdx2



Nanog



Oct4

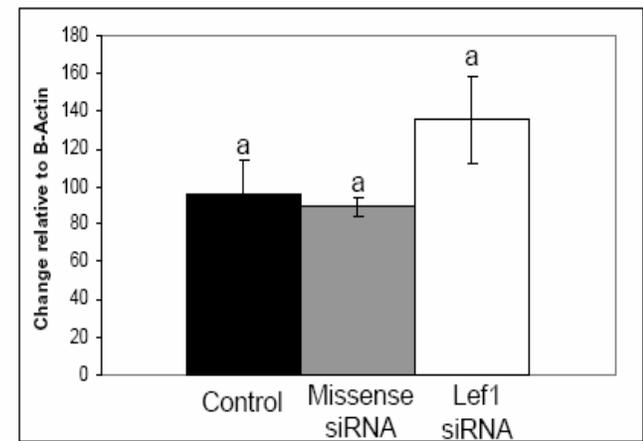


Table 3: Murine embryo cleavage rates after injection of siRNA

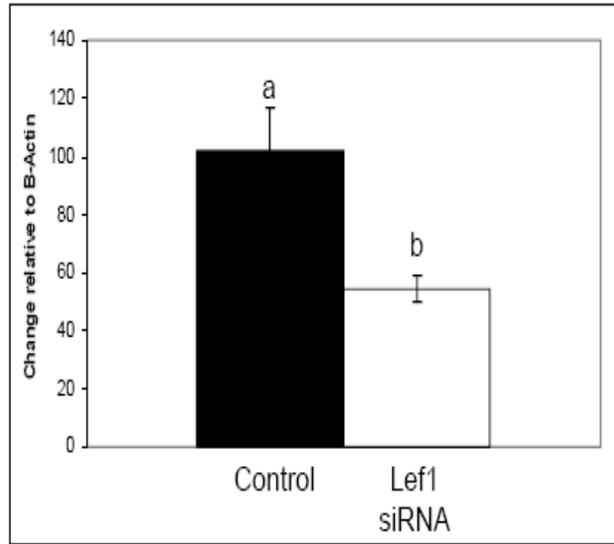
Below is a table indicating the total number of embryos used for each treatment group of injected embryos, as well as, the number of embryos cleaved the day after injections. Embryos that were lysed before cleaving into 2-cells were removed from the experiment and not taken into consideration for blastocyst rate. Also approximately 20 embryos from each treatment were removed at the 4-8 cell stage for processing. Different letters within column indicate treatments are significantly different at $P < 0.01$.

Treatment	Total	Cleaved	Percent cleaved	Percent to blastocysts
Control	139	134	96.4% ^a	70% ^a
Missense siRNA	183	106	57.9% ^c	48.5% ^b
Lef1 siRNA	175	128	73.1% ^b	52.4% ^{ab}

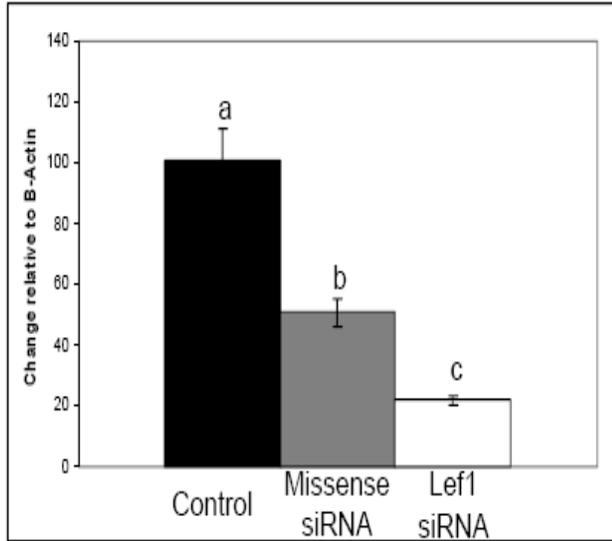
Figure 10: Mouse 4-8 cell embryos after Lef1 siRNA injection

Mouse 1-cell embryos were injected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control another group of cells were given no treatment (black). 4-8 cell embryos were collected 48-hours after injection and total RNA was isolated. Quantitative real-time PCR was used to determine expression of Lef1, Cdx2, and Oct4 in these cells. Presented results are the mean \pm SEM of the change in Lef1, Cdx2, and Oct4 relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin an average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

Lef1



Cdx2



Oct4

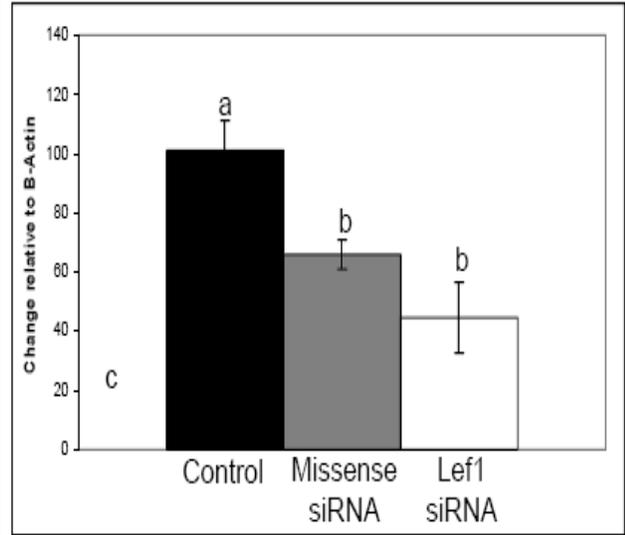


Figure 11: Murine blastocyst morphology after siRNA injection

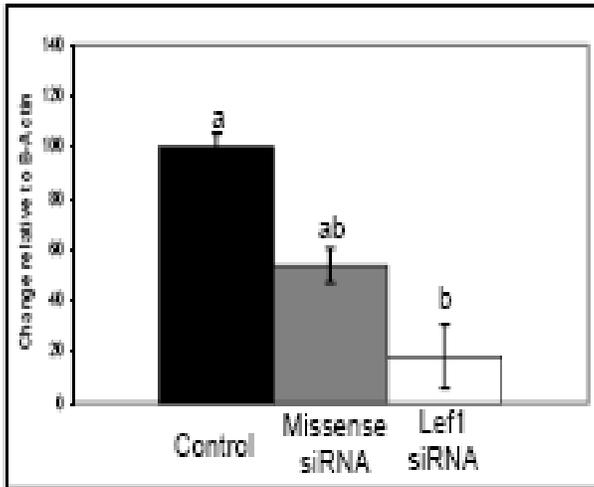
Embryos below are murine Day 4 blastocysts at 40x magnification. The embryo indicated by an “a” is from the control group in which no treatment was added. The “b” indicates an embryo from the missense group where missense siRNA was injected at the 1-cell stage. The “c” shows a representative from the Lef1 group that had Lef1 siRNA injected at the 1-cell stage.



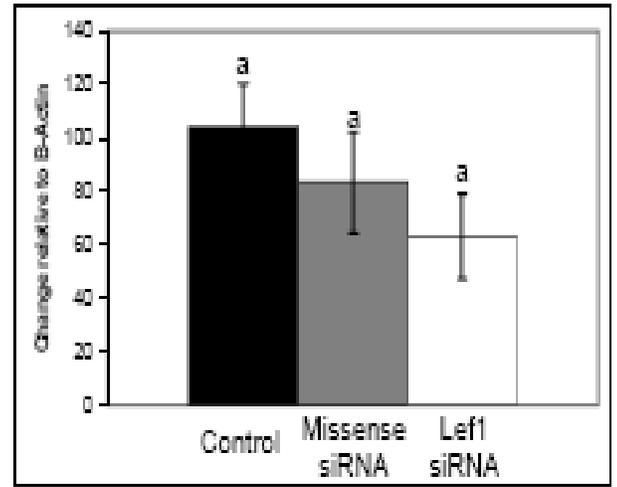
Figure 12: mRNA expression of key transcription factors in mouse blastocysts after siRNA injection

Mouse 1-cell embryos were injected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control another group of cells were given no treatment (black). Blastocysts were collected 3.5 days after injection and total RNA was isolated. Quantitative real-time PCR was used to determine expression of Lef1, Cdx2, Nanog and Oct4 in these cells. Presented results are the mean \pm SEM of the change in Lef1, Cdx2, Nanog, and Oct4 relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin and the average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

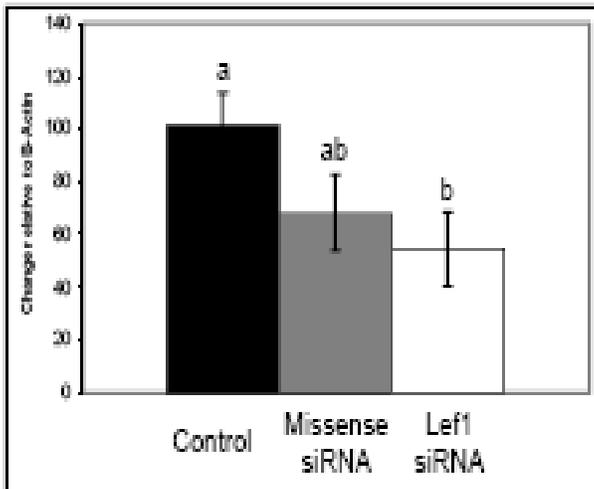
Lef1



Cdx2



Nanog



Oct4

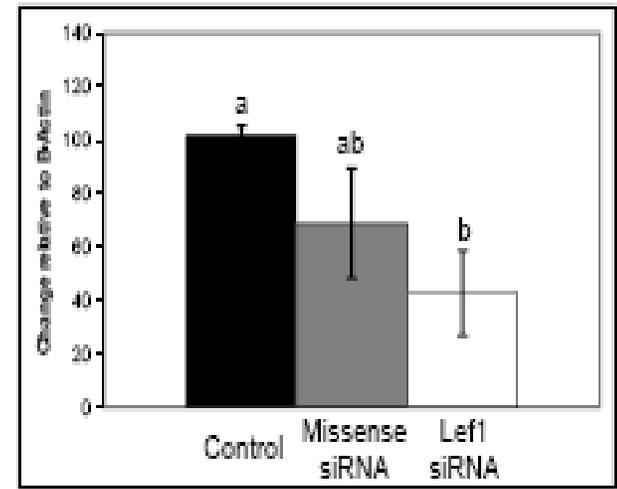
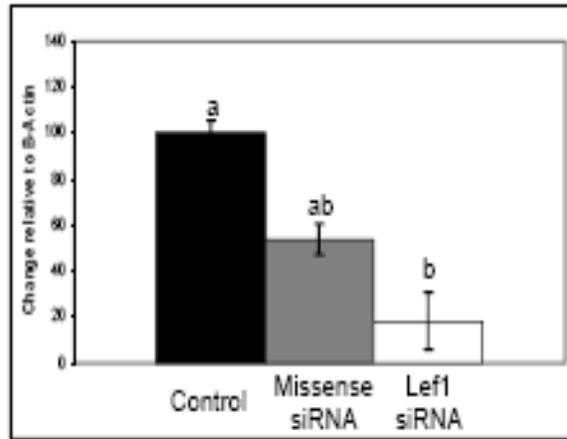


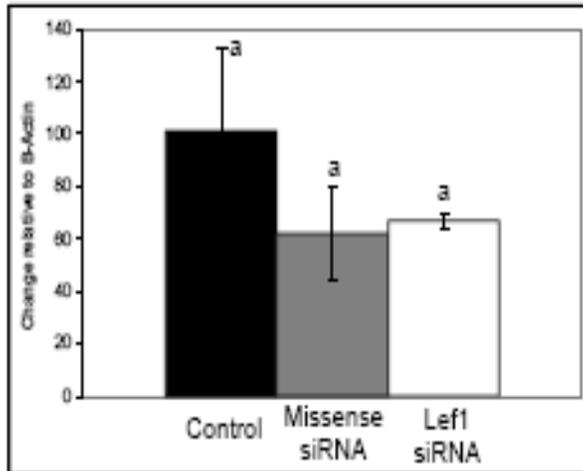
Figure 13: Expression of Tcf/Lef family members in mouse blastocysts after Lef1 siRNA injection

Mouse 1-cell embryos were injected with Lef1 specific siRNA (white) or non-specific missense siRNA (grey). As a control another group of cells were given no treatment (black). Blastocysts were collected 3.5 days after injection and total RNA was isolated. Quantitative real-time PCR was used to determine expression of Tcf3 and Tcf4 in these cells. Presented results are the mean \pm SEM of the change in Tcf3 and Tcf4 expression relative to β -Actin after transfection with missense or Lef1 siRNA. Both treatments were normalized to β -Actin and the average of the control treatments. Different letters within a gene indicate treatments are statistically different ($P \leq 0.05$).

Lef1



Tcf3



Tcf4

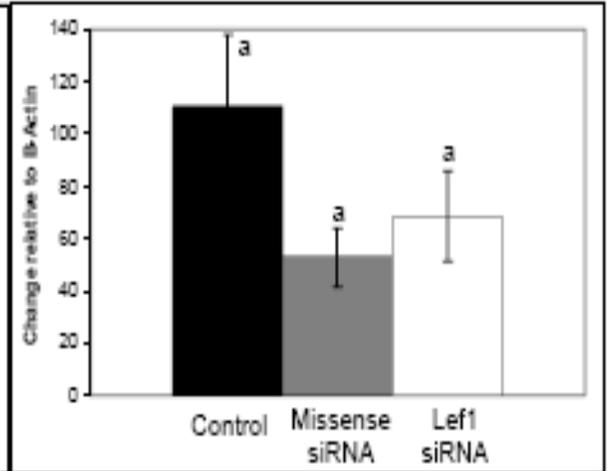


Figure 14: Summary of qRT-PCR data in the mouse

The table below is a summary of the transcription factors analyzed after transfection or injection of Lef1 siRNA in the different murine cells collected. A “↓” indicates mRNA expression was significantly decreased after addition of the Lef1 siRNA when compared to the control specified above. A “↔” indicates no statistical difference in mRNA expression when compared to the control specified above. The “N/A” indicates that particular transcription factor was not analyzed because the mRNA was not expressed in the cells collected. The star “*” denotes changes in expression were compared to the untreated control group because the missense control could not be analyzed in that set of data.

Murine	Embryonic stem cells <i>Change Relative to Control</i>	4-8 cell embryos <i>Change Relative to Missense Control</i>	Blastocysts <i>Change Relative to Missense Control</i>
Lef1	↓	↓	↓*
Cdx2	↔	↔	↓
Nanog	↑	↔	N/A
Oct4	↔	↔	↔
Tcf3	N/A	N/A	N/A
Tcf4	N/A	N/A	N/A

Appendices

Appendix Table 1: Numbers of bovine embryos collected to determine gene expression at different early development stages

	Oocyte	2-cell	4-8 cell	Morula	Blastocyst
Rep 1	20	20	20	20	9
Rep 2	20	20	20	20	-
Rep 3	20	20	20	20	10

Appendix Table 2: Numbers of bovine 4-8 cell embryos collected after siRNA injection

	Control	Lef1 siRNA	Missense siRNA
Rep 1	18	20	17
Rep 2	20	14	17
Rep 3	20	18	20
Rep 4	20	15	18

Appendix Table 3: Numbers of murine 4-8 cell embryos collected after siRNA injection

	Control	Lef1 siRNA	Missense siRNA
Rep 1	20	20	20
Rep 2	20	20	20
Rep 3	20	20	15

Appendix Table 4: Numbers of murine blastocysts collected after siRNA injection

	Control	Lef1 siRNA	Missense siRNA
Rep 1	15	10	3
Rep 2	6	9	5
Rep 3	11	7	9
Rep 4	15	14	13

Appendix Table 5: Bovine RT-PCR primer sequences

Primer	Sequence of forward and reverse primers 5'-3'	Annealing Temperature (°C)	Product size
β -Actin	TTGCTGACAGGATGCAGAAG TGATCCACATCTGCTGGAAG	56	147
Cdx2	AGTGAAAACCAGGACGAAAGACA CTCTGAGAGCCCCAGCGT	60	142
Lef1	GACGAGATGATCCCCTTCAA TTAGGTCGCTGTCAGTGTGG	56	760 & 670
Nanog	GGAAGTCTGGGGAAAATTA TACAAATCTTCAGGCTGTATGTTG	57	119
Oct4	AGGAGTCCCAGGACATCAA GCAGCTTACACATGTTCTTGAA	56	201

Appendix Table 6: Bovine qRT-PCR primer sequences

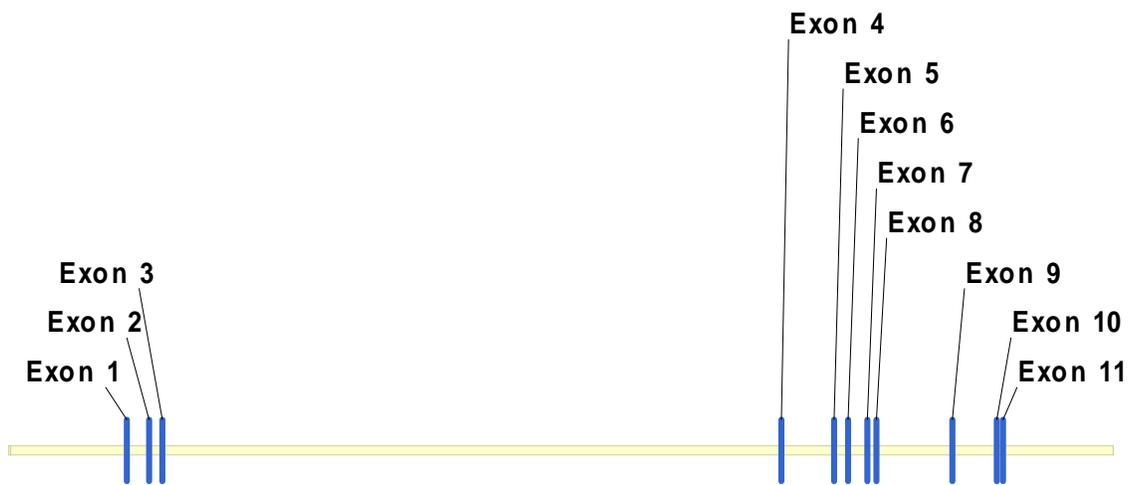
<u>Primer</u>	<u>Sequence of forward and reverse primers 5'-3'</u>	<u>Annealing Temperature (°C)</u>	<u>Product size</u>
β -Actin	TTGCTGACAGGATGCAGAAG TGATCCACATCTGCTGGAAG	56	147
Cdx2	AGTGAAAACCAGGACGAAAGACA CTCTGAGAGCCCCAGCGT	60	142
Lef1	GACGAGATGATCCCCTTCAA TGTCGGCTAAGTCACCTTCC	55	97
Nanog	GGAAGTGGCTGGGGAAAATTA TACAAATCTTCAGGCTGTATGTTG	57	119
Oct4	AGGAGTCCCAGGACATCAA GCAGCTTACACATGTTCTTGAA	56	201

Appendix Table 7: Mouse qRT-PCR primer sequences

<u>Primer</u>	<u>Sequence of forward and reverse primers 5'-3'</u>	<u>Annealing Temperature (°C)</u>	<u>Product size</u>
β -Actin	TTGCTGACAGGATGCAGAAG TGATCCACATCTGCTGGAAG	56	147
Cdx2	AGTGATTCCCTGGGGCTTCTT TGCCTCTGGCTCCTGTAGTT	59	278
Lef1 Total	TGAAGCCTCAACACGAACAG CGTGCACTCAGCTACGACAT	64	122
Nanog	CTCCAGAAGAGGGCGTCAGA CCTTTGGTCCCAGCATTGAG	61	285
Oct4	CCCGGAAGAGAAAGCGAACT ATAGCCTGGGGTGCCAAAGT	61	289
Tcf1	GCGAGGAACAGGACGATAAG CCTCGGCTTCTCCATGACT	55	~100
Tcf3	CTGTGCTCACCACCAAAC GGAGCGTAGCTGGGAAAGTA	55	~100
Tcf4	GATGACCTAGGCGCTAACGA GAATCGGAGGAGCTGTTTTG	55	~100

Appendix Figure 1: Bovine Lef1 gene

Below is the bovine Lef1 gene with exons and introns shown to scale using Vector NTI software.



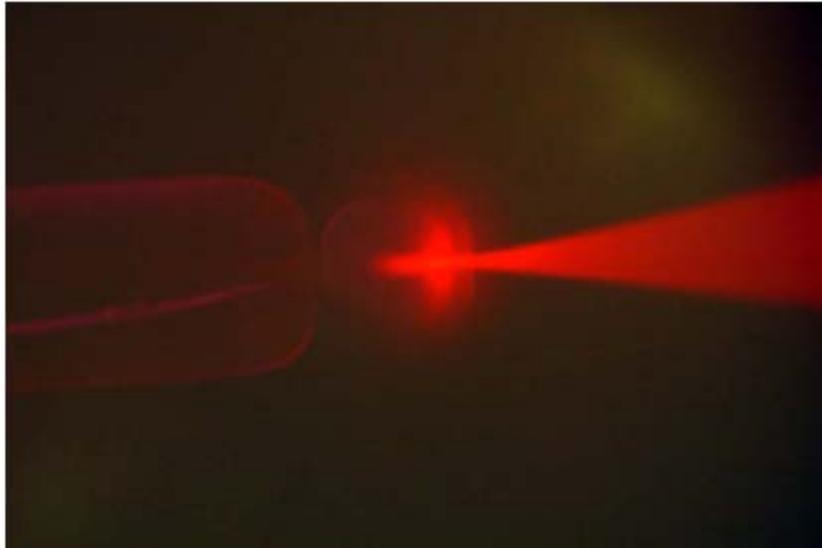

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human  AGGTTTTCCCATCATATGATTCCCGGTCTCCTGGTCCCCACACAACCTGGCATCCCTCAT 780
mouse  AGGTTTTCCCATCATATGATTCCCTGGTCCCCCTGGCCCCACACAACCTGGCATCCCTCAT 774
*****
bovine CCAGCCATTGTAACGCCTCAGGTCAAACAGGAACACCCCCACACTGACAGCGACCTAATG 840
human  CCAGCTATTGTAACACCTCAGGTCAAACAGGAACATCCCCACACTGACAGTGACCTAATG 840
mouse  CCAGCTATTGTAACACCTCAGGTCAAACAGGAGCACCCCCACACGGACAGTGACCTAATG 834
*****
bovine CACGTGAAGCCTCAGCATGAACAGAGAAAAGGAGCAGGAGCCAAAAAGACCTCACATTTAA 900
human  CACGTGAAGCCTCAGCATGAACAGAGAAAAGGAGCAGGAGCCAAAAAGACCTCACATTTAA 900
mouse  CACGTGAAGCCTCAACACGAACAGAGAAAAGGAGCAGGAGCCAAAAAGACCTCATATTTAA 894
*****
bovine AAGCCTCTGAATGCTTTTATGTTATACATGAAAGAAATGAGAGCGAATGTCGTAGCTGAG 960
human  AAGCCTCTGAATGCTTTTATGTTATACATGAAAGAAATGAGAGCGAATGTCGTTGCTGAG 960
mouse  AAGCCTCTGAATGCTTTTATGTTATATATGAAAGAAATGAGAGCGAATGTCGTAGCTGAG 954
*****
bovine TGTACTCTAAAGGAAAGTGCAGCTATCAACCAGATTCTAGGCAGAAGGTGGCATGCCCTC 1020
human  TGTACTCTAAAGGAAAGTGCAGCTATCAACCAGATTCTTGGCAGAAGGTGGCATGCCCTC 1020
mouse  TGCACGCTAAAGGAGAGTGCAGCTATCAACCAGATCCTGGGCAGAAGATGGCACGCCCTC 1014
** *
bovine TCTCGTGAAGAGCAGGCTAAATATTATGAATTAGCGCGGAAGGAAAGACAGCTACATATG 1080
human  TCCCGTGAAGAGCAGGCTAAATATTATGAATTAGCACGAAAGGAAAGACAGCTACATATG 1080
mouse  TCCCGGGAAGAGCAGGCCAAATACTATGAACTAGCACGAAAGAGAGACAGCTACACATG 1074
** *
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human  CAGCTTTATCCAGGCTGGTCTGCAAGAGACAATTATGGTAAGAAAAAGAAGAGGAAGAGA 1140
mouse  CAGCTTTATCCAGGCTGGTCTGCAAGAGACAATTATGGCAAGAAGAAGAAGAGGAAGAGA 1134
*****
bovine GAGAAGCTACAGGAATCCACATCAGGTACAGGTCCAAGAATGACAGCTGCCTACATCTGA 1200
human  GAGAACTACAGGAATCTGCATCAGGTACAGGTCCAAGAATGACAGCTGCCTACATCTGA 1200
mouse  GAGAAGCTACAGGAGTCGACTTCAGGTACAGGTCCCAGAATGACAGCTGCCTACATCTGA 1194
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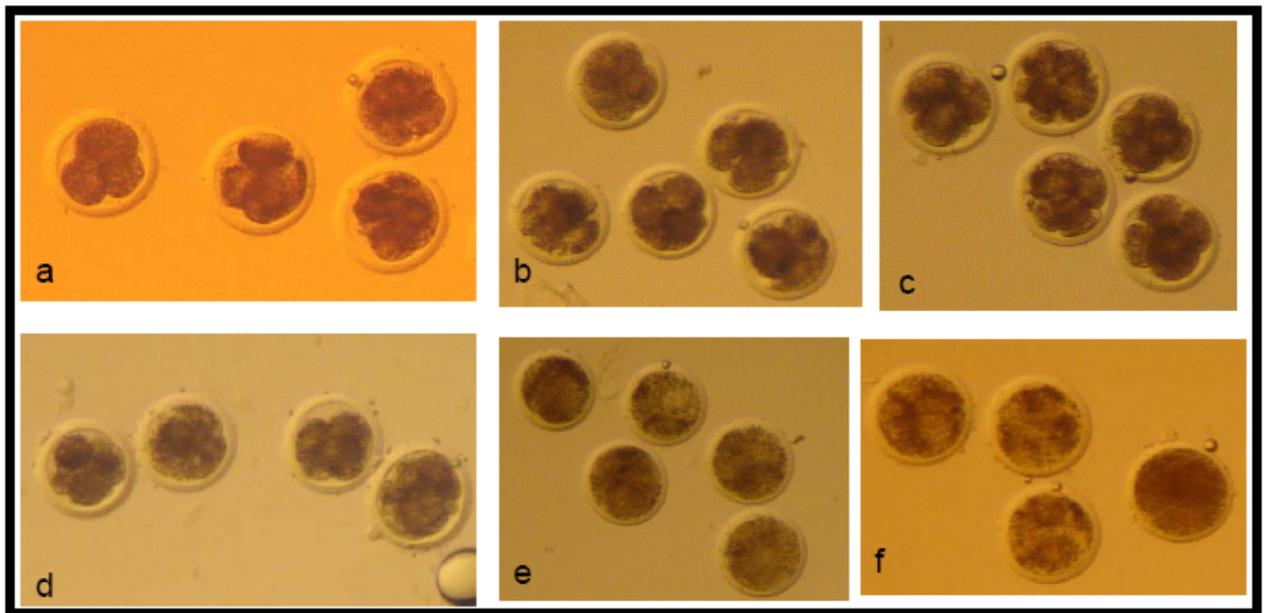
Appendix Figure 3: Injection of siRNA into bovine embryo

Below is a 40x magnification image of a bovine embryo being injected with siRNA-TRD. The siRNA-TRD diffuses throughout the cytoplasm as indicated by the red.



Appendix Figure 4: Direct incubation pilot study

Below are images of bovine embryos used in a pilot study to determine if bovine embryos could be cultured in 50 μ M of siRNA and survive. A-C are 4-cell embryos before the addition of siRNA to the 50 μ L culture drop of G1 media. D-E are the same embryos 22 hours after the addition of siRNA to the culture media. Groups A and D are the control embryos where nothing was added to the media. B and E had 50 μ M of non-specific missense siRNA added to the 50 μ L drop. C and F are the Lef1 group of embryos that had 50 μ M of Lef1 siRNA added to the 50 μ L drop.



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