COMPARISON OF GENE EXPRESSION IN PRE-IMPLANTATION BOVINE EMBRYOS EITHER INJECTED OR TRANSFECTED WITH A siRNA TARGETED AGAINST E-CADHERIN

A Dissertation

by

CAROL BAILEY MCCORMICK HANNA

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

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Committee,	Duane Kraemer
Committee Members,	William Foxworth
	Thomas Spencer
	Mark Westhusin
Head of Department,	Gary Acuff

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ABSTRACT

Comparison of Gene Expression in Pre-implantation Bovine Embryos Either Injected or Transfected with siRNA Targeted Against E-cadherin. (August 2008)

Carol Bailey McCormick Hanna, B.S., Colorado State University;

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Chair of Advisory Committee: Dr. Duane Kraemer

The ability to create transgenic livestock is a tremendous benefit in scientific research for many disciplines including functional genomics, pharmaceutical synthesis and development of enhanced production animals. Transgenes can either be stably or transiently expressed to alter gene function and obtain a specifically engineered phenotype. To create a transgenic bovine embryo, genetically altered somatic cells must be used in somatic cell nucleus transfer, or early 1-cell embryos (zygotes) must be microinjected with plasmid DNA or small interfering RNA (siRNA). Given the cost and skill associated with both methods, a preliminary investigation exploring alternative delivery techniques of siRNA (transient expression) into bovine zygotes with a non-homologous Cy3 labeled siRNA (Cy3-siRNA) was first performed. It was discovered that zygotes injected with more than 50 μmol L^{-1} of Cy3-siRNA fail to form a blastocoel and that, although bovine zygotes are not susceptible to chemical transfection, the trophectoderm cells of the blastocyst are. Based on this information, bovine E-cadherin gene expression was compared in day 9 blastocysts derived from either injected zygotes

(day 1) or transfected blastocysts (day 7) with a Cy3 labeled E-cadherin specific siRNA (Cy3-siEcad) to determine 1) if gene suppression in zygotes injected with 25 μmol L^{-1} Cy3-siEcad continues during embryo development up to hatching, and 2) if blastocysts transfected at a ratio of 9:6 with GeneJammer® truly experience gene knock down after siRNA transfection capable of maintaining suppression to day 9. Quantitative PCR indicated blastocysts transfected with Cy3-siEcad had a significant 15.3% decrease ($P < 0.05$) in E-cadherin mRNA at day 9 compared to the injected zygotes. Protein fluorescence analysis from immunocytochemistry of whole mounted day 9 blastocysts revealed injected zygotes accumulated significantly less E-cadherin protein (67.7%) than the transfected blastocysts ($P < 0.05$). From these data, it can be concluded that although siRNA injection may be capable of knocking down gene expression for the first 7 days of embryonic development, it does not persist to the hatching stage; however, blastocysts transfected at day 7 do express altered gene expression in the trophectoderm which can continue through embryonic hatching events.

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I would like to thank my committee chair, Dr. Duane. Kraemer, and my committee members, Dr. William Foxworth, Dr. Thomas Spencer, and Dr. Mark Westhusin, for their patience, guidance and support throughout the course of this research. I would also like to thank Dr. Charles Long for the endless time and direction he contributed to my project.

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Finally, thank you to the members of the Reproductive Sciences Laboratory for their comradery and support through out my tenure at Texas A&M.

NOMENCLATURE

dsRNA	Double Stranded RNA
ES Cells	Embryonic Stem Cells
GFP	Green Fluorescent Protein
IVP	*In Vitro* Produced (Embryo)
miRNA	Micro RNA
PCR	Polymerase Chain Reaction
PTGS	Post-transcriptional Gene Silencing
RNAi	Ribonucleic Acid Interference
SCNT	Somatic Cell Nucleus Transfer
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
ssRNA	Single Stranded RNA
USDA	United States Department of Agriculture
Zygote	One-cell Embryo

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CHAPTER I

INTRODUCTION

The first report describing the creation of a genetically modified animal was in 1975 (Jaenisch *et al.* 1975) when pronuclear stage zygotes were injected with foreign viral DNA to produce a viable transgenic mouse. A decade later, in 1985, the crossover from laboratory animals to livestock was made with the creation of the first transgenic rabbits, sheep and pigs (Hammer *et al.* 1985), leading the way for the production of other modified livestock species including cattle (Kuroiwa *et al.* 2004), goats (Reggio *et al.* 2001), chickens (Houdebine 2008), and even fish (Lee and Cole 2007) in the years to follow. These events have transformed research methodology, spurring transgenesis to become a powerful tool for biotechnological advancement in mammalian species, encompassing many fields in both science and medicine.

Applications for transgenic technology

Functional genomics studies rely heavily on transgenic mice to define gene targets and describe their function for translation into human genomics (Silvestri *et al.* 2008). In fact, several institutions have been established to provide researchers with specific gene modified mice including the Texas Institute of Genomic Medicine in College Station, Texas and The Jackson Laboratory in Bar Harbor, Maine. These transgenic mice are typically produced with a gene of interest knocked out either

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This dissertation follows the style of *Reproduction, Fertility, and Development*.

by an induced mutation which prevents gene expression, or by complete deletion of the gene. Phenotypic and sub-cellular differences may be detected which help define specific regulatory functions and pathways associated with a particular gene. Additionally, knockout mice are created to produce animal models which express symptoms analogous to human disorders allowing investigators to develop potential therapeutic strategies for disease treatments (Liu *et al.* 2008).

Pharming, a process where specific proteins are produced in the mammary glands of transgenic animals and purified from the collected milk, has been utilized in several species (Niemann and Kues 2007). Dairy animals such as cattle and goats are the most common types of transgenic animals produced due to the substantial yield of a single milking (Niemann and Kues 2007; Poirier and Blancho 2008). Several companies have been established which are dedicated to producing pharmed products for commercially available therapeutics. Netherlands based, *Pharming*, uses transgenic cows to produce Rhucin®, a recombinant human C-1 inhibitor, to treat hereditary angioedema. Similarly, *GTC Biotherapeutics* in Farmington, Massachusetts, uses transgenic goats to pharm ATryn®, a recombinant human antithrombin III to treat deep vein thrombosis. In addition to cattle and goats, mice (Nuijens *et al.* 1997), rabbits (Choi *et al.* 2007), and pigs (Houdebine 2008) have been successfully pharmed by commercial businesses to produce transgenic proteins, although their use is not as popular.

In some cases, the genetic modifications expressed in mammary tissues serve to benefit the animal. It has been demonstrated that milk nutrients in transgenic pigs can be

modified to include bovine alpha-lactalbumin which increases the amount of lactose available to the piglets during the first 12 days postpartum (Noble *et al.* 2002). Piglets from these transgenic gilts had more nutrient rich milk available to them, significantly increasing their growth and survival rates as compared to control piglets from non-transgenic gilts. In cattle, researchers at the USDA inserted a gene from *Staphylococcus simulans* which was designed to be expressed in the tissues of the mammary glands (Rexroad *et al.* 2007). The gene product enhanced the intramammary resistance to *Staphylococcus aureus* infection and reduced the animal's susceptibility to mastitis, a debilitating condition in the dairy industry. In both cases, the utilization of transgenic animals in a production setting greatly benefited animal health which ultimately may translate into an increase in economic gain.

Substantial progress has been made in the field of xenotransplantation since the incorporation of transgenic animals to produce suitable donors for organ xenografting. By knocking out the gene for native alpha 1,3-galactosyltransferase in pigs and inserting the human gene for alpha 1,2-fucosylosyltransferase, it may be possible to produce transgenic porcine models with organs that do not elicit hyperacute rejection in the recipient (Prather 2007). Although this approach has not yet been evaluated in human xenotransplant recipients, it is believed that eventually this transgenic strategy has the potential to circumvent delayed xenograft rejection events and greatly increase recipient survival rates (Ramsoondar *et al.* 2003).

Transgenic technology has also been realized as a solution to environmental concerns. Manure based phosphorus pollution into the environment from pig production

farms has become a problem. To address this issue, scientists in Canada have created transgenic pigs which carry the gene for the enzyme phytase, expressed in the saliva (Golovan *et al.* 2001). Phytase allows the pigs to digest the phytate in their diet which would otherwise pass undigested into the manure as phytate phosphorus and contribute to environmental pollution. When compared to non-transgenic controls, phytase pigs had 75% less phosphorus in their manure and it is expected that these environmentally friendly animals will enter the commercial market within the next few years (Niemann and Kues 2007).

Transgenic animal development

By definition, a transgenic animal possesses a segment of foreign DNA incorporated into the genome or has undergone genomic modification by artificial induction (Melo *et al.* 2007). Although there have been many strategies developed, transgenesis in the mouse is most commonly accomplished by targeting specific genes in pluripotent embryonic stem (ES) cell cultures, and then transferring the transgenic ES cells into an unaltered mouse blastocyst (Schindehutte *et al.* 2005). The blastocyst is then transferred into a recipient female and germline chimeric offspring are produced which are successively bred until a homozygous transgenic individual is obtained (Nagy *et al.* 2003).

However, ES cell cultures are difficult to establish in non-rodent models, eliminating the use of these pluripotent cells for gene targeting in most other species (Renard *et al.* 2007). Furthermore, it has been estimated that performing the necessary

multiple breedings in order to acquire the homozygous individual could take up to six years in cattle (Yang *et al.* 2004). Combined with the expense involved to maintain a breeding herd for that length of time, this strategy is not efficient enough to use in livestock based scientific research. However, a strategy was described where serial somatic cell nucleus transfer (SCNT) could be utilized with modified differentiated somatic cells to create transgenic cattle (Yang *et al.* 2004). Although the time to produce a homozygous animal was reduced to almost two years, this strategy is not preferential for most as it can only be performed in a specialized laboratory setting and is dependent on the inefficient process that is SCNT.

In 1998, a seminal paper was published which defined RNA interference (RNAi) for the first time as a technique for knocking down gene expression in *Caenorhabditis elegans* (Fire *et al.* 1998). This technology was quickly adapted for research in many species because it allowed for direct modification to somatic cells and embryos, and alleviated the need for successive breedings to obtain an affected individual. This ability to shorten production time hallmarked RNAi as the reasonably preferred technique to generate transgenic livestock models for scientific investigation.

Discovery of RNA interference

The first actual account of RNAi mediated gene suppression was reported in petunias and attributed to the unknown, but DNA methylation or paramutation between alleles was suspected (Napoli *et al.* 1990). Researchers were attempting to darken petal color by over expressing chalcone synthase (CHS), an enzyme responsible for

pigmentation, by introducing a chimeric petunia CHS gene. Instead of a darker violet color in the petals, researchers observed an ablation of color which they termed "co-suppression" and determined the cause to be a decrease in CHS RNA.

Shortly after the discovery in petunias, researchers introducing homologous sequences into the fungus *Neurospora crassa* were able to reverse albino coloration by silencing the al-1 and al-3 genes (Romano and Macion 1992). However, as with the petunia, the gene silencing pathway was not fully understood and the investigators termed the phenomenon as "quelling".

Finally, eight years after the first report in petunias, Fire *et al*. 1998 published a report in *Nature* identifying double stranded RNA (dsRNA) as a potent and specific inhibitor of gene expression and termed the process "RNA interference". The discovery of RNAi was serendipitous as the interfering double stranded RNA was actually the control in the experiments. Sense and antisense single stranded RNA (ssRNA) were under evaluation to determine the most effective ssRNA configuration to induce gene silencing. However, investigators discovered that the dsRNA control out performed the ssRNA sustaining much higher levels of gene suppression leading the investigation to eventually determine the structure and delivery of the interfering dsRNA. Since its discovery, RNAi has been incorporated in over 5,400 reports of gene modification studies and is widely considered a dependable and powerful tool in transgenic technology, encompassing many types of research models including, but not limited to, the mouse (Goa and Zhang 2007), rat (Berhanu and Rush 2008), fruit fly (Chen *et al.* 2008), cattle (Kobayahshi *et al.* 2007), zebrafish (Gruber *et al.* 2005), chicken

(Wakamatsu *et al.* 2007), and even humans (Nakanishi *et al.* 2008). In 2006, Drs. Andrew Fire and Craig Mello received the Nobel Prize in Medicine and Physiology for their discovery and description of siRNAs, thus lending credence to the colossal impact RNAi technology has had on science and transgenic research to date.

Pathways of RNAi expression

RNAi is a highly conserved evolutionary process which utilizes double stranded RNA to induce silencing of specific genes. Processing of the interfering RNA strands can initiate either in the nucleus or the cytoplasm, depending on the configuration and source of the RNA. The most common types of RNA molecules utilized in research are small interfering RNA (siRNA) or short hairpin RNA (shRNA), the latter of which is based on the structure of the innate RNAi particle, micro RNA (miRNA). Expression of these RNAs can be either transient or stable, depending on the characteristics of the target, delivery method, and experimental need. Additionally, depending on the target mRNA, the effects of RNAi may be reversible, irreversible and in some cases lethal.

Micro RNA participates in a highly conserved endogenous gene silencing pathway found in most species, and has been estimated to be directly involved in about 90% of gene regulation in humans (Perron and Provost 2008). Predictions based on bioinformatics suggest that miRNA genes constitute roughly 2% of known human genes, 50% of which are localized to noncoding RNA transcripts or are nested within the introns of other coding genes (Perron and Provost 2008; Ross *et al.* 2007). In the nucleus of a cell, RNA polymerase II transcribes the miRNA DNA sequence to produce

primary miRNA (pri-miRNA) which forms long tandem repeats of stem-loop RNA structures (Lee *et al.* 2004) (Figure 1). The ribonuclease, Drosha, then cleaves off individual stem-loop structures roughly 70 nucleotides (nt) in length and makes further modifications to form the miRNA precursors, pre-miRNAs (Lee *et al.* 2002). After formation, pre-miRNAs are shuttled to the cytoplasm by the protein Exportin-5 in what is considered the rate limiting event for miRNA guided gene silencing (Yi *et al.* 2005).

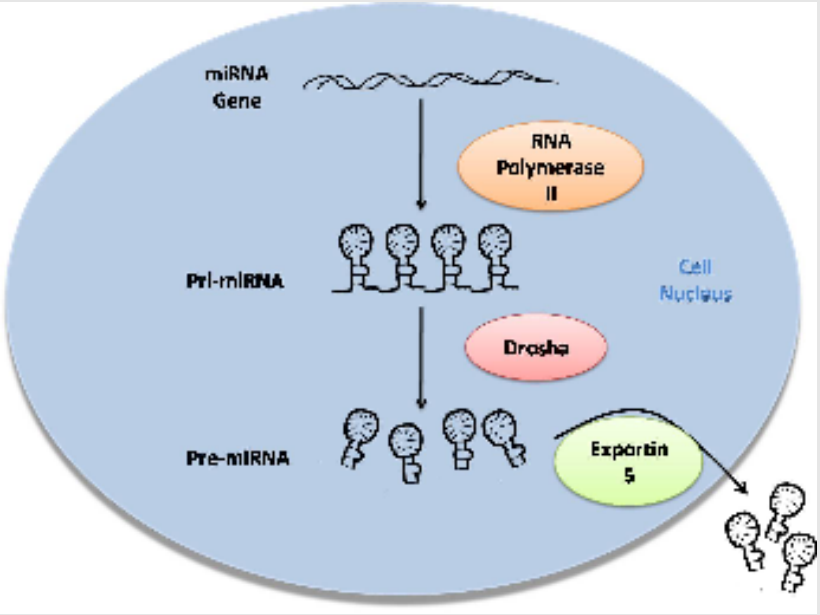

Fig. 1 MicroRNA processing in the nucleus. Genes encoding miRNA are transcribed by RNA polymerase II resulting in stem-loop formation of the pri-miRNA. Individual hairpins are cleaved by the RNase Drosha forming pre-miRNA and shuttled to the cytoplasm by Exportin 5.

Once in the cytoplasm, another ribonuclease, Dicer, generates about 21-23 nt dsRNA fragments now considered mature miRNA duplexes which typically have incomplete homology to their target mRNA (Bernstein *et al.* 2001) (Figure 2). Mature miRNA are loaded into the RNA-induced silencing complex (RISC), guide RISC to the target mRNA, and induce translational repression of the sequence (Martinez *et al.* 2002). Once inhibited, the mRNAs are transported to P-bodies where they are either rescued and reincorporated into a translational pathway, or ultimately, are degraded after an unspecified period of accumulation (Perron and Provost 2008).

Originally, post-transcriptional gene silencing (PTGS) mediated by siRNA was accomplished by introducing long dsRNA into the cell cytoplasm where the RNase III, Dicer, cleaves the long dsRNA into 21-25 nt duplexes to form the siRNA molecule (Zamore *et al.* 2000). However, in mammalian cells, the long dsRNA can resemble foreign viral RNA and elicit interferon-mediated nonspecific gene silencing (Kawasaki *et al.* 2005) activating the protein kinase PKR and initiating an immune response (Gao and Zhang 2007). To circumvent this reaction, synthetic siRNAs 21 nt long, with a 2 nt 3' overhang and complete homology to the target mRNA, can be directly produced and introduced into the cellular cytoplasm (Elbashir *et al.* 2001). Similar to miRNA, siRNA are loaded into RISC and identify the endogenous mRNA target (Figure 3). However, unlike miRNA, the complete homology of the siRNA signals degradation of the target by the RISC associated enzyme, Argonaute, which catalyzes the cleavage and destruction of the mRNA (Faehnie and Joshua-Tor 2007). siRNAs have become exceptionally popular and accessible for functional genomics studies and many

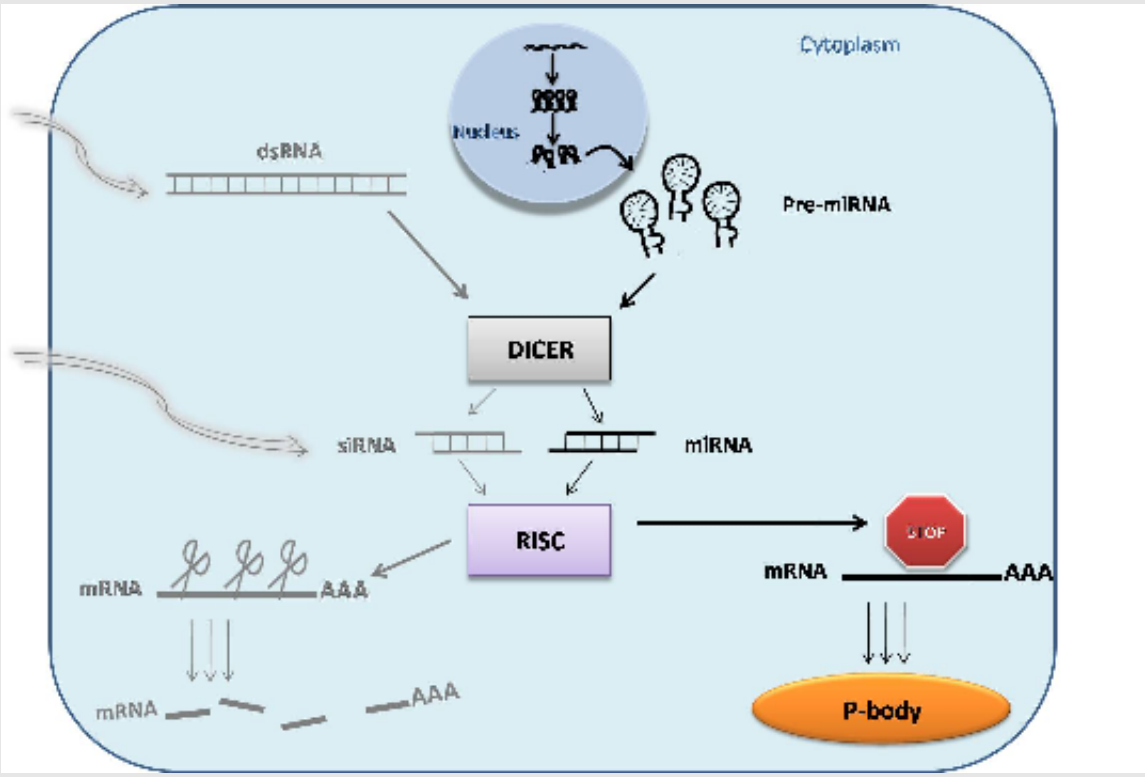

Fig. 2 MicroRNA processing in the cytoplasm. Dicer cleaves the loop off pre-miRNA to form mature miRNA that loads into RISC and prevents translation of the target mRNA sequence. Inhibited mRNAs are then stored in the P-body until degradation or re-circulation.

companies such as Ambion, Austin, Texas or Dharmacon, Chicago, Illinois now design and produce them as customized research tools. Furthermore, with an effective sequence, siRNAs are able to reduce gene expression in a multitude of cell types by an

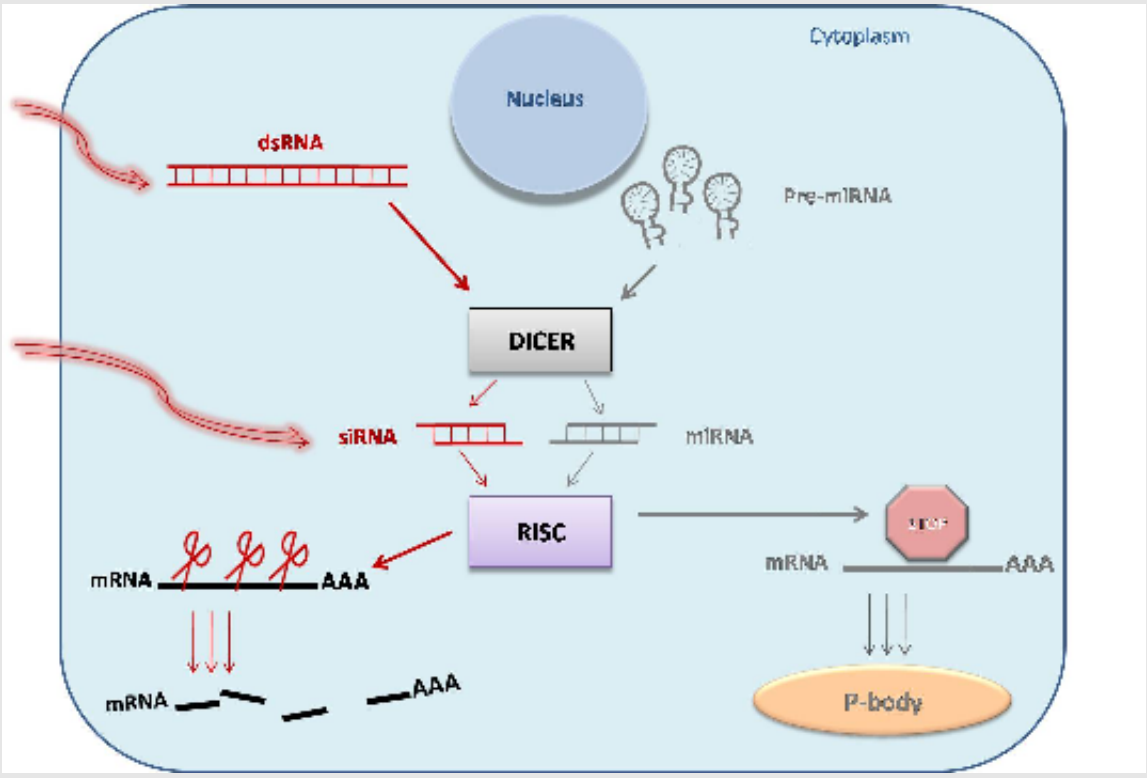

Fig. 3 siRNA processing in the cytoplasm. Double stranded RNA is cleaved by Dicer to form siRNAs which guide RISC to target mRNA for degradation preventing translation.

average of 75% (Gou *et al.* 2007) proving it to be a reliable method for research. Both miRNA and siRNA typically generate a transient effect in gene suppression, however, depending on the method of delivery a third type of RNAi inducing molecule known as shRNA, can provide either transient or stable PTGS. Short hairpin RNAs

have been found by some to be more efficient than siRNAs for inducing gene silencing, and because they are transcribed from a vector, can be continuously produced in the laboratory making them less costly than commercially produced siRNAs (Cheng and Chang 2007). Design and production of synthetic shRNAs incorporates nucleotide sequences found in miRNA to promote appropriate folding and loading of the shRNA into Dicer. A typical coding sequence for shRNA includes a common miRNA context region at both the 5' and 3' ends with a 22 nt sense-mi RNA loop-22 nt antisense sequence nestled in between (Figure 4), which when transcribed forms a RNA stem-loop structure similar to pre-miRNA (Paddison *et al.* 2004). This sequence is inserted into an expression vector under a RNA polymerase III promoter and may also include reporting sequences for fluorescent proteins or antibiotic resistance used in selection and diagnostics.

Fig. 4 RNA coding sequeances for shRNA. Common miRNA sequences form the 5' and 3' ends and the loop structure. Both the sense and antisense strands are included, but the antisense strand incorporates with RISC to induce translational silencing.

Transient expression of the shRNA requires similar processing as for miRNA (Figure 5). The shRNA vector is delivered to the cellular cytoplasm where the hairpin sequence is eventually transcribed *in vivo*, modified by Dicer to produce siRNA, and loaded into RISC for targeted mRNA translational inhibition (Chang *et al.* 2006).

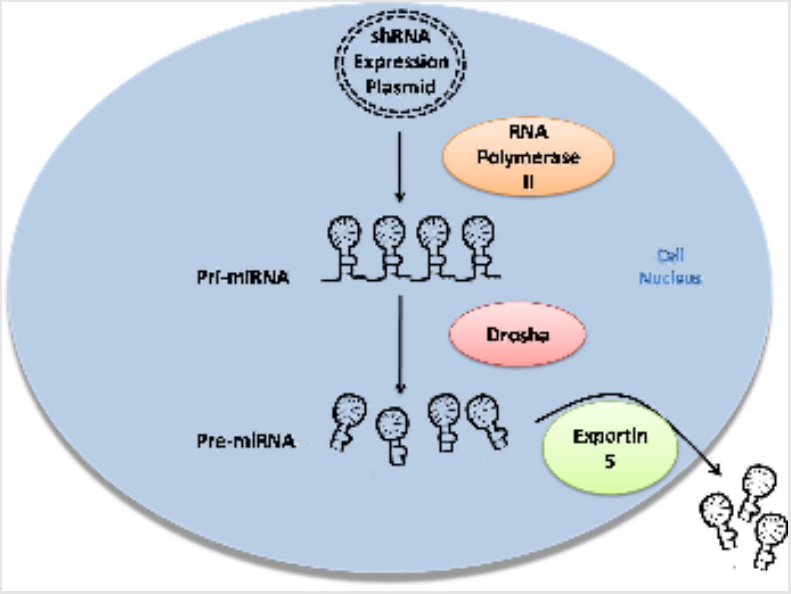

Fig. 5 shRNA expression plasmid processing. Plasmids containing a shRNA sequence are processed similar to miRNA by polymerase II to form pri-miRNA. Drosha cleaves individual pre-miRNAs which are transported to the cytoplasm by Exportin 5.

If stable expression of the shRNA is required, specific types of modified virus can be utilized to deliver and integrate the shRNA sequence into the host genome. The most

commonly used viral delivery system is the lentivirus, a member of the *retroviridae* family (Park 2007). Lentiviral vectors used in research are typically modified from the HIV-1 backbone sequence, are non-replicative, and capable of infecting both dividing and non-dividing cells. Along with the transfer plasmid containing the shRNA, two other plasmids containing the *gag/pol* and *env* viral gene sequences are co-transfected into human embryonic kidney 293 cells. The HEK293 cells transcribe the plasmids and produce multiple copies of the RNA hairpins and virus specific enzymes necessary for host genome integration, package them in the viral envelope, and release the infecting particles into the 293 culture medium (Tiscornia *et al.* 2006). Virus is collected from the medium and applied to various cell types including somatic cell cultures, oocytes, and embryos (Cockrell and Kafri 2007; Pfeifer *et al.* 2002). The shRNA sequence is integrated into the host genome and constitutively transcribed in the nucleus to produce the designed hairpin capable of entering the pre-miRNA processing pathway and successfully inducing post-transcriptional gene silencing (Figure 6).

Application for RNAi in livestock

Among the various fields of research, RNAi has become a focus for many interested in disease pathology, control and treatment. Translational research has demonstrated success utilizing small animal models such as rodents or diverse cell cultures to create disease models and evaluate RNAi based therapies for HIV, Hepatitis C virus, wound repair, angiogenesis, gene specific treatments, and bone related illnesses (Bhindi *et al.* 2007; Cheema *et al.* 2007; Hadj-Slimane *et al.* 2007; Li *et al.* 2007; Pan *et*

al. 2007; Yeung *et al.* 2007). However, the advent of RNAi technology has also allowed researchers to further explore livestock species as animal models due to the decreased production time required to obtain a genetically altered animal compared to more traditional methods.

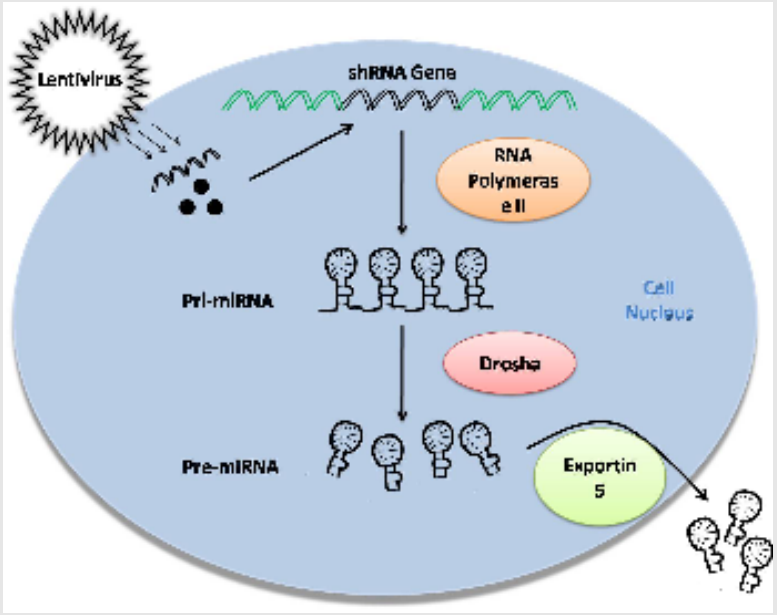

Fig. 6 Lentiviral delivery of shRNA. Viral enzymes for reverse transcription of the RNA sequence and integration into the host genome are delivered along with the shRNA sequence. Once integrated the shRNA gene sequence is transcribed and enters the miRNA processing pathway.

Lentiviral delivery of shRNAs targeted against the prion gene has been used to stably transform caprine somatic cell cultures for SCNT (Golding *et al.* 2006). The resulting conceptus expressed 90% less prion protein than the non-treated conceptus, suggesting that future animals generated by this protocol could be resistant to transmissible spongiform encephalopathy (TSE), a contagious and sometimes fatal disease in goats. Investigators have also begun defining protocols to establish prion knockout models in both bovine and ovine model systems using similar lentiviral delivery methods to create animals resistant to TSE and scrapie with similar success (Pfeifer *et al.* 2006; Sutou *et al.* 2007). New applications of RNAi in livestock research are emerging and as this trend continues, it can be expected that reports describing successful development of disease models and therapy directives are quickly approaching.

RNAi in ova and pre-implantation embryos

Lentivirus mediated delivery is an effective method to induce stable shRNA based gene silencing into cloned animals, however it is not a suitable protocol for all research scenarios. The logistics involved to produce a single cloned animal requires abundant time, money, and skill, resources which are not available to all laboratories. Therefore, using the porcine and bovine models, strategies have been developed to directly treat oocytes and early embryos with lentivirus. Pigs homogenously expressing green fluorescent protein (GFP) have been created by injecting lentivirus carrying the GFP gene into the perivitelline space of zygotes. Of the total zygotes injected, 65% expressed the transgene and resulting offspring from embryo transfers produced GFP in

the germ cells (Hofmann *et al.* 2003). Injection of a self-inactivating lentiviral vector containing an enhanced GFP reporter in the perivitelline space has been shown to successfully infect bovine oocytes and zygotes with 83% and 22%, respectively, expressing GFP and giving rise to live transgenic offspring which retained the transgene after 5 months of age (Hofmann *et al.* 2004). Further studies have shown that successful infection of both bovine oocytes and zygotes can also be achieved by compromising the zona with laser drilling and co-incubating with lentivirus. Although there was no observable difference in expression rates of the reporter gene (GFP) between microinjection below the zona and co-incubation in zygotes (26% and 26%, respectively), a greater proportion of subzonal virus injected oocytes expressed the transgene (67%) than those which were zona compromised (44%) (Ewerling *et al.* 2006). With all of these protocols in place to produce transgenic livestock through lentivirus mediated delivery, it becomes evident that it is a matter of time before farm animals are created with sustained knocked down gene expression derived from RNAi.

Although lentivirus delivery of RNAi sequences and other transgenes has proven successful in livestock species, persistent knock down of mRNA translation may not always be desirable for targeting transient or acute temporal gene expression in early embryos. Investigations of functional genomics involving oocyte development, embryonic stem cell differentiation, elongation or implantation could benefit from direct treatment of the oocytes or embryos with transiently expressed siRNA without the requirement of a lentivirus delivery system. Direct microinjection has been established as a useful method for delivery of dsRNA targeted against cyclin B1 into bovine

oocytes, although efficient knock down of gene expression has had limited success (Paradis *et al.* 2005). Successful microinjection of dsRNA into bovine zygotes has been reported to decrease E-cadherin gene expression by 80% in subsequent blastocysts (Nganvongpanit *et al.* 2006a) but has not been successful in knocking down Connexin-43 expression (Tesfaye *et al.* 2007). However, techniques for efficient delivery of siRNA into non-rodent embryos still needs further exploration, and attempts to do so with alternate methods in bovine zygotes are discussed in detail in Chapter II.

Experimental design

Since it is known that microinjected dsRNA can down regulate E-cadherin in bovine embryos up to day 7 in the blastocyst stage, it was decided to use the same target to determine if injected siRNA delivered at the zygote stage will persist through blastocyst expansion and hatching. This would make investigations into mechanisms such as apoptosis, which can greatly affect implantation and pregnancy rates, during hatching and post-hatching events possible (Jousan *et al.* 2008). In addition, recent studies, detailed in Chapter II, have established chemical transfection as a reliable method to deliver siRNA to trophectoderm cells in early blastocysts. Applying these two methods, gene expression of E-cadherin will be compared at day 9 of development between injected zygotes versus transfected early blastocysts with siRNA to evaluate efficacy of each treatment.

To accomplish this experiment, the complete coding sequence for bovine E-cadherin was first determined so that three different homologous siRNAs could be

designed. Synthesized siRNAs were validated by transfection into a cell culture to confirm silencing of the gene product and measured with quantitative PCR and western blot densitometry analysis. Once an effective siRNA was identified, it was injected into the cytoplasm of day 1 *in vitro* produced (IVP) zygotes or transfected into the trophectoderm cells of day 7 IVP blastocysts. On day 9 of development, all embryos were collected and E-cadherin expression was measured by quantitative PCR and densitometry measurements made from scanning confocal images of immunocytochemically labeled embryos (Figure 7). Comparisons of expression values between injected and transfected embryos were made to determine the most effective method to knock down gene expression in future post-hatching gene regulation studies.

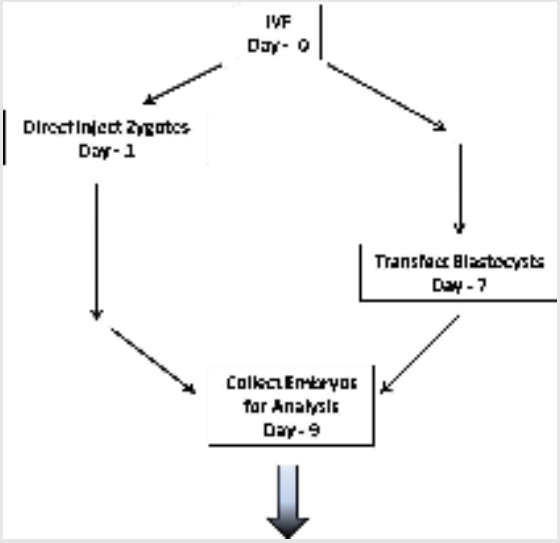

Fig. 7 Flow chart of experiments. IVF was performed on Day 0, followed by two independent embryo treatments at Day 1 or Day 7, and collection of embryos at Day 9.

CHAPTER II

PRELIMINARY INVESTIGATIONS: DELIVERY OF TRANSIENTLY EXPRESSED siRNA INTO BOVINE ZYGOTES

Pronuclear and cytoplasmic microinjection, or direct injection, has been well documented as a popular method for delivering transiently expressing foreign RNA/DNA into non-rodent oocytes and embryos (Dehennaut *et al.* 2008; Freitas *et al.* 2003; Nganvongpanit *et al.* 2006b; Verma *et al.* 2008) and is well established as an effective procedure in mouse zygotes to produce transgenic animals (Ittner and Götz 2007). Although microinjection is widely used in livestock species, there are limiting complications which still make it an inefficient process. Developmental rates to the blastocyst stage are often decreased due to technical error such as injection of too much fluid or lysis of the vitelline membrane with the injection pipette. Embryos of lesser quality which would otherwise develop in culture, often cannot recover from these insults and degrade in culture leaving only the better quality embryos to develop (Maga *et al.* 2003; Nganvongpanit *et al.* 2006a). In addition, integration before the first round of DNA replication or homologous distribution of the transgene throughout the cytoplasm does not always occur which can lead to mosaic expression during cell division in successively cleaving blastomeres (Rosochacki *et al.* 2003). Furthermore, successful application of microinjection requires expensive equipment and highly skilled technicians. The oocytes or embryos must be treated individually making the process not only expensive, but laborious and time consuming.

The initial aim of the research project was to bypass the problems associated with microinjection and devise a method to transiently transfect small groups of bovine zygotes in one treatment. Less invasive, grouped methods could eliminate the need for expensive equipment, advanced technical skills, limit the embryo treatment time out of culture and potentially alleviate the occurrence of chimerism associated with microinjection. Several embryo group treatment approaches to introduce siRNA into bovine zygotes were evaluated including chemical transfection, electroporation, and chemically induced vitelline membrane pore formation. Comparisons of transfection efficiency and blastocyst development were made for each treatment and ultimately compared to the control method of direct injection.

Microinjection

Before exploring alternative methods of siRNA delivery, developmental hindrance was assessed in bovine zygotes injected with siRNA. DNA plasmid injection has been routinely used in this laboratory to successfully express transgenes into the cytoplasm of zygotes. Therefore developmental rates of zygotes injected with siRNA were compared to previous developmental data collected from zygotes injected with one of three plasmids (at a concentration of 50 ng μl^{-1}) which possessed either the of fluorescent reporter genes, green fluorescent protein (GFP) or red fluorescent protein (RFP). Table 1 indicates that it is possible to obtain transcription of the cytoplasm injected transgenes from a plasmid in bovine zygotes, however, our experience is that

Table 1. Three plasmids used for cytoplasmic injection into bovine zygotes. Cleav %, cleavage rates at day 2 post fertilization; Blast %, blastocyst formation rates at day 7; Fluor %, percentage of total zygotes with fluorescent blastomeres at day 4 (8-cell). *All reported with mosaic expression.

Plasmid	Reporter	Expression Location	Emission λ nm	Total # Zygotes	Cleav %	Blast %	Fluor* %
Control	None	None	None	66	73	20	0
Fug-W	GFP	Cytoplasm	509	64	48	8	8
dsRed	RFP	Cytoplasm	579	56	35	0	13
phEFnGFP	GFP	Nucleus	509	56	45	0	16

once the embryos begin to cleave, reporter proteins reveal distribution among the blastomeres is unequal resulting in mosaic expression.

Experiment 1

To monitor delivery of the injected siRNA into zygotes, a Negative Control #1 siRNA (Cat# AM4621; Ambion, Inc., Austin, Texas) verified by Ambion to be non-homologous to any know bovine gene sequence was used. The siRNA was labeled on the 5' end with the red fluorescent dye Cy3 which permitted immediate visual confirmation of cytoplasmic delivery of the siRNA into the zygote under fluorescent excitation at 547 nm. This allowed for measurement of transfection efficiency and

developmental competence without incurring the expense of gene specific siRNA design and gene expression analysis.

Bovine zygotes were produced *in vitro* by standard lab operating procedures and cumulus cells were removed by vortexing in 200 µl of warmed TL-Hepes (GIBCO, BRL, Rockville, MD) for 2 minutes. After vortexing, zygotes were washed twice through warmed Holding medium consisting of TCM-199 with Hank's salts (GIBCO, BRL) supplemented with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT) and 1% gentamicin (50 mg ml^{-1} solution; GIBCO, BRL) and held until injection.

Lyophilized Cy3-siRNA was reconstituted to 100 µmol L^{-1} with the supplied RNase-DNase free water and then diluted to the desired final concentration in TE buffer (Appendix A). Injection plates were assembled by adding a 20 µl drop of Holding medium to the center of a 100 mm round tissue culture dish and a 5 µl drop of diluted Cy3-siRNA. Both drops were overlaid with warmed embryo culture tested light mineral oil (Sigma-Aldrich, St. Louis, MO) and ~30 zygotes were pipetted into the Holding drop. Embryos were visualized through a Zeiss Axiovert inverted microscope and held in place with a glass holding pipette (outer diameter: 180 – 200 µm, inner diameter: 40 – 60 µm). Injection pipettes were made by pulling glass capillary tubes with a Sutter Flaming/Brown P-97 puller to a filamentous tip [P=200, Heat=450, Pull=21, Vel=50, t=120] which was then broken against the holding pipette in the siRNA drop creating a lumen 3- 4 µm in diameter. Over a series of 2-3 replicates, embryos were injected once with 10 – 100 pl (enough to observe slight expansion of the cytoplasm) with one of the listed concentrations in Table 2, then washed through G1 (Vitrolife, Inc., Englewood,

CO) embryo culture medium supplemented with 8 mg ml^{-1} Pentax BSA (Miles Laboratories, Elkhart, IN) and 1 µl ml^{-1} gentamicin and returned to culture in 500 µl fresh supplemented G1 for 72 hours at 38°C with 5% CO_2, 5% O_2, and 90% N_2 humidified air (5/5/90). In addition to siRNA, some zygotes were injected with TE buffer alone to ascertain changes in development due to the actual injection procedure

Table 2. Development rates and transfection efficiencies of bovine zygotes injected with Cy3-siRNA. Cleav %, cleavage rates at day 2 post fertilization; Blast %, blastocyst formation rates at day 7; Fluor %, percentage of total zygotes with fluorescent blastomeres at day 4 (8-cell).

Treatment	Concentration	Total # Zygotes	Cleav %	Blast %	Fluor %
Control	None	56	52	23	0
TE	None	55	56	22	0
Cy3-siRNA	1 nmol L^{-1}	33	33	15	0
Cy3-siRNA	50 nmol L^{-1}	31	32	16	0
Cy3-siRNA	100 nmol L^{-1}	57	32	12	4
Cy3-siRNA	1 µmol L^{-1}	55	29	11	9
Cy3-siRNA	50 µmol L^{-1}	69	45	3	41
Cy3-siRNA	100 µmol L^{-1}	26	31	0	19

and other zygotes were not injected but rather moved directly into culture to assess the control development rates. After 72 hours, embryos with fluorescent blastomeres were recorded and then moved to 500 µl G2 (Vitrolife, Inc., Englewood, CO) similarly supplemented with G1 and returned to culture for an additional 72 hours at which point blastocyst development rates could be determined.

Forty minutes after the initial injections, all treated zygotes were observed under fluorescent light. Red fluorescence was visible in those injected with 100 nmol L^{-1} or greater but not in those treated with 1 or 50 nmol L^{-1}. After 3 days in culture, all embryos with red fluorescence had homogenous expression among all blastomeres and embryos injected with 50 µmol L^{-1} had the largest proportion of fluorescence still visible at the 8-cell stage. The injection process itself was not overtly detrimental to embryo development as noted by similar blastocyst formation rates between the control and TE injected zygotes. However, an increase in concentrations of the injected Cy3-siRNA resulted in a decrease in blastocyst formation. It is unclear the fate of the Cy3 once in the cell and if visual detection correlates to siRNA longevity. Therefore it is difficult to say by the 8-cell stage when fluorescence was evaluated again, if treatments in which there was no fluorescence detected was due to degradation of the Cy3 signal alone or if siRNAs were also depleted.

Given these results, it was determined that 1 - 50 µmol L^{-1} of Cy3-siRNA would be appropriate to deliver enough siRNA into bovine zygotes to sustain expression through subsequent cell divisions and still produce blastocysts for future gene expression

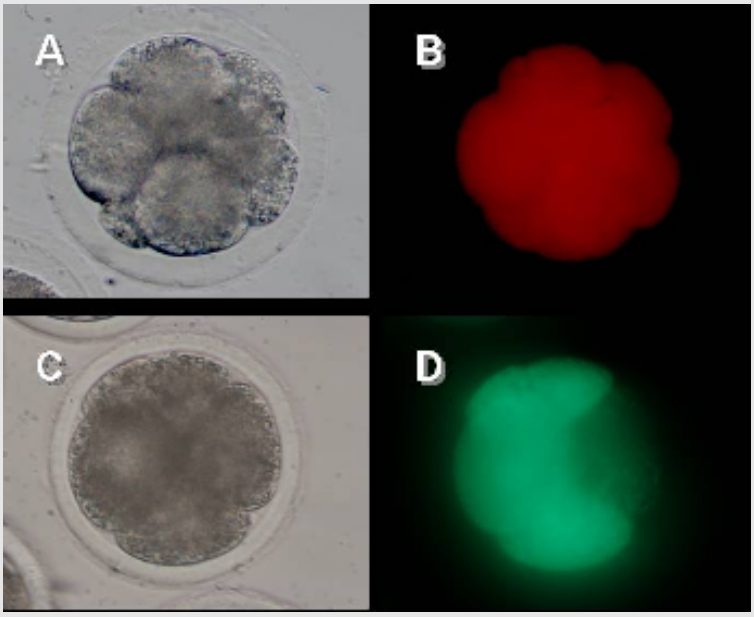

Fig. 8 Zygote microinjection. Bovine zygotes injected with Cy3-siRNA, A) observed with bright field, B) observed under fluorescence, or injected with Fug-W, C) observed with bright field, D) observed under fluorescence.

analysis studies. However, appropriate concentrations may vary for individual experiments depending on the abundance of the mRNA target.

Experiment 2

In a final evaluation, a comparison was made between bovine zygotes injected with 50 μmol L^{-1} Cy3-siRNA or 50 ng ml^{-1} Fug-W (the single plasmid demonstrated to

allow blastocyst development) to compare distribution of the injected transgene. Although blastocyst formation was lower in siRNA compared to plasmid treated embryos (2% and 9% respectively), 40% of the Cy3-siRNA injected embryos had homogenous fluorescence in all blastomeres and only 7% of the Fug-W treated embryos expressed GFP in a mosaic pattern (Figure 8).

The ability of the siRNA to consistently distribute evenly throughout the cytoplasm may be a result of RISC processing the molecule and shuttling it throughout the embryo in search of the mRNA target. Although shRNA transcribed from a plasmid are subject to the same distribution as siRNA, entire plasmids are not known to participate in any such pathway. Instead, they are subject to placement where they were injected and dependant on cellular metabolism and division events for distribution which may account for the mosaic expression often seen with the reporter protein. Therefore, it was concluded that for transient gene suppression studies, siRNAs are preferable to plasmid based gene silencing due to homogenous distribution during embryo cleavage. Although development is diminished when injected, siRNAs are a possible mechanism to induce transient gene silencing for studies in bovine early embryos and an acceptable control to compare to when developing other methods of siRNA delivery.

Chemical transfection

Chemical transfection is a common method used in both somatic and stem cell cultures to successfully introduce foreign RNA/DNA into the cytoplasm (Arnold *et al.* 2006; Tinsley *et al.* 2006; Zhang *et al.* 2007). There are numerous commercial kits

available for chemical transfections that utilize either cationic liposomes (termed lipofection) or cationic polymers. The cationic lipid based reagents FuGENE 6 (Cat# 11815091; Roche Applied Science, Indianapolis, IN) and Lipofectamine 2000 (Cat# 11668-027; Invitrogen, Carlsbad, California) bind the anionic RNA/DN into a liposome which then fuses with the eukaryotic cell membrane and passively transports the cargo into the cytoplasm. Cationic polymers such as GeneJammer (Cat# 204130; Stratagene, La Jolla, California) and ExGen 500 (Cat# R0511; Fermentas Life Sciences, Glen Burnie, Maryland) form an ionic bond with the RNA/DNA and through endocytosis are actively transported into the cell. These transfection reagents have all been developed in an effort to produce low cytotoxic effects while increasing transfection efficiency on a wide variation of cell types. In addition, preparation time of the RNA/DNA conjugates with transfection reagents is rapid and does not require expensive equipment to perform. Given the benefits of chemical transfection and the potential to treat many oocytes or embryos at one time, the possibility of delivering siRNAs via transfection into zygote stage embryos, as opposed to microinjection, was explored.

There are two reports indicating successful transfection in mouse oocytes and embryos using a commercial cationic liposome reagent. In the earliest report, immature oocytes to blastocyst stage embryos with either an intact or permeabilized zona were cultured in 50 µl of a plasmid DNA-FuGENE conjugate (125ng DNA-1 µl FuGENE diluted in 100 µl medium) for 3 hours (Carballada *et al.* 2000). Except for morulae with intact zonae, all treated oocytes and embryos exhibited varying degrees of success from 23.5% to100% positive transfection efficiencies (indicated by plasmid driven GFP

fluorescence) and subsequent developmental rates were not significantly different from the untreated controls. In a follow-up report, a DNA plasmid containing the *lacz* gene was successfully transfected into zona compromised murine pronuclear stage embryos using FuGENE (as described above) to produce transgenic offspring (Carballada *et al.* 2002). Although the transgene was detected in many tissues and organs in the transgenic mice, transfection efficiencies were extremely low and only 1.27% of the treated embryos that were transferred, survived.

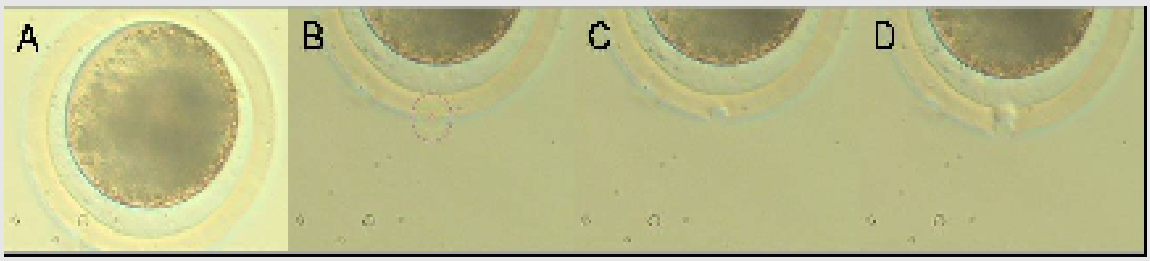

Fig. 9 Laser drilling a rent through bovine zygote zonae. A) intact zygote with a slightly shrunken membrane and rotated so polar body is not visible, B) target sights indicate placement of laser burst, C) first hole in zona has been drilled, D) completed drilling through zona with three successive laser bursts.

Experiment 3

Given the success transfecting zygotes in mice, FuGENE was the first transfection reagent evaluated at various ratios in co-culture with bovine zygotes for 48 hours. To compromise the zona, an objective mounted class I laser (Hamilton Thorne Biosciences, Beverly, MA) was used to drill a hole completely penetrating the zona (Figure 9). Cumulus cells were removed as previously described from presumptive zygotes which were transferred into 500 µl of warmed Holding medium supplemented with 75 mmol L^{-1} sucrose to slightly shrink the perivitelline membrane and reduce the risk of nicking the embryo with the laser. Zonae were drilled at 90% power with a 600 µsec pulse creating three successive holes, 20 µm in diameter, producing a rent that completely transversed the zona pellucida. After drilling, zygotes were washed twice through fresh Holding medium, once through G1, and moved to 450 µl of supplemented G1 culture medium (without gentamicin) until use.

To construct the FuGENE and siRNA conjugate, the specified amount of FuGENE was added to G1 (no BSA or gentamicin), mixed well, and allowed to incubate at room temperature for 5 minutes (Table 3). The specified quantity of siRNA (defined in Table 3) was then added from a 100 µmol L^{-1} stock solution to the G1, bringing the final volume up to 50 µl, held at room temperature for 15 additional minutes before drop wise addition to the zygotes in G1 (no gentamicin) while the dish was swirled, thoroughly mixing the zygotes with the conjugated transfection compounds. In addition, non-treated, FuGENE only, and Cy3-siRNA only controls were evaluated to determine the effects of each on embryo development. After 48 hours, embryos were moved to

fresh supplemented G1 and evaluated for fluorescence. After an additional 24 hours in G1, embryos were moved to supplemented G2 (G1/G2 sequential culture), cultured for an additional 72 hours after which blastocyst development rates were obtained. None of the evaluated ratios successfully transfected the embryos as indicated by lack of detectable fluorescence at 24 and 48 hours in any of

Table 3. Experiment 3 blastocyst formation. Lasered bovine zygotes were co-cultured for 48 hours with transfection complex. No fluorescence was detected in any of the treatments.

Treatment	FuGENE:siRNA	Total # Zygotes	Blastocyst %
Control	None	21	43
FuGENE Only	1 µl FuGENE	30	7
Cy3-siRNA Only	1 µg Cy3-siRNA	30	10
Transfect	1 µl:125 ng	31	16
Transfect	1 µl:500 ng	33	18
Transfect	3 µl:1 µg	36	6
Transfect	3 µl:2 µg	34	15
Transfect	6 µl:1 µg	35	9

the treatments. Exposure to FuGENE for 48 hours and use of the laser with sucrose appeared to have a negative effect on embryo development as blastocyst rates for all treatments (range 7-18%) were noticeable lower than the control (43%). From these data, it was concluded that greater care needed to be taken when drilling the zonae and a higher concentration of sucrose should be considered to further shrink the vitelline membrane increasing the distance between it and the laser and decreasing the risk of damage on the cytoplasm. Furthermore, it was suspected that 48 hours exposure to FuGENE was toxic to the embryos and shorter exposure times warranted evaluation.

Experiment 4

Similar ratios were evaluated as detailed in Experiment 3, however the sucrose concentration in which zygotes were lasered was increased to 200 mmol L^{-1} and zygotes were incubated directly in the transfection complexes for 3 hours at 38.5ºC in 5/5/90 humidified air. After incubation, zygotes were washed through supplemented G1 and cultured in sequential G1/G2 for 6 days. Fluorescence was evaluated directly after 3 hours of co-culture with the transfection complex and again after 24 hours. As observed in Experiment 3, none of the treatments successfully transfected the bovine zygotes. Blastocyst rates were variable and not overtly attributable to any one factor (Table 4).

Experiment 5

Increased concentrations of FuGENE transfection reagent and siRNA were evaluated simultaneously with a second lipid based transfection chemical, Lipofectamine

2000 (Table 5). Zygotes were prepared as described in Experiment 2 and treated similarly, except incubation in either transfection compound was increased to 4 hours. Embryos were evaluated for fluorescence immediately after incubation in the complex and again after 24 hours of culture. After 4 hours, zygotes cultured in a 9 µl:6 µg ratio of FuGENE to siRNA obtained 48% fluorescence and in those treated with 12 µl:8 µg, 4% expressed the Cy3-siRNA throughout the cytoplasm. However, after 24 hours of

Table 4. Experiment 4 blastocyst formation. Lasered bovine zygotes were incubated in transfection complex for 3 hours. No fluorescence was detected in any of the treatments.

Treatment	FuGENE:siRNA	Total # Zygotes	Blastocyst %
Control	None	24	25
FuGENE Only	1 µl FuGENE	24	29
Cy3-siRNA Only	1 µg Cy3-siRNA	26	27
Transfect	1 µl:125 ng	26	4
Transfect	1 µl:500 ng	25	20
Transfect	3 µl:1 µg	25	8
Transfect	3 µl:2 µg	24	33
Transfect	6 µl:1 µg	24	4

culture in G1, those which previously fluoresced red did not continue to develop and the cytoplasm appeared to be in early stages of degeneration and most likely were already dead when put into co-culture. Furthermore, blastocyst formation was ablated at all levels tested in FuGENE and in the highest concentration of Lipofectamine. Based on these results, it was decided to continue evaluating delivery methods with FuGENE and to reduce the amount used in culture with the zygotes.

Table 5. Experiment 5 blastocyst formation and transfection efficiencies. Lasered bovine zygotes were incubated in transfection complex for 4 hours. Lipofect, Lipofectamine 2000.

Treatment	Reagent:siRNA	Total # Zygotes	Blastocyst %	Fluorescence % At 4 Hours
Control	None	30	17	0
FuGENE Only	9 µl	21	0	0
FuGENE Transfect	9 µl:6 µg	25	0	48
FuGENE Transfect	12 µl:8 µg	28	0	4
Lipofect Only	9 µl	25	8	0
Lipofect Transfect	9 µl:6 µg	26	12	0
Lipofect Transfect	12 µl:8 µg	24	0	0

Experiment 6

To maintain closer proximity to the cytoplasmic membrane, 100-150 pl of three different FuGENE transfection ratios were directly injected into the perivitelline space of zona intact zygotes (Table 6). Additionally, the same ratios were also used to laser the zygotes directly in the complex and continuing with 3 hours of co-culture to take advantage of any gradient differentials which might force the transfection compound into the perivitelline space (Table 6). After co-culture, all embryos were moved into G1/G2 sequential embryo culture for 6 days. As reported in Experiments 1 and 2, none of the treatments produced successful transfection in bovine zygotes following 3 hours of culture in transfection complex.

Experiment 7

To determine if the rent created by the laser was simply not allowing adequate access of FuGENE to the zygotic membrane, zonae were either weakened with acid Tyrodes (pH 2.5; Sigma-Aldrich, St. Louis, MO) or completely removed with 0.5% Pronase E in Holding medium (Table 7). For each treatment, cumulus free zygotes were transferred to 500 µl of either solution for 3 minutes, then washed three times through fresh Holding medium before incubation for 3 hours directly in the FuGENE complex with a ratio of 3 µl of FuGENE to 4 µg of Cy3-siRNA. Neither of the zona treatments appeared to have a positive effect on transfection efficiencies as there was no fluorescence detected for either method after 3 hours of incubation in transfection compound. Blastocyst formation rates for Pronase E treated embryos was much lower

than the control due to recombination of blastomeres from multiple embryos creating several giant blastocysts.

Table 6. Experiment 6 blastocyst formation. Bovine zygote development after injection of the transfection complex into the perivitelline space (PVSI) or laser drilling of the zonae in the transfection complex (DIC) followed by co-culture for 3 hours.

Treatment	FuGENE:siRNA	Total # Zygotes	Blastocyst %
Control	None	37	46
FuGENE Only	3 µl	39	54
PVSI	1 µl:125 ng	39	41
PVSI	3 µl:2 ng	36	69
PVSI	9 µl:6 µg	41	46
Control	None	40	23
FuGENE Only	3 µl	42	14
DIC	1 µl:125 ng	41	10
DIC	3 µl:2 ng	40	15
DIC	9 µl:6 µg	43	7

Experiment 8

Cumulus free ova were injected subzonally with transfection complex to ascertain if the cortical granule envelope which forms around the periphery of the ooplasm immediately after fertilization could be preventing successful transfection. Cumulus cells were removed by pipetting the ova through a fine bore glass needle and 100-150 pl of transfection compound was injected beneath the zona. Ova were subjected to standard *in vitro* fertilization protocols and cultured in sequential G1/G2 media to the blastocyst stage. After 24 hours, none of the treated ova exhibited fluorescence indicating that the cortical granule envelope may not be the block preventing successful transfections of bovine zygotes. Blastocyst rates were also greater in the treated oocytes indicating that the micromanipulation, although subtle, may have induced parthenogenic activation (Table 8).

Table 7. Experiment 7 blastocyst formation. Embryo development after zona weakening with acid Tyrode's or zona removal with Pronase E followed by 3 hours incubation directly in FuGENE transfection complex.

Treatment	FuGENE:siRNA	Total # Zygotes	Blastocyst %
Control	None	50	50
Acid Tyrode's	3 µl:4 µg	50	10
Pronase E	3 µl:4 µg	49	41

Table 8. Experiment 8 blastocyst formation. Embryo development after injection of FuGENE below the zona of cumulus free bovine ova.

Treatment	FuGENE:siRNA	Total # Zygotes	Blastocyst %
Control	None	53	9
Cumulus Free Control	None	52	25
Cumulus Free FuGENE Only	3 µl	53	23
Cumulus Free Transfect	3 µl:2 µg	49	35

Experiment 9

An alternate transfection reagent, ExGen 500, was evaluated in bovine zygotes. Different from FuGENE and Lipofectamine 2000, ExGen 500 is a cationic polymer which induces active transport of the siRNA across the cell membrane where it becomes a proton sponge lysing the endosome and releasing the siRNA before it is transported and degraded in a lysosome. Zygotes were laser drilled in 200 mmol L^{-1} sucrose and transferred to 450 µl supplemented G1 (without gentamicin). The Cy3-siRNA was diluted into 50 µl of 150 mmol L^{-1} sodium chloride and vortexed. ExGen 500 was then added and the sample was vortexed for 10 seconds, held at room temperature for 10 minutes, then applied drop wise to the zygotes in G1. The following ratios of ExGen 500 to Cy3-siRNA were evaluated: 1:3, 1:6, 1:9, 2:6, 2:12, and 2:18 as well as non-treated, laser treated only, and 9 µl of ExGen only controls. After 24 hours of co-

culture, not only were there no fluorescence detected in any embryos, but none of the embryos treated with ExGen cleaved, unlike the controls without ExGen which had 25 and 12% cleavage. Based on these data ExGen was not considered for future transfection experiments.

Experiment 10

Results from Experiments 3-9 demonstrate the difficulty associated with chemically transfecting bovine zygotes, however, it was not known if any developmental stage other than zygotes would preferentially take up the transfection regents. Therefore, chemical transfection with two different reagents, either FuGENE (lipid based) or GeneJammer (cationic polyamine) were tested on ova and with all stages of embryo development through blastocyst formation (Table 9). All ova and embryos were laser drilled in 200 mmol L^{-1} sucrose and moved to either 450 µl of TCM-199 with Earle's salts and 10% v/v fetal bovine serum (ova) or supplemented G1 (no gentamicin) (embryos). Transfection complexes were constructed by adding 9 µl of transfection reagent to 38 µl of TCM-199 or G1 and incubated at room temperature for 5 minutes.

Table 9. Experiment 10 transfection efficiencies. Two chemical reagents electroporated into bovine ova and embryos with laser compromised zona or zona intact blastocysts.

		FuGENE		GeneJammer	
Stage	**Zona**	**No. Transfected**	**% Transfected**	**No. Transfected**	**% Transfected**
Ova	Drilled	38	0	39	0
Zygote	Drilled	35	0	36	0
2 Cell	Drilled	32	0	33	0
4-6 Cell	Drilled	28	0	29	0
8 Cell	Drilled	30	0	30	0
16 Cell	Drilled	12	0	12	0
Morula	Drilled	26	0	26	0
Blastocyst	Drilled	24	100	30	67
Blastocyst	Intact	15	88	12	92

Then 3 µl Cy3-siRNA was added and the solution was allowed to sit for 15 minutes at room temperature before addition to the ova/embryos in culture medium. Once the transfection solution was added, the ova/embryos were co-cultured for 24 hours and evaluated for fluorescence indicating successful Cy3-siRNA transfection. Results indicated that transfection at any stage was not achieved until blastocyst formation at which point those transfected in FuGENE or GeneJammer had 100 and 97% transfection efficiencies, respectively. In these blastocysts it appeared that the inner cell mass (ICM)

remained unaffected; however, further research is required to verify this observation. Following these results, zona intact blastocysts were co-cultured with transfection reagents as described above to evaluate the necessity of compromising the zona before transfection (Table 9). Although transfection efficiencies were slightly diminished, blastocysts transfected with FuGENE or GeneJammer still achieved 88 and 92% transfection in embryos. Chi Square analysis on combined blastocyst transfection data from both reagents indicates no difference in transfection efficiencies between zona drilled (98%) and zona intact (90%) treatments ($P = 0.24$).

Considering all these data, it was concluded that current techniques for oocyte and embryo chemical transfection which have otherwise proven successful in the mouse, do not translate into the bovine. Transfection of siRNA is inhibited in early stage bovine embryos until differentiation occurs at the blastocyst stage, likely due to changes in cellular architecture and polarization. Either lipid based or cationic polymers are effective sirNA transfecting agents into blastocysts and although the laser drilling of the zona is not necessary, it can increase transfection efficiencies.

Electroporation

Electroporation based transfection applies unipolar electric field pulses to create transient aqueous pores through which the charged RNA/DNA molecules may pass (Schmotzer *et al.* 2003). Parameters such as voltage, pulse length (μsec), the number of pulses, and how many series applied can be manipulated to determine the optimal settings for successful electroporation. In the mouse, dsRNA targeted against both *c-*

mos and GFP has been successfully delivered into oocytes and transgenic embryos (zygotes and 4-cell), respectively, using electroporation (Grabarek *et al.* 2002). Spatial and temporal knock down of gene expression has also been accomplished in postimplantation mouse embryos removed from the uterus and electroporated *in vitro* (Mellitzer *et al.* 2002). There are no known reports utilizing this technique in bovine embryos, therefore delivery of a GFP plasmid and Cy3-siRNA was attempted based on previous reports in the mouse.

Experiment 11

Zonae from cumulus free bovine zygotes were laser drilled as previously described in 200 mmol L^{-1} sucrose and maintained in Holding medium until use. Prior to electroporation, zygotes were washed three times through 20 mmol L^{-1} Hepes Buffered Saline (HBS) and transferred to HBS containing 5 µg ml^{-1} Fug-W plasmid. Embryos were then moved to a 3.2 mm square wire fusion chamber filled with 700 µl of HBS + Fug-W and electroporated with the parameters outlined in Table 10. After treatment, embryos were washed twice through HBS and placed in G1/G2 sequential culture. In addition, non-treated, laser only, and sucrose and HBS exposed embryos were cultured as controls. None of the treatments successfully transfected the embryos as indicated by lack of green fluorescence among any group. Except for embryos electroporated with 35 volts, all other treatments had noticeably reduced blastocyst development.

Table 10. Experiment 11 parameters and blastocyst formation. Embryo development rates following bovine zygote electroporation with 50 mg ml^{-1} of Fug-W plasmid. Blast, Blastocyst.

Treatment	Voltage	Time (µs)	# Pulses	# Series	# Zygotes	% Blast
Control	___	___	___	___	22	14
Sucrose/HBS	___	___	___	___	30	30
Laser Only	___	___	___	___	26	8
Fug-W	20	100	2	3	26	8
Fug	35	100	2	3	26	23
Fug	50	100	2	3	24	8
Fug	100	100	2	3	24	8
Fug	200	100	2	3	28	4

Experiment 12

Based on results in Experiment 11, the range of voltage used in Experiment 12 was tailored to evaluate electroporation with 10, 30, or 60 volts and pulse time was decreased from 100 to 50 µsec for some of the trials (Table 11). A 1.0 mm round wire chamber was used and 50 µmol L^{-1} of Cy3-siRNA was diluted into the HBS for electroporation. After treatment, embryos were moved to sequential G1/G2 medium.

No cytoplasmic fluorescence was detectable in any of the embryos after treatment, however at 30 and 60 voltage for 100 µsec, a red glow was visible under fluorescent

Table 11. Experiment 12 parameters and blastocyst formation. Embryo development rates following electroporation of bovine zygotes with 50 µmol L^{-1} of Cy3-siRNA. Blast, Blastocyst.

Treatment	Voltage	Time (µs)	# Pulses	# Series	# Zygotes	% Blast
Control	___	___	___	___	23	30
Cy3-siRNA	10	50	2	3	23	9
Cy3-siRNA	30	50	2	3	14	0
Cy3-siRNA	60	50	2	3	29	21
Cy3-siRNA	10	100	2	3	19	21
Cy3-siRNA	30	100	2	3	26	8
Cy3-siRNA	60	100	2	3	35	6

light in the perivitelline space indicating that the siRNA was moved from the surrounding medium to the embryos, but did not penetrate the cellular membrane. After 24 hours culture of these embryos Cy3 fluorescence was no longer detectable. Blastocyst development was varied across all treatments with a specific contributing factor unidentifiable.

Table 12. Experiment 13 parameters and blastocyst formation. Embryo development rates following electroporation of bovine zygotes injected subzonally with FuGENE complexed Cy3-siRNA FuGENE, injected with only transfection reagent; FuGENE:si, injected with compounded transfection reagent and Cy3-siRNA; Blast, Blastocyst.

Treatment	Voltage	Time (µs)	# Pulses	# Series	# Zygotes	% Blast
Control	___	___	___	___	46	0
FuGENE	10	50	3	3	31	0
FuGENE	30	50	3	3	26	0
FuGENE	60	50	3	3	27	0
FuGENE:si	10	50	3	3	25	0
FuGENE:si	30	50	3	3	29	0
FuGENE:si	60	50	3	3	22	0

Experiment 13

In a final effort to confine the siRNA near the embryo during electroporation, FuGENE was compounded with Cy3-siRNA in a 3:2 ratio and 100-150 pl were injected beneath the zona of cumulus free bovine zygotes. Injected zygotes were electroporated in HBS in a 1.0 mm round wire chamber with parameters listed in Table 12 and then cultured in sequential G1/G2. None of the treated groups exhibited red fluorescence nor did any group develop to the blastocyst stage.

Given the time and multiple steps involved in the electroporation process coupled with the lack of success, no further experiments were attempted. Electroporation was

deemed too injurious and as it was not effective to create transgenic bovine embryos, no further resources were allocated to this project.

Streptolysin-O

Streptolysin-O (SLO) has been successfully used to deliver large molecules up to 100 kDa in size through the membranes of cell cultures. SLO can reversibly permeabilized cell membranes by forming pores in the lipid rafts allowing molecules through that would otherwise be too large for sodium pump channels. Experimental parameters were based on previous work where SLO was utilized to permeabilized cellular membranes in fetal fibroblasts prior to SCNT (Sullivan *et al.* 2004). Bovine zygotes with either the zona intact, zona drilled, or zona removed were cultured with 1, 10, 50, 100, and 500 IU of SLO (Sigma-Aldrich, St. Louis, MO) for 30, 60, 90, and 120 minutes in a calcium and magnesium free TCM199 with Earle's salts. Propidium iodide was added to the culture as an indicator of pore formation as uptake would cause red fluorescence in the zygote cytoplasm. After 30 minutes, lysis of zygotes had already begun in random treatment groups, and continued to increase as exposure time increased. By 120 minutes, few red zygotes were visible in all treatments; however these zygotes looked deformed with cell membranes blebbing out. After exposure to SLO, zygotes were washed through supplemented 199-Earle's with 2 mmol L^{-1} calcium chloride to seal the pores and moved to sequential G1/G2 medium. None of the zygotes exposed to the evaluated levels of SLO cleaved but rather had begun degrading. The obvious failure of this experiment prevented any further exploration.

Conclusion

Direct injection continues to be the only method which is capable of successfully delivering siRNA into bovine zygotes while grouped embryo methods are thus far unsuccessful. Injection of siRNA appears to alleviate distribution problems in the cytoplasm of zygotes as mosaic expression was reduced compared to those injected with plasmids. None of the chemical methods evaluated effectively transfected zygotes or any other developmental stage for that matter until the blastocyst formation when trophectoderm cells were easily transfected. The inner cell mass of these blastocysts did not appear to incorporate the Cy3-siRNA, although further validation is required. Neither electroporation nor pore formation was beneficial at the parameters tested. Due to obvious deleterious effects early in the investigation, further variants of these experiments were not pursued. For future experiments in bovine early embryo targeting specific genes, direct injection or trophectoderm transfection could be appropriate tools for expression knockdown with siRNAs.

CHAPTER III

MATERIALS AND METHODS

Preliminary experiments in Chapter II investigating delivery of siRNA into bovine embryos suggested that microinjection into the cytoplasm of bovine zygotes or transfection of the trophectoderm in blastocysts could successfully deliver the Cy3-siRNA. Therefore, experiments were performed to determine if siRNAs targeting bovine E-cadherin could successfully knockdown gene expression after delivery by microinjection or transfection and evaluated for efficacy in sustaining gene knockdown in day 9 embryos.

RNA isolation for gene sequencing

Small one inch strips of bovine kidney tissue collected from a slaughtered cow were immediately immersed in liquid nitrogen (LN_2) after collection, wrapped in aluminum foil for storage, and maintained at -80ºC. On dry ice, 50-100 mg of kidney tissue were shaved off in small flakes and collected into 1 ml of chilled RNA Stat-60 (Tel-Test, Woodlands, Texas) in a 7 ml Dounce tissue grinder. Flakes were ground until no tissue mass was detectable in a consistent homogenate, and then allowed to sit on ice for 5 minutes. The homogenate was transferred into a 2 ml micro tube and 200 µl of chloroform was added. The tube was shaken vigorously for 15 seconds to mix the chloroform and homogenate and then allowed to set undisturbed for 3 minutes at room temperature. Samples were centrifuged for 15 minutes at 12,000 x g to separate into two

phases: the lower red phase containing protein topped with a white buffy coat of DNA, and the upper colorless aqueous phase containing RNA. The aqueous phase was transferred to a clean 2 ml micro tube and 500 µl of isopropanol was added. The samples were maintained at room temperature for 10 minutes and were then centrifuged at 12,000 x g for 10 minutes to form a white RNA pellet at the bottom of the tube. After centrifugation, the supernatant was aspirated off leaving the RNA pellet behind which was washed with 1 ml of 75% ethanol, vortexed, and centrifuged again at 7,500 x g for 5 minutes. Supernatant was aspirated and the RNA pellet air dried for 5 minutes, after which 150 µl of nuclease free water was added to resuspend the pellet. Isolated RNA samples were stored at -80ºC until use.

mRNA purification for gene sequencing

To extract the mRNA from the previously isolated total RNA, the Poly(A) Purist Kit from Ambion® (Austin, Texas) was used as directed. Briefly, total RNA volume was brought up to 250 µl with nuclease free water to which 250 µl of 2x Binding Solution was added. Samples were mixed and each was added to a column of oligo(dt) cellulose in a 2 ml micro tube, vortexed to mix, and incubated at 70ºC for 5 minutes. Columns were rocked at room temperature for 1 hour then centrifuged at 3,000 x g for 3 minutes. Supernatant was discarded and the cellulose column was washed with 500 µl of Wash Solution I followed by vortexing and centrifugation at 3,000 x g for 3 minutes. Filtrate was discarded and washed once more followed by 3 additional washes with Wash Solution II, discarding the filtrate after each wash. The cellulose column was

transferred to a clean micro tube and 200 µl of warmed RNA Storage Solution was added, vortexed to mix, centrifuged at 5,000 x g for 2 minutes to extract the mRNA from the cellulose, and repeated once more. To the filtrate 40 µl NH_4Ac, 1 µl glycogen, and 1.1 ml of 100% ethanol was added to precipitate the mRNA. The samples were stored on dry ice for 30 minutes and centrifuged at 12,000 x g for 25 minutes to form a white mRNA pellet at the bottom of the tube. Supernatant was discarded the pellet was washed with and 1 ml of 70% ethanol, vortexed, and centrifuged at 12,000 x g for 10 minutes. Again the supernatant was removed and the mRNA pellet was resuspended in 50 µl of RNA Storage Solution and stored at -20ºC until use.

Primer specifications for reverse transcription and gene sequencing

Primer sets for bovine E-cadherin (CHD1) were designed using the on-line software "Net Primer" and based on a human E-cadherin coding sequence listed in Genbank (Accession# NM_004360). All primers were designed with a melting temperature (Tm) between 40-70ºC, around 50% GC content, hairpin formations <-4 kcal/mol, primer diamerizations <-4 kcal/mol, and similar homology with <50 sequences in a BLAST report. Primers were ordered on-line from Integrated DNA Technologies with standard desalting and rehydrated with nuclease free water to 100 µmol L^{-1}. Two gene specific primers were used for mRNA reverse transcription and 3 sets of overlapping primers were required for PCR amplification of the entire bovine E-cadherin coding sequence (Table 13).

Reverse transcription, PCR amplification and gel electrophoresis for gene sequencing

The Enhanced Avian Reverse Transcription kit (Sigma-Aldrich, St. Louis, MO) was used to amplify cDNA from the purified mRNA as directed. Briefly, 5 µl of mRNA, 1 µl dNTP mix (500 µmol L^{-1} each dNTP), 1 µl of either "Af" or "Dr" primer (10 µmol L^{-1}), and 3 µl of PCR grade water were combined in a 200 µl PCR micro tube and gently mixed. Tubes were heated for 10 minutes at 70ºC to denature any secondary structures in the RNA, and then cooled to 4ºC to stop the reaction.

Table 13. E-cadherin primer sequences for reverse transcription (RT) and PCR amplification. Rxn, Reaction; Prod. (nt), size of amplicon.

Primer	Sequence	Rxn	Prod. (nt)	Anneal Temp.
Af	5' GCTTGCGGAAGTCAGTTCAG 3'	RT	____	50ºC
Dr	5' CTGGCTCAAGTCAAAGTCCTG 3'	RT	____	50ºC
Red	5' GCTTGCGGAAGTCAGTTCAG 3' 5' ACCACACTCACAGTGACTGATGC 3'	PCR	1276	51ºC
C1	5' TGAACACCTACAATGCCGCCA 3' 5' TGACCACCTCTCTCCTCCGA 3'	PCR	1334	58ºC
Blue	5' CGTGAGTCTCTGATTTTGAAGCC 3' 5' AGGCGGCGAGGACGACTAG 3'	PCR	700	55ºC

Tubes were placed on ice and to each was added 2 µl 10x Buffer, 1 µl Enhanced AMV-RT enzyme, 1 µl RNase inhibitor (20 units/µl), and 6 µl PCR grade water. All samples were incubated at 50ºC for 50 minutes, cooled to 4ºC, and stored at -20ºC until use. Additionally, reactions omitting the primers were performed to detect any DNA contamination in the mRNA samples.

To PCR amplify the bovine E-cadherin coding sequence from cDNA, the Advantage® GC2 Polymerase Kit (Clontech, Mountainview, CA) was used. For each reaction in a 200 µl micro tube, the following reagents were combined: 25 µl PCR grade water, 10 µl 5x GC2 PCR buffer, 10 µl GC Melt (5 mol L^{-1}), 1 µl 50x dNTP (10 mmol L^{-1}), 1 µl GC2 Polymerase, 1 µl cDNA, and 2 µl gene specific primer mix (10 µmol L^{-1} for each primer). Each tube was placed in a thermocycler and reactions were performed under the following conditions: 94 ºC for 6 minutes (initial denature), 94 ºC for 30 seconds (step 1 of cycle), specific primer annealing temperature (Table 13) for 30 seconds (step 2 of cycle), 68 ºC for 1.5 minutes (step 3 of cycle), cycle repeated 35 times, then heated to 68 ºC for 6 minutes (final extension) and held at 4 ºC upon completion. Additional reactions omitting either the cDNA or the primers were performed to detect any contamination in the primer stocks or cDNA.

Amplified 50 µl samples were prepared for gel electrophoresis by adding 5 µl of Blue Juice™ loading buffer (Invitrogen, Carlsbad, CA) and mixing well. A 10-well, 1.2% w/v agarose gel made with 1X TAE buffer (Appendix B) and stained with ethidium bromide was prepared and allowed to solidify. A total of 25 µl of each sample was loaded into the gel and 10 µl of Tri-Dye 2-log DNA ladder (New England Biolabs,

Ipswich, MA) was included to approximate DNA amplicon size. Samples were electrophoresed at 60 mAmps for 30 minutes and then DNA migration was visualized under UV light in a light-tight box.

DNA bands of appropriate size were excised from the gel and purified with a QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) as directed. The excised agarose was placed into a 1.5 ml tube and 1 ml of Buffer QX1 was added and heated for 5 minutes at 50 ºC or until agarose completely melted. To the agarose solution, 10 µl of QIAEX II was added, vortexed, and incubated at 50 ºC for 10 minutes. The solution was centrifuged at 13,000 x g for 30 seconds and supernatant was aspirated leaving the DNA pellet behind. The pellet was washed twice with 500 µl of Buffer PE followed by centrifugation for 30 seconds and allowed to air dry for 10 minutes. Following the washes, collected DNA was resuspended in 20 µl of nuclease free water, incubated at room temperature for 5 minutes, centrifuged at 13,000 x g for 30 seconds, and the supernatant containing the DNA was collected into a fresh micro tube and sent to the Gene Technologies Laboratory at Texas A&M University for sequencing.

E-cadherin siRNA sequences

Once the complete coding sequence for bovine E-cadherin was determined, it was submitted to Ambion (Austin, TX) for analysis. Three siRNA sequences were produced (Table 14) with standard purification in 5 nmol L^{-1}. Using nuclease free water, lyophilized samples were rehydrated to 100 µmol L^{-1} and stored at -80ºC until validation in transfected MDBK cells with quantitative PCR and western blot analysis.

MDBK cell transfection

Mandin-Darby Bovine Kidney (MDBK) cells were cultured to ~70% confluence in 6-well tissue culture dishes in 2.0 ml of Glutamax (Gibco BRL, Rockville, MD) supplemented with 2.0 mmol L^{-1} glutamine, 10 % v/v fetal bovine serum (FBS), and 1 µl ml^{-1} gentamicin (50 mg ml^{-1}) at 37 ºC in humidified 5% CO_2 air. To transfect the cells, 1 µl of either the negative control Cy3-siRNA (described in Chapter II),

Table 14. E-cadherin siRNA sequences designed by Ambion ® .

Sequence	Length	Sense
siEcad-12	21 bp	5'GGCAUCCUUGCUUUUCUGAtt 3' 5' UCAGAAAAGCAAGGAUGCCtc 3'
siEcad-13	21 bp	5' GCUAAGUUUUCUUGUCCAUtt 3' 5' AUGGACAAGAAAACUUAGCtc 3'
siEcad-14	21 bp	5' GGAGGUGGAGAAGAAGAUCtt 3' 5' GAUCUUCUUCUCCACCUCCtt 3'

siEcad-12, siEcad-13, or siEcad-14 was combined with 6.3 µl of 2.0 mol L^{-1} calcium chloride and 42.7 µl nuclease free water and mixed well. To this solution, 50 µl of 2X Hepes Buffered Saline (HBS; Appendix C) was added drop wise while constantly flicking the tube to prevent precipitate from forming. Once mixed the transfection solution was added to the cell cultures, mixed thoroughly into the culture medium, and

co-cultured for 20 hours. After co-culture, cells were washed with calcium and magnesium free Dubelco's Phosphate Buffered Saline (DPBS; Gibco BRL, Rockville, MD), given 2.0 ml of fresh supplemented Glutamax, and returned to the incubator for 48 additional hours. After 48 hours, cells were washed again with DPBS and collected for either RNA or protein isolation along with untreated cell samples for controls.

Transfection efficiency in each replicate was determined in two separately transfected cell cultures with the negative control Cy3-siRNA. Prior to harvesting of cells for analysis, 8µl of 1 mg ml^{-1} Hoechst 33342 was added to the cells and incubated at 37ºC for 5 minutes. Five digital images of each cell culture were taken at 20X with both UV and fluorescent light. The total number of cells in each image was considered equivalent to the total number of fluorescent nuclei labeled with Hoechst. The number of Cy3 fluorescent cells for each image was determined and divided by the total number of cells to obtain the proportion of cells which were successfully transfected per culture. Transfection efficiencies between the two samples were then averaged to obtain the presumed transfection efficiency for that replicate.

Bovine zygote microinjection and blastocyst transfection

As described in Chapter II, bovine zygotes were produced *in vitro* by standard laboratory operating procedures and cumulus cells were removed by vortexing in 200 µl of warmed TL-Hepes (GIBCO, BRL, Rockville, MD) for 2 minutes. After vortexing, zygotes were washed twice through warmed Holding medium consisting of TCM-199 with Hank's salts (GIBCO, BRL) supplemented with 10% v/v fetal bovine serum (FBS;

HyClone, Logan, UT) and 1% gentamicin (50 mg ml^{-1} solution; GIBCO, BRL) and either held for direct injection or cultured to the blastocyst stage in G1/G2 sequential medium (Vitrolife, Littleton, CO) supplemented with 8 mg ml^{-1} Pentax BSA (Miles Laboratories, Elkhart, IN) and 1 µl ml^{-1} gentamicin at 38.5 ºC in 5% CO_2, 5% O_2, 90% N_2 humidified air.

Microinjection procedures detailed in Chapter II were followed and presumptive zygotes were injected with 25 µmol L^{-1} of either the negative Cy3-siRNA control or a Cy3 labeled siEcad in Holding medium under oil. After injection, zygotes were washed once through G1 and cultured in G1/G2 sequential medium. Day 7 blastocysts were transferred to fresh G2 and cultured until day 9 when non-treated and injected blastocysts were collected for analysis.

Chemical transfections were performed on day 7 *in vitro* produced blastocysts zona compromised by laser drilling in Holding medium supplemented with 200 µmol L^{-1} sucrose as previously described in Chapter II. Transfection complexes were constructed by combining 9 µl of GeneJammer with 38 µl of G2 (no BSA or gentamicin) and incubating at room temperature for 5 minutes. Either 3 µl of the negative Cy3-siRNA control or 3 µl of the Cy3-siEcad were added to the transfection complex, mixed gently, and incubated at room temperature for 20 minutes. After incubation, 50 µl of the transfection compound were added to the lasered blastocysts in 450 µl of G2 (no gentamicin) and incubated for 20 hours. Blastocysts were then washed twice and transferred into fresh G2 medium and further cultured until collection on day 9 for analysis.

RNA isolation and cDNA synthesis for quantitative PCR (qPCR)

For qPCR analysis, the RNeasy Kit (QIAGEN, Valencia, CA) was used as directed with a few modifications. To isolate RNA from MDBK cells, 350 µl of RLT RNA lysis buffer (no β-mercaptoethanol) was applied directly on DPBS washed cells and allowed to set for 1 minute. The lysed cells and solution were pipetted into a 1.5 ml micro tube, passed through a 25 guage needle five times to further disrupt the cell membranes, then snap frozen in liquid nitrogen (LN_2) and stored at -80ºC until purification. To isolate RNA from bovine blastocysts, 10 blastocysts were washed through DPBS and transferred in as little fluid as possible into 75 µl of RLT (no β-mercaptoethanol) in a 1.5 ml micro tube, vortexed for 2 minutes, then snap frozen in LN_2, and stored at -80ºC.

Prior to RNA purification, samples were thawed on ice and vortexed for 2 minutes. To each sample of cells/**blastocysts,** 350/ **75** µl of 80% v/v ethanol was added, mixed by pipetting, and transferred into an RNeasy spin column. Columns were centrifuged for 30 seconds at 10,000 x g and flow through was discarded. The column was washed with 350 µl Buffer RW1 and centrifuged for 30 seconds at 10,000 x g, again discarding flow through. In a clean 500 µl micro tube, 10 µl of DNase I and 70 µl of buffer RDD were mixed and then applied to the top of the filter membrane in the spin column and allowed to incubate at room temperature for 15 minutes. After incubation, 350 µl of buffer RW1 was added to the column which was then centrifuged for 30 seconds at 10,000 x g. The flow through was discarded, 500 µl of buffer RPE was added to the column and centrifuged for 30 seconds at 10,000 x g. Flow through was again

discarded and 500 µl of 80% ethanol applied to the column and centrifuged for 2 minutes at 10,000 x g. The column was then removed from the micro tube and directly centrifuged at full speed with the column lid open for 5 minutes to allow the membrane to dry. After centrifugation, the column was placed in a clean 1.5 ml mini tube and 30/**20** µl of nuclease free water was added directly on top of the membrane. The column was centrifuged once more at full speed for 1 minute to collect the purified RNA and stored at -80ºC until use.

Table 15. E-cadherin and GAPDH primer and probe sequences for qPCR. BHQ, Black Hole Quencher.

Gene	Primer/ Probe	Total Bases	Amplicon Size	Sequence
E-cadherin	Primers	20	76 bp	5' GGTGTTTGATTATGAAGGAA 3'
		17		5' GGTCTTGGTCTGACTCT 3'
E-cadherin	Probe	23	____	5' /FAM/ TGGTTCCGAAGCTGCTA CTCTGA /BHQ/ 3'
GAPDH	Primers	18	73 bp	5' GGCATTCTAGGCTACACT 3'
		19		5' CGAAGGTAGAAGAGTGAGT 3'
GAPDH	Probe	22	____	5' /FAM/ AGGACCAGGTTGTCTC CTGCGA /BHQ/ 3'

To synthesize cDNA, the iScript cDNA Synthesis Kit (Biorad, Hercules, CA) was used as directed. Briefly, 1 µg of MDBK RNA or the entire blastocyst RNA eluate were mixed with 4 µl 5x iScript Reaction Mix and Q.S. to 20 µl with nuclease free

water. The samples were incubated for 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, and held at 4ºC until storage at -20ºC.

Quantitative PCR

Primer sets for bovine E-cadherin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 15) were ordered online through Integrated DNA Technologies and dual-labeled (fluorophore FAM and Black Hole Quencher) TaqMan® probes were ordered online from Biosearch Technologies (Novato, CA). Lyophilized primers and probes were resuspended to 100 μmol L^{-1} with DNase-free water, immediately aliquoted into single use doses to limit possible contamination, and stored at -20ºC until use.

Synthesized cDNA from MDBK cells was subjected to SYBR® Green detection and samples from blastocysts were amplified with TaqMan® and dual labeled probes. Three experimental replicates were analyzed and all samples were performed in triplicate to detect E-cadherin and GAPDH in an ABI StepOne™ 48-well detection system. Purified RNA from each sample was also directly amplified in triplicate to detect possible DNA contamination in the cDNA and reactions omitting cDNA were performed for each primer set to establish purity of primer and probe stocks. In addition, a standard curve consisting of 5 samples increasing in concentration by a factor of 10 for each primer set was analyzed to validate appropriate primer performance.

For SYBR® Green detection, each reaction was performed in a 20 μl total volume and contained 10 μl Power SYBR® Green Master Mix (Applied Biosystems,

Foster City, CA), 2.5 µl each of sense and antisense primers (900 nmol L^{-1}), and 5 µl of cDNA diluted 1:10 in TE buffer and loaded into a 48-well plate covered with adhesive film. Samples were then heated to 95ºC for 10 minutes and cycled 40 times at 95ºC for 15 seconds followed by 60ºC for 1 minute. A disassociation curve was also performed at the end of each qPCR experiment for all samples to detect non-specific PCR amplification by heating the samples at 95ºC for 15 seconds, decreasing the temperature to 60ºC for 1 minute, and then increasing the temperature in 0.3ºC increments back up to 95ºC.

For TaqMan® probe based detection, 20µl total volume reactions containing 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 1.25 µl each of sense and antisense primers (900 nmol L^{-1}), 2.5 µl of probe (250 nmol L^{-1}) and 5 µl of cDNA diluted 1:10 in TE buffer and were loaded into a 48-well plate covered with adhesive film. Quantitative PCR reactions were heated to 50ºC for 2 minutes, 95ºC for 10 minutes, and then cycled 40 times at 95ºC for 15 seconds followed by 60ºC for 1 minute.

Protein isolation and western blot analysis

MDBK cells washed with DPBS were coated with 300 µl Protein Lysis Buffer (PLB; Appendix D), agitated until cells detached from the culture dish, then transferred into a 1.5 ml micro tube. Groups of 20 bovine blastocysts from similar treatment groups were washed through DPBS and transferred in as little fluid as possible into 30 µl of PLB in a 1.5 ml micro tube and vortexed for 2 minutes. All samples in PLB were

repeatedly snap frozen in LN_2 then thawed at 37ºC five times and stored at -80ºC. Prior to use, protein samples were thawed and centrifuged at 13,000 x g at 4ºC for 30 minutes. Supernatant was pipetted into a fresh micro tube and heated at 95ºC for 5 minutes to denature the proteins, then cooled to 4ºC. For MDBK protein samples 20 µg of protein were combined with 5 µl of 4X Sample Buffer (Appendix E) and Q.S. to 20 µl. Blastocyst samples were prepared by combining 22 µl of isolated protein with 8 µl 4X Sample Buffer.

Buffered protein samples were electrophoresed at 100 volts for 1 hour in a 1 mm thick 5% polyacrylamide stacking gel layered on top of a 10% polyacrylamide resolving gel (Appendix F) in 1X Electrode Buffer (Appendix G). For each gel, 20 µl of Precision Plus Protein Standards (Bio-Rad, Hercules, CA) was included for size approximation of migrated protein bands. Gels were transferred to a PVDF membrane in Semi-Dry Anode Buffer (Bio-Rad, Hercules, CA) with a semi-dry transfer apparatus at 10 volts for 20 minutes. [All following washes and incubations were carried out in an orbital shaker or rocker]. PVDF membranes were blocked in 5% non-fat dry milk in tris buffered saline + 0.05% Tween (TBST) (Appendix H) for 1 hour at room temperature followed by overnight incubation in primary antibody targeted against E-cadherin (Table 16) diluted in 2% non-fat dry milk in TBST at 4ºC. Membranes were washed 3 consecutive times for 10 minutes each wash in TBST and incubated for 1 hour at room temperature in the secondary antibody diluted in 2% non-fat dry milk in TBST. The blots were then washed three more times in TBST for 10 minutes each wash and incubated 5 minutes at room temperature in 10 ml total of SuperSignal® West Pico Chemiluminescent

Substrate (Pierce, Rockford, IL) while protecting from light. Membranes were placed inside a clear sheet protector and imaged in an AlphaInnotech light tight system. Densities of acquired chemiluminescent bands were measured with AlphaInnotech Alpha Ease Software v. 4.0. After imaging, blots were washed twice in TBST for 10 minutes at room temperature, and then incubated at 60ºC in 20 ml of Stripping Buffer (Appendix I) to remove antibodies. Blots were re-blocked and incubated with antibodies (Table 16) for the selected endogenous control genes (either GAPDH or β-actin) and then analyzed as described above. Controls for MDBK cells omitted incubation in the primary antibody to asses any non-specific binding of the secondary antibody.

Immunocytochemistry with bovine blastocysts

Prior to fixing, blastocysts were washed once through DPBS then held for 3 minutes in 500 µl of warmed 0.5% Pronase E in TCM199 with Hank's salts + 10% FBS (199-Hank's) to remove the zona. Zona-free embryos were then washed three times through 500 µl of 199-Hank's and twice more through DPBS. Successive cold methanol (MeOH) dilutions (-20ºC) in DPBS were used to fix the embryos by first holding them in 1:1 MeOH:DPBS for 2 minutes, then in 2:1 MeOH:DPBS for an additional 2 minutes. Blastocysts were then washed for 5 minutes in DPBS + 0 .01% Tween (PBST) on an orbital rocker before overnight incubation at 4ºC in Blocking Buffer (Appendix J). Embryos were incubated in primary E-cadherin antibody (Table 17) diluted 1:50 in Blocking Buffer at a density of 1 embryo/10 µl of buffer for 4 hours at room temp, followed by 6 washes for 20 minutes each through fresh Blocking Buffer on an orbital

shaker. The secondary antibody (Table 17) was diluted 1:100 in PBST and embryos were incubated overnight at 4 ºC in a similar density as the primary antibody.

Table 16. Antibodies used for western blot analysis.

Gene	Antibody	Source	Application – Dilution
E-cadherin	1º Anti-Rabbit Monoclonal	Millipore #07-697 (Burlington, MA)	MDBK Cells – 1:5,000 Blastocysts – 1:5,000
GAPDH	1º Anti-Rabbit Polyclonal	Affinity Bioreagent #PA3-16782 (Golden, CO)	MDBK Cells – 1:5,000
E-cadherin GAPDH	2º Goat Anti-Rabbit IgG H&L	Invitrogen #G-21234 (Carlsbad, CA)	MDBK Cells – 1:10,000
β-actin	1º Anti-Mouse Monoclonal AC-15	Abcam #ab6279 (Cambridge, MA)	Blastocysts – 1:5,000
β-actin	2º Goat Anti-Mouse IgG H&L	Invitrogen #M30007 (Carlsbad,CA)	Blastocysts – 1:10,000

Upon completion of antibody labeling, embryos were washed twice through PBST and mounted on a glass microscope slide in 10 µl of Mounting Medium (Appendix K). A

glass cover slip was very gently layered on top of the blastocysts and sealed with clear nail polish. Scanning confocal images were taken by Dr. Roula Mouneimne at the Texas A&M College of Veterinary Medicine Image Analysis Lab to detect fluorescence intensity of labeled E-cadherin in each embryo. A Meridian Ultima Confocal Microscope was used for image capture at 20x by excitation of the fluorophore with an argon laser at 488 nm and emission at 515-530 nm in a total of 35 z-series sectionals, 1 µm apart. Confocal images of embryo area and intensity were measured with Adobe Photoshop® to determine an average intensity of E-cadherin expression for each embryo evaluated.

Table 17. Antibodies used for immunocytochemistry.

Gene	**Antibody**	**Source**	**Application - Dilution**
E-cadherin	1º Mouse Monoclonal IgG2a	BD Biosciences #610F1 (San Jose, CA)	Blastocysts – 1:50
E-cadherin	2º Goat Anti-Mouse Alexa Fluor 488	Imgenex #A11001 (San Diego, CA)	Blastocysts – 1:100

Statistical analysis

The Ct values from similar replicates collected during qPCR were averaged and the ΔΔCt method (Hettinger *et al.* 2001) was used to determine relative quantitation of E-cadherin gene expression compared to the endogenous control gene GAPDH. A Student's T-test was performed with Microsoft Excel software to determine significant differences in E-cadherin gene expression with $P < 0.05$. Density measurements were taken of western blot bands within a set pixel area to obtain an average light density measurement for E-cadherin and GAPDH. Relative density for each sample was determined and subjected to ANOVA analysis using JMP 5.1 statistical software to detect significance with $P < 0.05$ and a Dunnett's test with $P < 0.05$ significance was used for comparisons of each treatment to the control when ANOVA indicated differences within the data. Fluorescence intensity of E-cadherin was averaged among all ICC processed embryos in similar treatment groups and compared with ANOVA and Student's T-tests with significance in expression differences considered when $P < 0.05$.

CHAPTER IV

RESULTS

Bovine E-cadherin coding sequence

Amplicon sequences from three overlapping primer sets were compiled to establish the coding sequence (linear mRNA) for bovine E-cadherin. The resulting gene was submitted to GenBank and assigned the accession number AY508164 on January 17th, 2004. The coding portion of the gene contains 2649 base pairs which translate into an 882 amino acid protein and the nucleotide sequence is given below in Figure 10.

5'ATGGGCCCTTGGAGCCGCAGCCTCTCTGCGCTCTGCTGCTGCTGCAGGTGT
AATCCGTGGCTCTGCCGGGAGCCGGAGCCCTGCATTCCTGGCTTTGGTGCTG
AGAGTTACACGTTCACCGTGCCCCGGCGGAACTTGGAGAGAGGGCGAGTCC
TAGGCAGAGTGAGTTTTGAAGGATGTGCTGGCCTACCAAGGACAGTCTATGT
TTCTGATGACACCCGATTCAAAGTGCACACAGATGGCGTGCTTACAGTCAGA
CGACCTGTACACCTTCATCGTCCAGAGCTAAGTTTTCTTGTCCATGCCTGGGA
CTCCACCCACAGGAAGCTCTCCACCAAAGTGACACTGGAGGTATCAGCGCA
CCACCACCACCACCACAGTCATCATGACTCTCCCTCTGGAACCCAGACAGAA
GTGCTCACATTTCCTGGCCCCCACCATGGTCTCAGGAGACAGAAGAGAGACT
GGGTTATTCCTCCTATCAGCTGCCCAGAAAATGAGAAAGGCCCATTTCCTAA

Fig. 10. Bovine E-cadherin coding sequence.

GTCGCTGGTCCAGATCAAATCTAACAAGGAGAAAGAAACCCAAGTTTTCTAC

AGCATCACTGGCCAACGAGCTGATACACCCCCTGTCGGTGTTTTTATTATTG

AAAGAGAAACAGGATGGTTAAAAGTGACACAGCCTCTGGATAGAGAACAGA

TTGCCAAGTACATTCTCTTCTCTCATGCCGTGTCTTCAAATGGACAAGCCATT

GAAGAGCCTATGGAGATTGTGATCACCGTGACCGACCAGAATGACAACAAG

CCCCAGTTCACCCAGGAGGTCTTCAAGGCGTCTGCCCTGGAAGGCGCTCTTC

CAGGAACCTCTGTGATGCAGGTCACGGCCACAGATATAGATGACGAGGTGA

ACACCTACACCGCTGCCATCGGTTACACAATCCCAGCCCAAGATCCCATGCT

GCCGCACAACAAAATGTTCACCATCAACAAGGAAACAGGCGTCATCAGTGT

GCTCACCACCGGGCTGGACCGTGAGAGTTTTCCCACATACACCCTGATGGTC

CAAGCAGCAGACCTTAACGGCGAAGGCTTGAGCACAACTGCAACGGCCGTG

ATCACAGTCTTGGACACCAATGATAATGCTCCCAGATTCAACCCAACCACGT

ACGTGGGGTCGGTGCCTGAGAACGAGGCTAATGTGGCCATCACCACACTCA

CAGTGACTGATGCCGACGACCCCAACACCCCGGCATGGGAGGCTGTTTACAC

AGTATTAAATGATAACGAGAAGCAATTTATCGTCGTCACAGACCCAGTCACC

AATGAAGGCACTCTGAAAACAGCTAAGGGCTTGGATTTTGAGGCCAAGCAG

CAGTACATCCTGTACGTGGCAGTGACAAATGTGGCCCCCTTTGAAGTCACTC

TCCCCACTTCCACAGCCACCGTCACTGTGGATGTGATAGATGTGAATGAAGC

CCCCATCTTTGTGCCTCCTCAAAAGAGAGTGGAAGTGCCCGAGGACTTTGGC

GTGGGCCTGGAGATCACATCCTATACTGCCCGGGAGCCAGACACATTTATGG

AACAGAAGATCACGTATCGGATTTGGAGGGACACTGCCAACTGGCTGGAGA

Fig. 10. Continued.

TTAATCCAGAAACGGGTGCCATTTCCACTCGGGCTGAGTTGGACAGAGAGG
ATGTCGATCATGTGAAGAACAGCACGTACACGGCCCTCATTATAGCCACTGA
CAATGGTTCTCCACCTGCCACTGGGACAGGCACCCTGCTCTTGTTCCTCGATG
ATGTGAATGACAATGGCCCCGTACCAGAACCCCGGACCATGGACTTCTGCCA
GAGGAATCCTGAGCCTCATATCATCAACATCAATGATCCTGATCTCCCTCCG
AACACCTCCCCCTTTACAGCAGAACTGACACATGGGGCGAGTGTCAATTGGA
CCATTGAGTACAATGACCAAGAACGTGAGTCTCTGATTTTGAAGCCAAAGAA
AACCTTAGAGCTGGGTGACCACAAAATCAATCTCAAGCTCATAGACAACCA
GAACAAAGACCAGGTGACCACACTTGATGTGCACGTGTGTGACTGTGATGG
GATCGTCAGCAACTGCAGGAAGGCACGGCCTGCTGAAGCAGGATTGCAAGT
TCCCGCCATCCTGGGGATCCTTGGAGGCATCCTTGCTTTTCTGATCCTTATTT
TGCTGCTTCTGCTACTTGTTCGGAGGAGAAGGGTGGTCAAAGAGCCCTTACT
GCCCCCAGAAGATGACACCCGGGACAATGTGTATTACTATGATGAAGAAGG
AGGTGGAGAAGAAGATCAGGACTTTGACTTGAGCCAGTTACATAGGGGCCT
GGATGCTCGGCCTGAAGTGACTCGCAATGACGTGGCACCAACCCTCATGAGT
GTGCCCCAGTACCGACCCCGCCCTGCCAATCCTGATGAAATTGGAAACTTTA
TTGATGAAAACCTGAAGGCAGCTGATAGTGACCCCACTGCCCCACCCTATGA
CTCTCTGCTGGTGTTTGATTATGAAGGAAGTGGTTCCGAAGCTGCTACTCTG
AGCTCCCTGAACTCCTCAGAGTCAGACCAAGACCAGGACTATGACTACCTGA
ATGAATGGGGCAATCGCTTCAAGAAGCTGGCGGACATGTATGGAGGCGGCG
AGGACGACTAG 3’

Fig. 10. Coninued.

E-cadherin specific siRNA validation

The three siRNA sequences developed by Ambion® (Table 14) based on the above E-cadherin sequence and a null Cy3-siRNA were transfected into MDBK cells to identify which custom designed siRNA induced the greatest knockdown of mRNA and protein expression as assessed by qPCR and western blot analysis. Transfections were performed in three replicates for each method of assessment and transfection efficiencies based on duplicated Cy3 fluorescent cells in each replicate ranged from 92.5-95.0%. Expression levels were quantified individually in each replicate, and then averaged by treatment across all experiments for comparison and statistical analysis.

SYBR® Green based qPCR analysis measured the Ct value for E-cadherin and GAPH in each replicate and the Ct mean and ΔCt mean were calculated and reported in Appendix L. All ΔCt mean values in similar treatments from all replicates were combined into ΔΔCt calculations to obtain the fold difference between treatments which was then log10 transformed to ascertain relative changes in gene expression and standard error of the mean (± S.E.M.). A Student's T-test was performed to determine significance ($P < 0.05$) between each treatment and the non-treated Control and Cy3-siRNA null. There was no difference in relative E-cadherin gene expression in any of the treatments compared to the Control (1.02 ± 0.26) (Figure 11). However, siEcad-13 and siEcad-14 (0.62 ± 0.37 and 0.84 ± 0.22, respectively) had significantly diminished expression compared to the null, Cy3-siRNA (1.19 ± 0.25), which was not different from

siEcad-12 (1.21 ± 0.45). Disassociation curves for each replicate confirmed appropriate

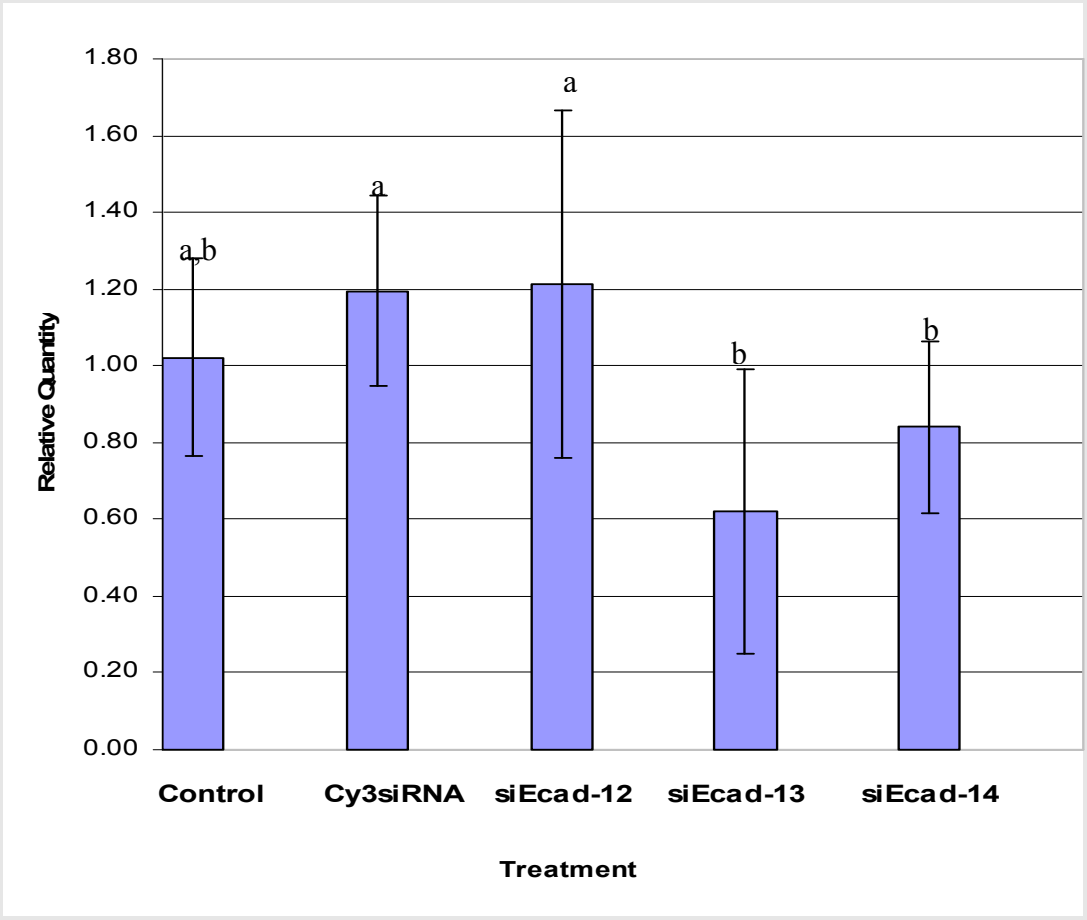

Fig. 11. Expression levels of E-cadherin mRNA. MDBK cells were transfected with a null siRNA (Cy3-siRNA) or with sequence specific siRNAs. Bars indicate standard error of the mean (± S.E.M.). Differences in letters indicate significance at $P < 0.05$.

SYBR® Green signal amplification and negative controls indicated no contamination in the cDNA or primers.

Density measurements within a fixed pixel area were obtained for E-cadherin and GAPDH in MDBK transfected cells from western blot image analysis (Figure12). Two protein bands were consistently detected with E-cadherin antibody labeling between 100-150 kDa. The larger protein did not appear to be affected by E-cadherin siRNA interaction, while the smaller appeared around 106 kDa, the presumed size for E-cadherin, and responded appropriately to treatment with siRNAs. GAPDH proteins were detected at the expected size, approximately 38 kDa, in every replicate. Density ratios and relative protein expression levels were calculated and reported in Appendix M and ANOVA analysis indicated significant differences in E-cadherin expression between treatments ($P < 0.0001$). A Dunnett's test further indicated all three E-cadherin specific siRNAs significantly decreased protein expression by 69% (siEcad-12), 73% (siEcad-13), and 72% (siEcad-14) when compared to the non-treated Control (Figure 13), and by 49, 53, and 52%, respectively, when compared to the null Cy3-siRNA.

Based on these data, siRNA-13 was identified as an appropriate sequence for experiments in bovine embryos. A new sequence was produced by Ambion® with a Cy3 label on the 5' end and is further referred to as Cy3-siEcad.

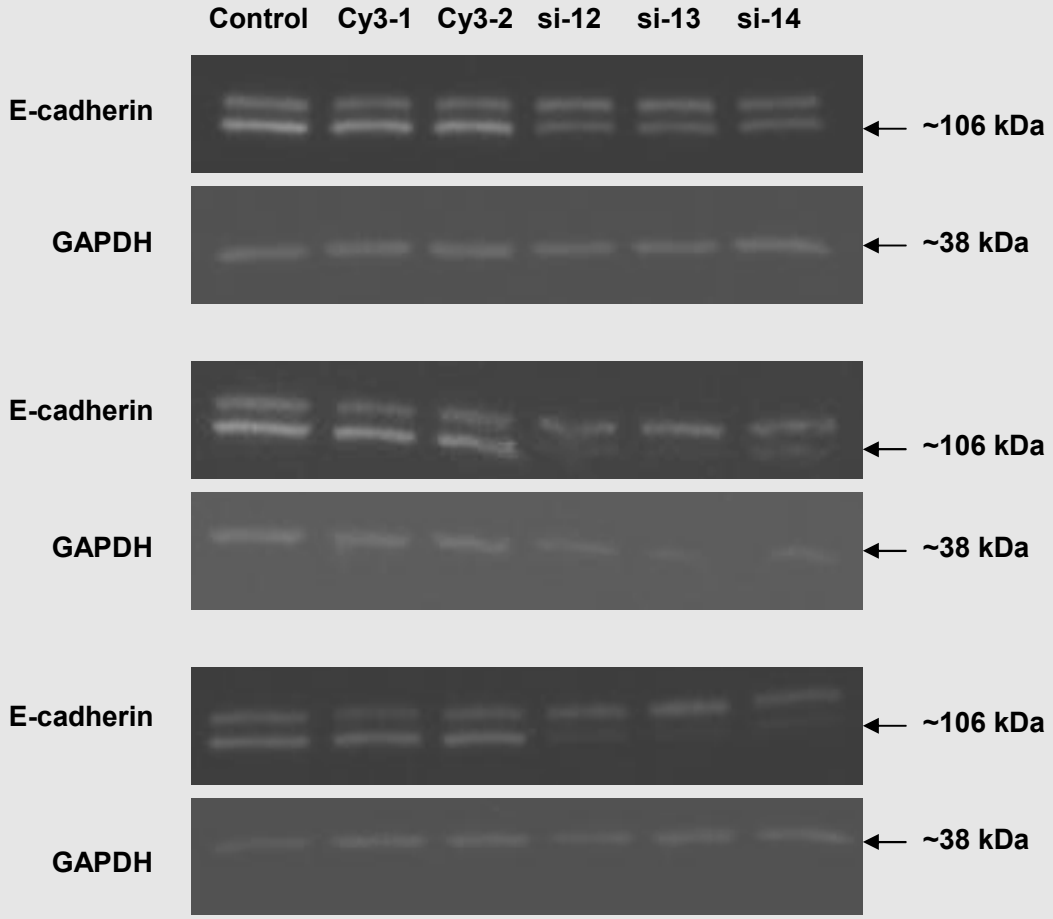

Fig. 12. Western blot analysis with transfected MDBK cells. Three replicates transfected with null Cy3-siRNA (Cy3-1, Cy3-2) or E-cadherin specific siRNA (si-12, si-13, si-14). Expected E-cadherin proteins are approximately 106 kDa and GAPDH about 38 kDa.

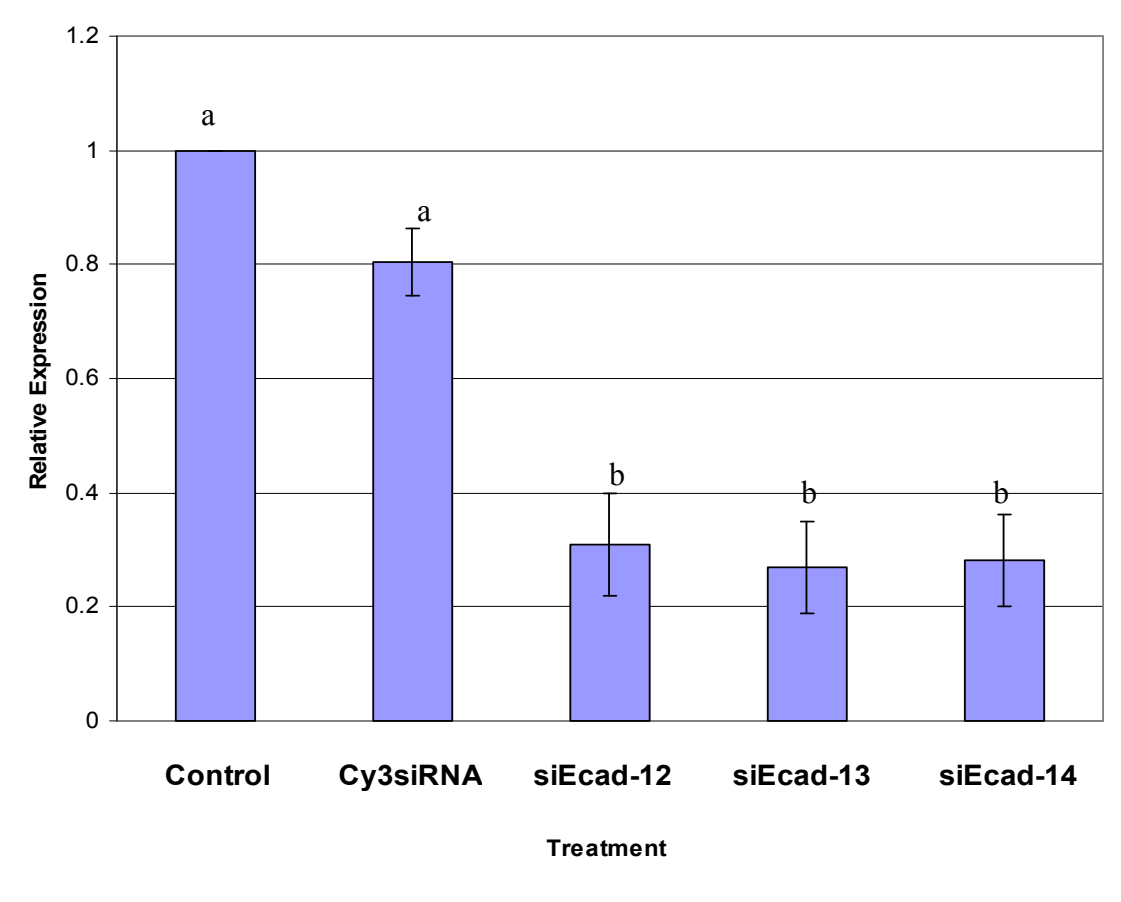

Fig. 13. Relative E-cadherin protein expression in transfected MDBK cells. Null siRNA (Cy3-siRNA) or sequence specific siRNAs are compared to the non-treated Control. Bars indicate standard error of the mean (± S.E.M.) and different letters indicate a significant difference with $P < 0.0001$

Analysis of E-cadherin expression in Cy3-siEcad treated bovine embryos

Day 1 bovine zygotes were injected and day 7 blastocysts were transfected with null Cy3-siRNA or E-cadherin specific Cy3-siEcad siRNAs. Groups of 10 similarly treated blastocysts were collected on day 9 and analyzed for E-cadherin and GAPDH gene expression utilizing TaqMan® based qPCR. The Ct values were measured and the ΔCt for each replicate was determined, averaged by treatment (Appendix N), and then combined into a ΔΔCt analysis for all replicates to obtain relative expression levels (Figure 14). Statistical analysis was performed with a Student's T-test and significance was considered when $P < 0.05$. Compared to the Control (2.83 ± 0.01), there was no difference in E-cadherin expression in either of the null Cy3-siRNA treatments (2.99 ± 0.09, injected and 2.87 ± 0.11, transfected), however Cy3-siEcad injected embryos had significantly more mRNA expression (3.01 ± 0.01; $P < 0.005$). There was significantly less E-cadherin detected in Cy3-siEcad transfected embryos (2.55 ± 0.09) compared to the non-treated and null Cy3-siRNA transfection control embryos ($P < 0.05$).

Groups of 20 blastocysts were collected from each treatment and relative E-cadherin protein expression was compared to β-actin. Three replicates were performed, however, only one appropriately labeled both proteins and is therefore demonstrative of proof-of-concept only and not subject to statistical analysis. Graphical representation in Figure 15 illustrates that there was very little variation in relative protein expression between treatments with expression ratios ranging from 0.99 – 1.09.

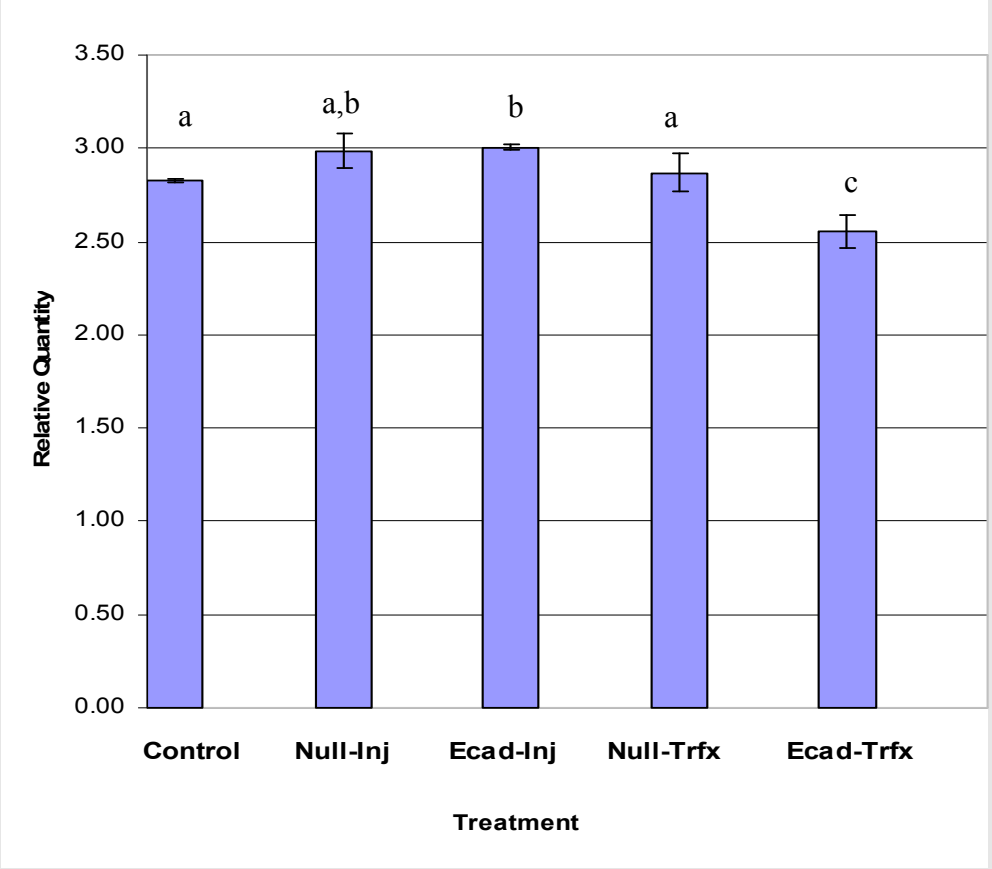

Fig. 14. Relative expression of E-cadherin mRNA in day 9 blastocysts. Embryos were either injected at the zygote stage (Null-Inj and Ecad-Inj) or transfected at day 7 (Null-Trfx and Ecad-Trfx). Different letters indicate significant changes in expression with $P < 0.05$. Error bars represent the standard error of the mean (± S.E.M.)

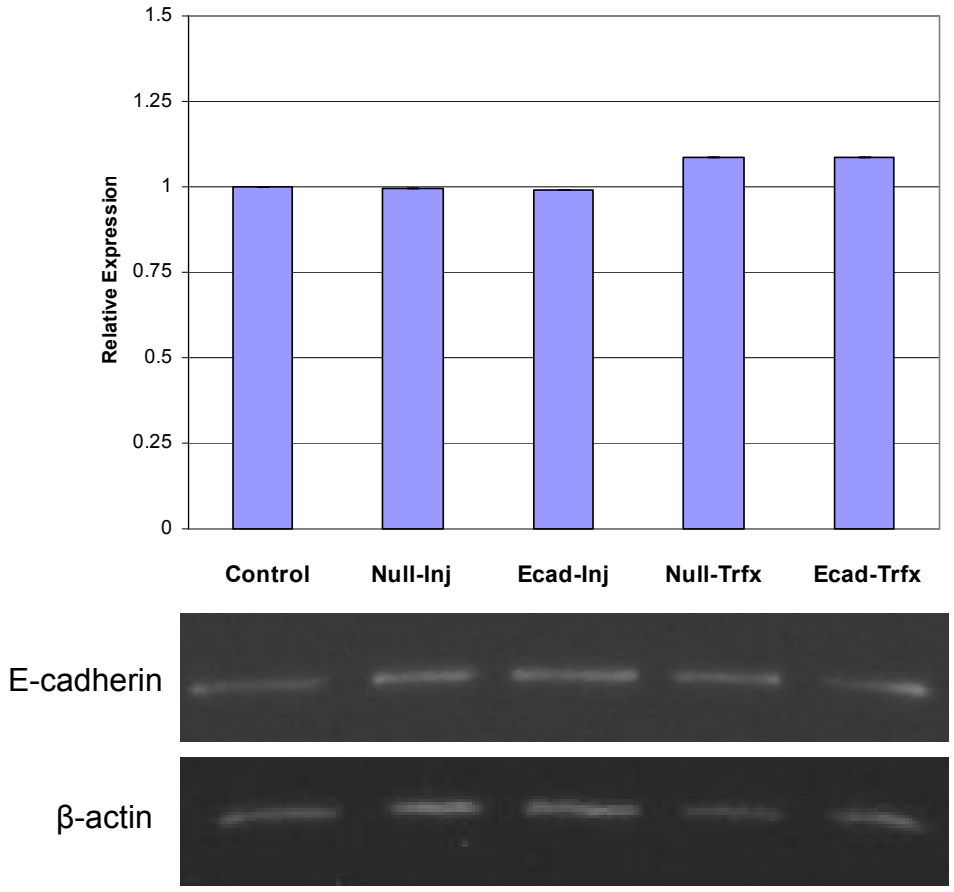

Fig. 15. Western blot display and relative expression of E-cadherin in day 9 blastocysts. Bovine embryos were either injected (Inj) or transfected (Trfx) with Cy3-siRNA (null) or Cy3-siEcad (Ecad).

To further evaluate protein expression, a more sensitive method was used to detect changes in E-cadherin intensity in individual embryos. Four to five blastocysts from each treatment were subjected to immunocytochemistry and E-cadherin antibody fluorescence intensity was measured with scanning confocal microscopy. Intensity measurements were averaged within each treatment and analyzed with ANOVA and Student's T-test with $P < 0.05$ (Figure 16). Compared to the Control, there was no difference in intensity among any of the treated groups. Although there was a tendency in Cy3-siEcad injected embryos to express less E-cadherin than the Cy3-siRNA null, the overall intensities of these treatments was not significantly different. Furthermore, E-cadherin expression did not differ between the blastocysts transfected with the null and E-cadherin siRNAs. The only statistical difference in these data was between the two Cy3-siEcad treatments where injected embryos had decreased protein expression compared to those which were transfected ($P < 0.05$).

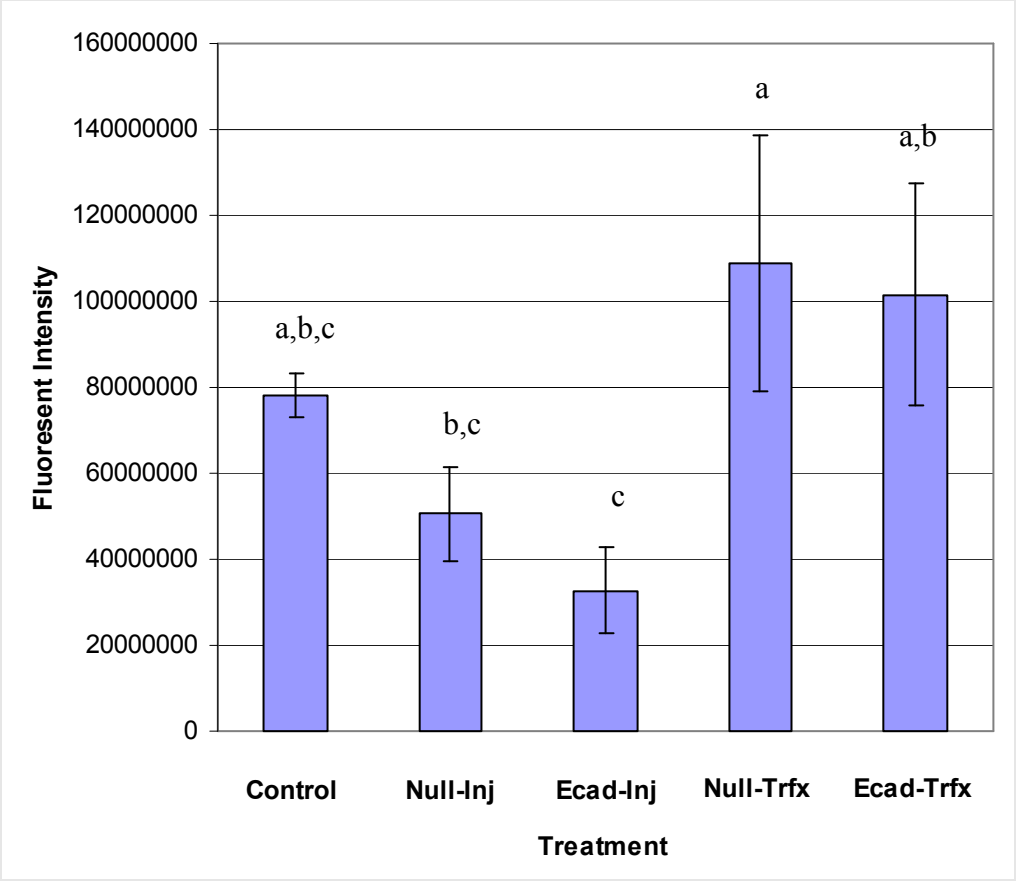

Fig.16. Fluorescence intensity of E-cadherin in ICC labeled day 9 blastocysts. Bovine embryos were either injected or transfected with null or E-cadherin specific siRNAs. Error bars indicate the standard error of the mean (± S.E.M.) with $P < 0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

E-cadherin forms the main component of the adherent junctions between trophectoderm cells and has been demonstrated to be absolutely required for cellular differentiation and blastocoel formation in bovine embryos (Barcroft *et al.* 1998). Therefore, E-cadherin was selected as the target gene to compare two methods for delivery of siRNAs to knock down gene expression in hatched (day 9) bovine embryos for several reasons. First, there is an obvious knockout phenotype which is easily recognizable upon visualization. Second, bovine E-cadherin has been characterized in early embryo trophectoderm (Barcroft *et al.* 1998) and antibodies have been developed and validated for appropriate detection. Third, previous experiments in both the mouse and cow have demonstrated the ability to effectively decrease E-cadherin gene expression up to day 7 (Nganvongpanit *et al.* 2006a; Wianny and Zernicka-Goetz 2000) in embryos by microinjecting dsRNA.

Since publication of the complete coding sequence for bovine E-cadherin in GenBank (Figure 10), there has been one additional entry describing the complete mRNA sequence (Accession# BC147914) obtained from bovine fetal fibroblast RNA. A ClustalW alignment between the two sequences indicates 99.02% homology with 2623/2649 base pairs aligning in a similar arrangement. In addition, Nganvongpanit *et al*. 2006, designed long dsRNA based on the gene sequence detailed here and

demonstrated successful targeting of bovine E-cadherin in early stage embryos. Given these reports, it is with confidence that the coding sequence presented within (Figure 10) is at least 99% accurate. Furthermore, properly designed siRNAs targeting bovine E-cadherin based on this sequence has a 99% chance to be completely homologous to its target and can be expected to induce post transcriptional gene silencing.

Ambion® guarantees that two out of the three gene specific siRNA sequences they design will induce at least 80% protein expression knock down in most mammalian cell lines. All three of the siRNA sequences evaluated in this report against E-cadherin in MDBK cells decreased protein expression by approximately 70% compared to the control and by 50% when compared to the null transfected cells. However mRNA expression profiles were somewhat different after MDBK cell transfection. Both siEcad(13) and siEcad(14) significantly reduced detectable E-cadherin mRNA compared to the null Cy3-siRNA control, but siEcad(12) treated cells were not different from either the control or null treated cell populations. This can be attributed to one of the qPCR replicates for siEcad(12) which produced a much lower ΔCt Mean value, most likely due to a pipetting error when setting up the reaction, skewing the relative quantity value slightly higher. Although removal of this replicate from the ΔΔCt calculations would have lowered the overall relative quantity of siEcad(12), this difference compared to the Cy3siRNA control was calculated not to have been significant and therefore the replicate values remained in the final expression profile report. Based on these data, a Cy3 labeled siEcad(13) (Cy3-siEcad) was chosen to attempt knock down of E-cadherin in zygote and blastocyst stage bovine embryos.

The expected outcome after introduction of siEcad(13) into early bovine embryos was that both protein and mRNA expression would be noticeably altered in the injected zygotes and transfected blastocysts, similar to what was observed in the MDBK cells. However, by day 9 of embryonic development, there was no observable difference in E-cadherin mRNA expression in Cy3-siEcad injected zygotes as opposed to those injected with the null Cy3-siRNA (Figure 14). There was however a significant increase in expression when compared to the non-treated control, but this probably is due more to a difference in the quality of embryos rather than interactions of the siRNA. Zygotes which are capable of continued development after microinjection tend to derive from better quality oocytes creating a population of embryos that are predominantly healthier than a control population which may include a mixed assortment, some of the embryos representing inferior quality detectable in qPCR analysis.

Conversely, day 7 blastocysts transfected with Cy3-siEcad did express a significant decrease in mRNA on day 9 compared to either control (Figure 14), a result which was expected. Cy3 fluorescence provided visual confirmation of successful transfection into the trophectoderm and was still slightly visible at the time of blastocyst collection for analysis, indicating the potential for sustained siRNA activity. Therefore it was reasonable to expect to see a significant decrease in E-cadherin mRNA after 48 hours of siRNA interaction, similar to what was observed in the MDBK cells. It is interesting that significantly less E-cadherin mRNA was detected in blastocysts transfected with siEcad(13) than in zygotes injected with siEcad(13). This would seem to demonstrate that the continuance of injected siRNAs at 25 μmol L^{-1} into zygotes does

not persist more than 7 days, suggesting targeted suppression of genes associated with hatching and elongation is unlikely to succeed with this technique. Not only did these data indicate E-cadherin mRNA was successfully targeted, but also siEcad(13) was 100% homologous to the target inducing mRNA degradation presumably through the siRNA processing pathway detailed in Figure 3. Additionally, this further validates the accuracy of the E-cadherin coding sequence presented in Chapter IV (Figure 10). To date, this is the first report describing successful post transcriptional gene silencing in bovine blastocyst trophectoderm cells using transfected siRNAs.

Given the results from mRNA analysis the complete lack of protein expression knockdown in similarly treated embryos (Figure 15) was surprising. Western blot analysis protein quantification detected virtually no difference in E-cadherin expression levels among any of the treatments in the one replicate which was available for analysis. However, in the remaining two replicates in which β-actin was not detectable, making quantification data unobtainable, E-cadherin density measurements were consistently similar to what was observed in the first replicate. This would suggest that changes to protein levels in the blastocysts truly were not detectable with western blot.

Since western blots do not always provide sensitive detection of minute changes in protein intensity; ICC was utilized to measure E-cadherin protein expression in individually immuno-labeled blastocysts. Although there was no statistical difference in the protein levels between the Control and injected embryo treatments (Figure 16), there was a tendency for Cy3-siEcad injected zygotes to exhibit less fluorescent emissions than the Control or null injected embryos. Neither the null nor siEcad(13) transfected

blastocysts differed from the Control or each other, however, unlike the mRNA analysis, there was a significant amount more of E-cadherin protein detected in the transfected embryos than in the Cy3-siEcad injected zygotes. It is strange that mRNA but not protein levels would be affected by blastocyst transfection. The half-life of E-cadherin in confluent epithelial cells is 5-10 hours (Gumbiner 2000) and therefore it is expected that protein levels in the embryos would diminish within 24 hours after siRNA transfection. However, the most likely reason for no detectable change in E-cadherin protein expression is probably due to a flawed method of blastocyst collection. In the Cy3-siEcad transfected blastocysts, trophectoderm cells were already beginning to disassociate blastocysts on day 9. These unincorporated cells however were not collected along with the blastocyst for analysis so that E-cadherin and GAPDH levels were only measured in the healthy or unaffected cells which remained in the trophectoderm, thus protein levels appeared to be unaltered.

These E-cadherin protein expression data would also reaffirm that injected siRNAs are depleted in developing embryos well before day 9 when E-cadherin expression levels are evaluated. E-cadherin transcript levels apparently are able to re-establish normal quantities and embryo development continues in a belated time line, as represented in Figure 17, which depicts the delayed blastocoel development in Cy3-siEcad injected zygotes. On day 7, both the Control and null Cy3-siRNA blastocysts had an obvious blastocoel formed and were beginning to expand. The Cy3-siEcad injected embryos did not have a blastocoel and appeared similar to a large morula before compaction. By day 8, blastocysts from both control groups had begun to hatch whereas

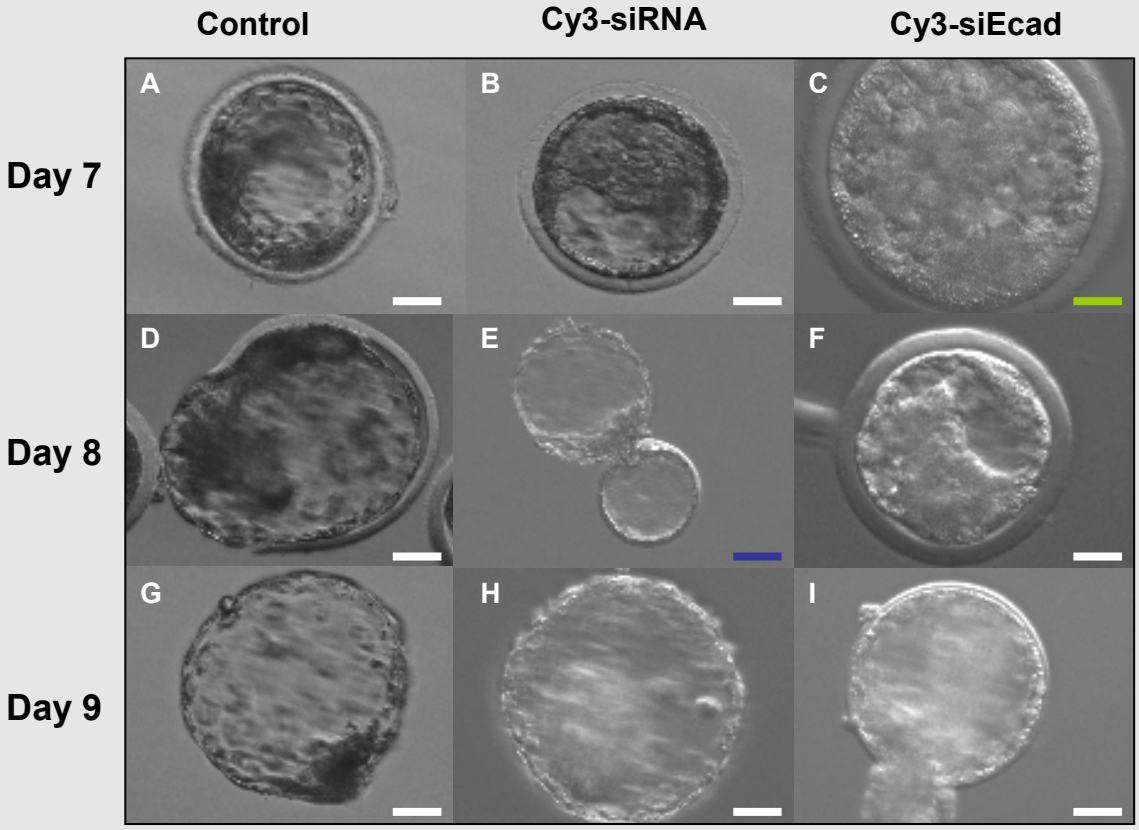

Fig. 17. Embryo development in injected zygotes. Blastocyst formation in day 7, 8, and 9 bovine embryos from non-treated Control, Cy3-siRNA injected, or Cy3-Ecad injected zygotes. Both Control and Cy3-siRNA embryos display normal progression from blastocoel formation on day 7 (A and B) to hatching on day 8 (D and E), and fully hatched blastocysts by day 9 (G and H). Cy3-siEcad injected zygotes have delayed development with blastocoel formation beginning on day 8 (F) and hatching initiating on day 9 (I). White scale bars = 50 µm at 20X, blue scale bar (E) = 100 µm at 10X, and green scale bar (C) = 25 µm at 40X.

the Cy3-siEcad embryos just begun blastocoel formation and did not initiate hatching until day 9 by which point the control embryos were completely hatched.

Although protein expression levels appeared unaffected in embryos by the siRNAs, transfected blastocysts which were allowed to continue in culture for an additional 24 hours to day 10 underwent complete trophectoderm disintegration, presumably as a result of siRNA induced PTGS (Figure 18). On the day of data collection for this project (day 9), Cy3-siEcad transfected blastocysts already had begun to exhibit signs of trophectoderm cell adhesion loss whereas the null control appeared normal, comparable to an *in vitro* derived embryo. By day 10 of culture, while the Cy3-siRNA transfected blastocysts continued to expand, the trophectoderm of E-cadherin targeted blastocysts had completely disassociated leaving behind multiple inner cell mass structures. This would indicate that the siRNAs were actually functioning as expected, but protein degradation in embryos appears to be delayed compared to epithelial cell cultures.

Undoubtedly, these presented data demonstrate efforts towards altering gene expression in hatching and elongating bovine embryos with siRNAs should be attempted by transfection at the blastocyst stage once cell differentiation has occurred. Although it is possible that higher concentrations of injected siRNAs could sustain gene silencing to the blastocyst stage, early studies reported in Chapter II indicate decreased development with injected concentrations of 50 $\mu mol\ L^{-1}$ or greater. Unfortunately, at this time, current techniques for blastocyst transfection do not appear to affect the inner cell mass (ICM). The mechanisms for chemical transfection described in Chapter II involve

endocytotic and membrane fusion pathways which conclude in the cytoplasm and do not employ exocytosis. Therefore it is not possible for the chemical transfection reagents to pass completely through the trophectoderm and then attempt to further transfect cells in the ICM (Rossant 2007). However, this limited access to the ICM associated with blastocyst transfection could be beneficial in altering trophectoderm gene function without contaminating the embryo with transgenes. Gene expression anomalies could potentially be rescued and functional genomics during elongation could be assessed with relatively simple treatments to the embryos before transfer.

Future directions for this project may include developing treatments for bovine blastocysts with siRNAs targeted against apoptotic genes, such as *bax,* before embryo transfer to reduce the incidence of apoptosis and increase implantation rates in a commercial production setting (Yang and Rajamahendran 2002). siRNA transfection may also be useful in treating cloned embryos before transfer to reduce highly aberrant gene regulation of DNA methyltransferase 1 which can lead to improper epigenetic modifications, failed development and implantation (Giraldo *et al.* 2008). Treatment of blastocysts with siRNAs targeting E-cadherin could also server as an alternative to immunosurgery for isolating ICM cells for stem cell isolation. Figure 18(D) depicts the complete removal of trophectoderm cells surrounding the inner cell mass after transfection with Cy3-siEcad. This resulting morphology would alleviate the need for complicated embryo manipulation associated with immunosurgery, although further modifications would warrant investigation to reduce structural damage to the ICM

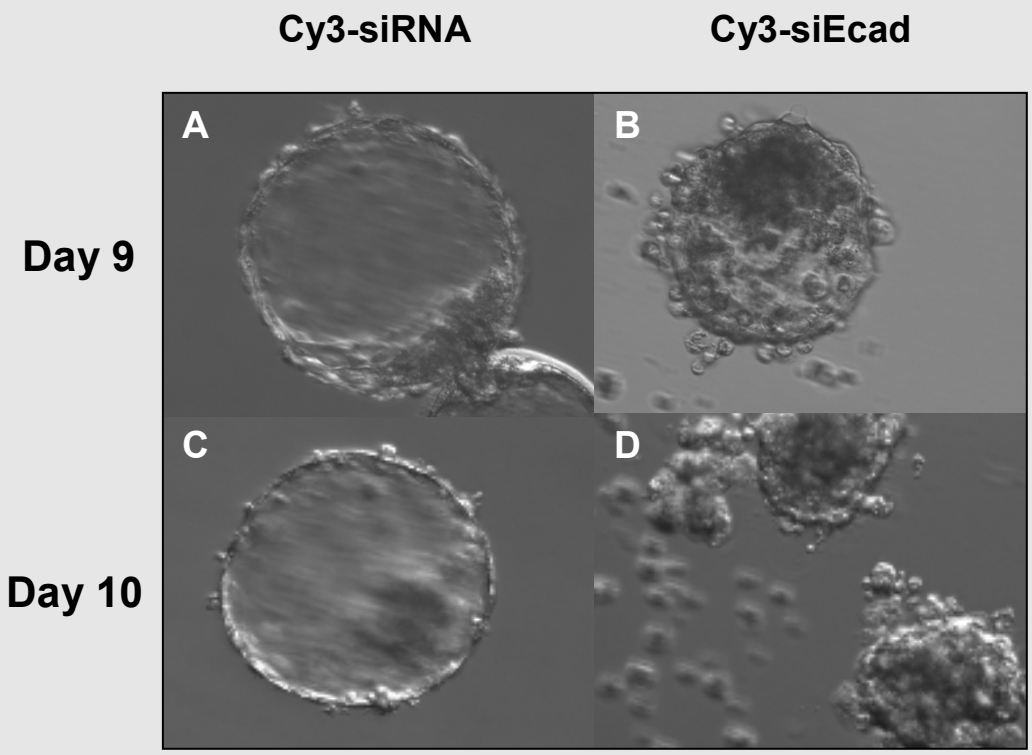

Fig. 18. Trophoblast morphology in transfected blastocysts. Bovine embryos transfected on day 7 with the null Cy3-siRNA or gene specific Cy3-siEcad and cultured to day 10. Null treated blastocysts continue to expand (A and B)while the trophectoderm cells in E-cadherin targeted blastocysts begin to disassociate on day 9 (B) until the trophectoderm disintegrates leaving only the inner cell mass behind (D).

GeneJammer has been used to optimize adenovirus gene delivery into porcine mesenchymal stem cells and it is possible that similar success could result with lentivirus mediated gene transfer into the trophectoderm of bovine blastocysts (Bosch *et al.* 2006).

Such stable gene knockdown would make genes involved in implantation and pregnancy maintenance more accessible for study or regulation. Alternatively, lentiviral delivered shRNA targeting viral transcripts could protect the transferred embryo by preventing infectious contamination within the uterine environment of the surrogate. Ultimately, the potential exists to successfully create transgenic livestock for development of animal models for biomedical and pharmaceutical evaluation, and to increase production efficiency in a commercial setting by targeting gene expression in the early embryo.

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APPENDIX A

1X TE Buffer

0.1579 g Tris-HCL (10mmol L^{-1})

0.0038 g EDTA (0.1 mmol L^{-1})

Q.S. 100 ml with ultrapure H_2O

Mix all until salts are dissolved and pH to 7.3 - 7.4, then filter through a 0.2 μm filter.

Aliquot and store in -20ºC until use.

APPENDIX B

50X TAE Buffer

242 g Tris base (2 mol L^{-1})

57.1 ml glacial acetic acid (1 mol L^{-1})

100 ml 0.5 M Na_2 EDTA (pH 8.0)

Q.S. up to 1000 ml with ultrapure H_2O

APPENDIX C

2X Hepes Buffered Saline

8.0 g NaCl

0.37 g KCl

0.099 g Na_2HPO_4

1.0 g Dextrose

5.0 g Hepes

400 ml dd H_20

Mix above thoroughly and pH to excatly 7.2. Q.S. to 500 ml with dd H_2O and filter sterilize.

APPENDIX D

Protein Lysis Buffer

1% v/v Triton X-100

0.5% v/v NP-40

150 mmol L^{-1} NaCl

10 mmol L^{-1} EDTA

1 mmol L^{-1} EGTA

0.2 mmol L^{-1} Na_3Vo_4

0.2 mmol L^{-1} PMSF

50 mmol L^{-1} NaF

30 mmol L^{-1} $Na_4P_2O_7$

1 μg ml^{-1} Leupeptin

1 μg ml^{-1} Pepstatin

Mix all chemicals into desired volume of dd H_2O, aliquot and store at -20ºC.

APPENDIX E

4X Sample Buffer

7.5 ml dH2O

760 mg Tris-base

2.0 g SDS

Adjust pH to 7.0

10 ml glycerol

Adjust pH to 6.8

5 ml 2-mercaptoethanol

300 µl 2% bromphenol blue (in 100% ethanol)

Aliquot and store at -20ºC.

APPENDIX F

Polyacrlyamide Gel Formulation

Reagent	**10% Resolving Gel**	**5% Stacking Gel**
Water	3.6 ml	2.3 ml
4X Separating Buffer[1]	2.0 ml	1.0 ml
40% Acrylamide Bis*	2.0 ml	500 µl
Ammonium Persulfate*[2]	400 µl	200 µl

*Bio-Rad, Hercules, CA

[1]4X Separating Buffer

18.16 g Tris-base

0.4 g SDS

200 µl TEMED

75 ml dH_2O

Adjust pH to 8.8 then Q.S. to 100 ml with dH_2O. Aliquot in 4 ml and Store at -20ºC.

[2]Ammonium Persulfate (APS)

100 mg APS

1 ml dH2O

Make fresh before casting each gel and protect from light.

APPENDIX G

10X Electrode Buffer

30.3 g Tris-base

144.2 g glycine

10.0 g DSD

Adjust pH to 8.3

Q.S. to 1 liter with dH_2O

Dilute 50 ml of 10X buffer into 450 ml dH_2O for 1X working concentration.

APPENDIX H

Tirs Buffered Saline with 0.05% Tween (TBST)

2.42 g Tris-base

800 ml dH_2O

Adjust pH to 7.5

8.01 g NaCl

500 µl Tween 20

Q.S. to 1 liter with dH_2O

APPENDIX I

Stripping Buffer

5.12 g Tris-HCl

450 ml dH_2O

Adjust pH to 6.7

3.9 ml β-meraptoethanol

10 g SDS

Q.S. to 500 ml

APPENDIX J

Blocking Buffer

0.1 g BSA (Do not use Fatty Acid Free)

200 µl of serum (From same species as secondary antibody was raised in)

9.8 ml DPBS

Heat to 37ºC to melt BSA and store at 4ºC. Centrifuge before use.

APPENDIX K

Mounting Medium

10 ml of 2X PBS-DABCO

10 ml 40% Glycerol Solution

4 µl ml^{-1} Hoechst 44432 from 500 µg stock (Optional)

Aliquot and store in dark amber tubes at 4 ºC.

2X PBS-DABCO

80 ml dH_2O

0.04 g KCl

0.04 g KH2PO4

1.60 g NaCl

0.23 g Na2HPO4-H2O

20.0 g DABCO

Q.S. to 100 ml with dH_2O, pH to 9.0 and filter sterilize and store at 4ºC.

40% Glycerol Solution

4 ml Glycerol

6 ml dH_2O

APPENDIX L

Replicate 1 – Ct values from qPCR with transfected MDBK cells.

Treatment	Target Name	Cт	Cт Mean	ΔCт Mean	ΔCт Mean AVERAGE
Control	E-cadherin	27.90323639			
Control	E-cadherin	27.91489029			
Control	E-cadherin	28.05664253	27.95825768	9.772618294	
Control	GAPDH	18.13887787			
Control	GAPDH	18.15245628			
Control	GAPDH	18.26557922	18.18563843		
Cy3-siRNA 1	E-cadherin	27.29890251			
Cy3-siRNA 1	E-cadherin	27.22384644			
Cy3-siRNA 1	E-cadherin	28.32833099	27.61702728	9.582667351	
Cy3-siRNA 1	GAPDH	17.96418762			
Cy3-siRNA 1	GAPDH	17.97932053			
Cy3-siRNA 1	GAPDH	18.15957069	18.03436089		9.395053
Cy3-siRNA 2	E-cadherin	27.53307152			
Cy3-siRNA 2	E-cadherin	28.13148308			
Cy3-siRNA 2	E-cadherin	27.39345932	27.68600464	9.207438469	
Cy3-siRNA 2	GAPDH	18.39346695			
Cy3-siRNA 2	GAPDH	18.49491501			
Cy3-siRNA 2	GAPDH	18.5473156	18.47856522		
siEcad-12	E-cadherin	27.29037094			
siEcad-12	E-cadherin	29.43343925			
siEcad-12	E-cadherin	29.5652771	28.76303101	6.120476246	
siEcad-12	GAPDH	20.57524872			
siEcad-12	GAPDH	20.3726635			
siEcad-12	GAPDH	26.97974586	22.64255333		
siEcad-13	E-cadherin	30.7974987			
siEcad-13	E-cadherin	31.02691078			
siEcad-13	E-cadherin	30.76859665	30.86433601	11.00736237	
siEcad-13	GAPDH	19.67303085			
siEcad-13	GAPDH	19.98696518			
siEcad-13	GAPDH	19.910923	19.85697365		
siEcad-14	E-cadherin	28.12215424			
siEcad-14	E-cadherin	30.5201931			
siEcad-14	E-cadherin	28.35874367	29.0003643	10.16623974	
siEcad-14	GAPDH	18.78666496			
siEcad-14	GAPDH	18.86982727			
siEcad-14	GAPDH	18.8458786	18.83412361		

Replicate 2 – Ct values from qPCR with transfected MDBK cells.

Treatment	Target Name	Cт	Cт Mean	ΔCт Mean	
Control	E-cadherin	29.55807114			
Control	E-cadherin	29.64612579			
Control	E-cadherin	29.44480896	29.54966736	10.88727379	
Control	GAPDH	18.75847054			
Control	GAPDH	18.60272408			ΔCт Mean
Control	GAPDH	18.62598991	18.66239357		AVERAGE
Cy3-siRNA 1	E-cadherin	29.15526581			
Cy3-siRNA 1	E-cadherin	29.04409027			
Cy3-siRNA 1	E-cadherin	28.96968269	29.05634499	10.15249062	
Cy3-siRNA 1	GAPDH	18.82888222			
Cy3-siRNA 1	GAPDH	18.88990593			
Cy3-siRNA 1	GAPDH	18.99277878	18.90385437		10.14112
Cy3-siRNA 2	E-cadherin	29.17858887			
Cy3-siRNA 2	E-cadherin	29.45907402			
Cy3-siRNA 2	E-cadherin	29.12443924	29.25403404	10.12975216	
Cy3-siRNA 2	GAPDH	19.12789536			
Cy3-siRNA 2	GAPDH	19.20157051			
Cy3-siRNA 2	GAPDH	19.04337883	19.12428093		
siEcad-12	E-cadherin	30.20763016			
siEcad-12	E-cadherin	30.85931969			
siEcad-12	E-cadherin	30.5081749	30.52503967	11.26663685	
siEcad-12	GAPDH	19.39923477			
siEcad-12	GAPDH	19.22440338			
siEcad-12	GAPDH	19.151577	19.25840569		
siEcad-13	E-cadherin	30.80695152			
siEcad-13	E-cadherin	30.64473534			
siEcad-13	E-cadherin	30.98952293	30.81373596	12.94279003	
siEcad-13	GAPDH	17.95928574			
siEcad-13	GAPDH	17.95530701			
siEcad-13	GAPDH	17.69824791	17.87094688		
siEcad-14	E-cadherin	28.78746605			
siEcad-14	E-cadherin	28.9896431			
siEcad-14	E-cadherin	29.82163239	29.19957924	11.43390751	
siEcad-14	GAPDH	17.9648819			
siEcad-14	GAPDH	17.83406067			
siEcad-14	GAPDH	17.49807739	17.76567268		

Replicate 3 – Ct values from qPCR with transfected MDBK cells.

Treatment	Target Name	Cт	Cт Mean	ΔCт Mean	
Control	E-cadherin	30.61294746			
Control	E-cadherin	30.9888649			
Control	E-cadherin	30.78809929	30.79663658	7.968657017	
Control	GAPDH	22.67020798			
Control	GAPDH	22.91531372			ΔCт Mean
Control	GAPDH	22.89841843	22.82798004		AVERAGE
Cy3-siRNA 1	E-cadherin	32.19010162			
Cy3-siRNA 1	E-cadherin	29.82148552			
Cy3-siRNA 1	E-cadherin	30.98059654	30.99739647	6.940715313	
Cy3-siRNA 1	GAPDH	23.765028			
Cy3-siRNA 1	GAPDH	24.20433807			
Cy3-siRNA 1	GAPDH	24.20067215	24.05667877		7.384953
Cy3-siRNA 2	E-cadherin	30.11410332			
Cy3-siRNA 2	E-cadherin	Undetermined			
Cy3-siRNA 2	E-cadherin	Undetermined	30.11410332	7.829189777	
Cy3-siRNA 2	GAPDH	21.99886703			
Cy3-siRNA 2	GAPDH	22.38411522			
Cy3-siRNA 2	GAPDH	22.47175789	22.28491211		
siEcad-12	E-cadherin	31.97445297			
siEcad-12	E-cadherin	31.51188278			
siEcad-12	E-cadherin	31.48143005	31.65592194	9.329821587	
siEcad-12	GAPDH	22.66463661			
siEcad-12	GAPDH	21.6977787			
siEcad-12	GAPDH	22.61588669	22.3261013		
siEcad-13	E-cadherin	30.29426765			
siEcad-13	E-cadherin	30.3830986			
siEcad-13	E-cadherin	30.44544601	30.37426949	8.687010765	
siEcad-13	GAPDH	21.38614464			
siEcad-13	GAPDH	21.66250229			
siEcad-13	GAPDH	22.0131321	21.68726158		
siEcad-14	E-cadherin	29.70878029			
siEcad-14	E-cadherin	29.77229691			
siEcad-14	E-cadherin	29.98179817	29.82095909	8.845119476	
siEcad-14	GAPDH	20.94114494			
siEcad-14	GAPDH	20.95035934			
siEcad-14	GAPDH	21.03601265	20.97583961		

APPENDIX M

Density measurements and relative protein expression from three replicates of western blot analysis with MDBK transfected cells.

	Average Density		Ecad/GAPDH	Relative Ecad	Relative Ecad Expression
	E-cadherin	GAPDH	Ratio	Expression	Average
Control	1522	958	1.59	1.00	
	902	862	1.05	1	
	5527	1262	4.38	1	1
Cy3-siRNA-1	1438	890	1.62	1.02	
	883	1420	0.62	0.59	
	4424	1215	3.64	0.83	
Cy3-siRNA-2	1373	1077	1.27	0.80	
	974	1314	0.74	0.71	
	4592	1203	3.82	0.87	0.80
siEcad-12	273	621	0.44	0.28	
	197	1055	0.19	0.18	
	2104	1022	2.06	0.47	0.31
siEcad-13	218	427	0.51	0.32	
	134	1191	0.11	0.11	
	1904	1141	1.67	0.38	0.27
siEcad-14	332	581	0.57	0.36	
	176	1362	0.13	0.12	
	2033	1297	1.57	0.36	0.28

APPENDIX N

Replicate 1 – Ct values from qPCR analysis with injected or transfected embryos.

Treatment	Target Name	CT	CT Mean	ΔCT Mean
Control	E-cadherin	31.49744034		
Control	E-cadherin	31.7396965		
Control	E-cadherin	31.94904137	31.72872353	3.536716461
Control	GAPDH	28.21177292		
Control	GAPDH	27.9911232		
Control	GAPDH	28.37313271	28.19201088	
Null-Inj	E-cadherin	31.71483803		
Null-Inj	E-cadherin	31.20245171		
Null-Inj	E-cadherin	30.76796532	31.22841835	3.566733599
Null-Inj	GAPDH	27.47844124		
Null-Inj	GAPDH	27.91827583		
Null-Inj	GAPDH	27.58833694	27.66168404	
Ecad-Inj	E-cadherin	34.94485092		
Ecad-Inj	E-cadherin	34.85758972		
Ecad-Inj	E-cadherin	34.38339233	34.72861099	2.8738029
Ecad-Inj	GAPDH	31.92241096		
Ecad-Inj	GAPDH	31.95448112		
Ecad-Inj	GAPDH	31.68753242	31.8548069	
Null-Trfx	E-cadherin	31.96861649		
Null-Trfx	E-cadherin	31.82393074		
Null-Trfx	E-cadherin	31.10855865	31.63370323	3.689622879
Null-Trfx	GAPDH	27.9709816		
Null-Trfx	GAPDH	27.95827866		
Null-Trfx	GAPDH	27.90297699	27.94407845	
Ecad-Trfx	E-cadherin	34.94418335		
Ecad-Trfx	E-cadherin	34.63793564		
Ecad-Trfx	E-cadherin	34.67087555	34.75099564	4.565057278
Ecad-Trfx	GAPDH	30.20593452		
Ecad-Trfx	GAPDH	30.01963425		
Ecad-Trfx	GAPDH	30.33225441	30.1859417	

Replicate 2 – Ct values from qPCR analysis with injected or transfected embryos.

Treatment	Target Name	C_T	C_T Mean	ΔC_T Mean
Control	E-cadherin	35.95948792		
Control	E-cadherin	35.84313965		
Control	E-cadherin	35.121521	35.64138412	3.521244049
Control	GAPDH	31.97899246		
Control	GAPDH	31.96207428		
Control	GAPDH	32.41934967	32.12013626	
Null-Inj	E-cadherin	34.64260864		
Null-Inj	E-cadherin	33.96315002		
Null-Inj	E-cadherin	34.0406723	34.21547699	2.505097151
Null-Inj	GAPDH	31.73567772		
Null-Inj	GAPDH	31.91873932		
Null-Inj	GAPDH	31.47672272	31.71038055	
Ecad-Inj	E-cadherin	34.13925171		
Ecad-Inj	E-cadherin	35.05111694		
Ecad-Inj	E-cadherin	34.40056229	34.53030777	3.029754639
Ecad-Inj	GAPDH	31.59681702		
Ecad-Inj	GAPDH	31.53313637		
Ecad-Inj	GAPDH	31.37171364	31.50055504	
Null-Trfx	E-cadherin	34.34132385		
Null-Trfx	E-cadherin	34.04854202		
Null-Trfx	E-cadherin	33.99695969	34.12894058	3.822823286
Null-Trfx	GAPDH	29.82579231		
Null-Trfx	GAPDH	30.53089142		
Null-Trfx	GAPDH	30.56167221	30.30611992	
Ecad-Trfx	E-cadherin	34.84516907		
Ecad-Trfx	E-cadherin	35.08021545		
Ecad-Trfx	E-cadherin	34.98668671	34.97069168	4.892755508
Ecad-Trfx	GAPDH	29.56757927		
Ecad-Trfx	GAPDH	30.34253502		
Ecad-Trfx	GAPDH	30.32369041	30.07793427	

Replicate 3 – Ct values from qPCR analysis with injected or transfected embryos.

Treatment	Target Name	Cт	Cт Mean	ΔCт Mean
Control	E-cadherin	32.30030441		
Control	E-cadherin	32.71348572		
Control	E-cadherin	32.75072861	32.58817673	3.586159468
Control	GAPDH	28.9414978		
Control	GAPDH	28.87763977		
Control	GAPDH	29.186903	29.00201416	
Null-Inj	E-cadherin	31.44138336		
Null-Inj	E-cadherin	31.12998581	31.28568459	2.993611574
Null-Inj	GAPDH	28.2148304		
Null-Inj	GAPDH	28.28322029		
Null-Inj	GAPDH	28.37816811	28.2920742	
Null-Inj	E-cadherin	31.28365326		
Ecad-Inj	E-cadherin	31.27362633		
Ecad-Inj	E-cadherin	30.86239624	31.13989258	2.94906044
Ecad-Inj	GAPDH	27.94904327		
Ecad-Inj	GAPDH	28.56986809		
Ecad-Inj	GAPDH	28.05358315	28.19083214	
Ecad-Inj	E-cadherin	31.96383095		
Null-Trfx	E-cadherin	32.51134491		
Null-Trfx	E-cadherin	32.31225204	32.26247406	2.701194048
Null-Trfx	GAPDH	29.63020706		
Null-Trfx	GAPDH	29.59121895		
Null-Trfx	GAPDH	29.46241951	29.5612812	
Null-Trfx	E-cadherin	33.42828751		
Ecad-Trfx	E-cadherin	33.55495071		
Ecad-Trfx	E-cadherin	33.85670471	33.61331177	3.926681519
Ecad-Trfx	GAPDH	30.18994141		
Ecad-Trfx	GAPDH	29.7347393		
Ecad-Trfx	GAPDH	29.13521767	29.68663216	

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