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Effect of culture conditions on gene expression in manipulated bovine embryos

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EFFECT OF CULTURE CONDITIONS ON GENE EXPRESSION IN
MANIPULATED BOVINE EMBRYOS

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in
The Interdepartmental Program of
Animal and Dairy Sciences

by
Megan N. Purpera
B.S., Louisiana State University, 2005
December 2007
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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technologies</td>
</tr>
<tr>
<td>BME</td>
<td>Basal Medium Eagle</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CIDR</td>
<td>Controlled Intravaginal Releasing Device</td>
</tr>
<tr>
<td>COCs</td>
<td>Cumulus Oocyte Complexes</td>
</tr>
<tr>
<td>CS</td>
<td>Calf Serum</td>
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<tr>
<td>CT</td>
<td>Threshold Cycle</td>
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<tr>
<td>CTT</td>
<td>Threshold Cycle for the Reference Gene</td>
</tr>
<tr>
<td>CTgT</td>
<td>Threshold Cycle for the Target Gene</td>
</tr>
<tr>
<td>dPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>Glut-1</td>
<td>Glucose Transporter-1</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours Post-Insemination</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
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<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
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<td>IVF</td>
<td>In Vitro Fertilization</td>
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<td>IVM</td>
<td>In Vitro Matured</td>
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<tr>
<td>IVP</td>
<td>In Vitro Production</td>
</tr>
<tr>
<td>KSOM</td>
<td>Potassium Simplex Optimized Medium</td>
</tr>
<tr>
<td>KSOMaa</td>
<td>KSOM supplemented with amino acids</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LOS</td>
<td>Large Offspring Syndrome</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
</tr>
<tr>
<td>MM</td>
<td>Master Mix</td>
</tr>
<tr>
<td>MMM</td>
<td>Master Master Mix</td>
</tr>
<tr>
<td>MPF</td>
<td>Maturation Promoting Factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NT</td>
<td>Nuclear Transfer</td>
</tr>
<tr>
<td>OMM</td>
<td>Oocyte Maturation Medium</td>
</tr>
<tr>
<td>Poly A</td>
<td>Poly(A) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PHE</td>
<td>Penicillamine Hypotaurine Epinephrine</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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</table>
P/S ...................................................................................................................... Penicillin/Streptomycin
PVA ........................................................................................................................ Polyvinyl Alcohol
Q-PCR .............................................................. Quantitative or Real-Time Polymerase Chain Reaction
RNA ...................................................................................................................... Ribonucleic Acid
RT ...................................................................................................................... Reverse Transcription
RT-PCR .............................................................. Reverse Transcriptase Polymerase Chain Reaction
SCNT ...................................................................................................................... Somatic Cell Nuclear Transfer
SDS ...................................................................................................................... Sodium Dodecyl Sulfate
SEM ...................................................................................................................... Standard Error of the Mean
SOF ...................................................................................................................... Synthetic Oviductal Fluid
SOFaa ........................................................................................................ SOF supplemented with amino acids
TALP ...................................................................................................................... Tyrode’s Albumin Lactate Pyruvate
TCM-199 ........................................................................................................ Tissue Culture Medium-199
TE ...................................................................................................................... Trophectoderm
TE Buffer ........................................................................................................ Tris EDTA
TL ...................................................................................................................... Tyrode’s Lactate
TUGA ........................................................................................................ Transvaginal Ultrasound-Guided Aspiration
ABSTRACT

Numerous studies have reported aberrant gene expression levels attributed to suboptimal in vitro culture conditions presented to embryos. Since the culture environment is a common aspect of both in vitro production (IVP) and nuclear transfer (NT), research focusing on the in vitro culture system will have the potential to improve both techniques. This study investigated the effects of different culture systems and protein sources on the developmental competence of IVP embryos measured by cleavage and blastocyst rates, cell number, and relative abundance of oct-4, nanog, connexin 43, and GLUT-1 transcripts when compared to in vivo embryos. Experiment 1 compared IVP embryos cultured in either synthetic oviductal fluid (SOFaa) or potassium simplex optimized medium (KSOMaa) supplemented with amino acids. Experiment 2 compared the same two culture systems with and without the addition of calf serum (CS). Results from both experiments indicated that despite similar developmental rates, significant differences were observed at the mRNA level. In Experiment 1, oct-4 was the only transcript to have a mean abundance level significantly higher in KSOMaa blastocysts when compared with both SOFaa and in vivo embryos. The same pattern of upregulation of oct-4 in KSOMaa or KSOMaa with CS blastocysts was noted in Experiment 2. There were no significant alterations of the ICM specific transcript nanog in either experiment. In contrast to reports by others, connexin 43 was not expressed at detectable levels in in vivo embryos analyzed in our studies. Connexin 43 was not detected in IVP blastocysts used in Experiment 1. Connexin 43 was detected in KSOMaa, SOFaa, and SOFaa with CS blastocysts in Experiment 2. Blastocysts cultured in SOFaa with CS or KSOMaa had a significant upregulation of GLUT-1 when compared with other treatments and in vivo embryos. Overall, the transcript levels of the majority of the genes analyzed were significantly altered by an in vitro culture condition. Differences continue to be observed between in vitro cultured and in vivo embryos, and until these differences are minimized, aberrations in in vitro development will continue to arise.
CHAPTER I

INTRODUCTION

The advent of *in vitro* production (IVP) (or *in vitro* fertilization – IVF) and nuclear transfer (NT) of bovine embryos has introduced alternate methods to conventional superovulation techniques (Bavister, 1995). IVP embryonic development is dependent on oocyte maturation, fertilization and subsequent culture. In particular, the 5 to 6 day post fertilization period of IVP embryos is the time during which various developmentally important events occur including the first cleavage division, activation of embryonic genome (Memili and First, 2000), compaction of the morula, and blastocyst formation (Tesfaye et al., 2004).

NT offers the ability to precisely manipulate the genome of livestock resulting in offspring with predetermined genetic modifications. However, the success of NT is very low with only 0.2 to 3.4% of manipulated embryos resulting in cloned offspring with few exceptions (Yanagimachi, 2002).

The inefficiency of these biotechnologies is likely related to the abnormal gene expression levels seen in these manipulated embryos. Previously proposed hypotheses for the aberrant gene expression observed in these embryos/offspring include: abnormal expression of transcription factors, epigenetic effects such as DNA methylation, reprogramming of the somatic cell nucleus, chromatin remodeling, and a particular hypothesis of our laboratory which focuses on the embryo culture environment. A great deal of research has been dedicated to embryo culture conditions. However, no one particular culture environment has been determined to be the best or optimal medium. We hypothesize that it is a suboptimal culture medium that is initiating abnormal transcript levels in bovine IVF embryos. Therefore, we feel that there is still much to learn about embryonic culture conditions and the knowledge that can be gained from these experiments will lead to a better understanding of the bovine embryo culture environment.
Most experiments determine developmental competency of embryos simply by utilizing morphological evaluations such as cleavage rates, blastocyst rates, or less frequently cell number data. While these endpoints are useful, they fail to accurately determine viability of the embryo post transfer into a recipient. The only true measure of viability is evident by the production of a healthy offspring. More quantitative measurements such as polymerase chain reaction (PCR) that measures gene expression levels in embryos should enable a more detailed picture of developmental competence than simple morphological assessments. Numerous studies have detected aberrant expression levels attributed to suboptimal culture conditions (Wrenzycki et al., 1996, 1999, 2001, 2004; Eckert and Niemann, 1998; Lazzari et al., 2002; Rizos et al., 2002, 2003; Lonergan et al., 2003). Therefore, we believe that PCR analysis of suitable candidate genes for developmental competence would be a valuable tool to test a select group of embryos prior to transfer.

The overall goal of our research is to increase the efficiency of embryo biotechnologies through a better understanding of the transcriptional mechanisms controlling gene expression in bovine embryos. More specifically, we hope to determine if alterations to current culture conditions will affect gene expression and/or make embryo biotechnologies more proficient. The results of these studies may formulate a more suitable embryo culture environment for manipulated bovine embryos.
CHAPTER II
LITERATURE REVIEW

Mammalian Embryonic Development

Embryogenesis, the period from fertilization to implantation, involves various morphological, cellular, and biochemical changes related to genomic activity. Embryonic cells undergo both proliferation and differentiation processes to form the fetus and placental tissues throughout early embryogenesis. After fertilization, embryonic development involves protein synthesis, proliferation, differentiation, and the formation of fetal and extra-embryonic tissues. During this period, various changes take place with a genetic contribution to the fetus. Such events lead to morphologic elongation of embryonic tissues, cell-cell contact between the mother and the embryo/fetus, and placentation (reviewed by Ushizawa et al., 2004). It is assumed that a network of regulatory genes controls the transformation of genetic information from the parental source to the embryo (Scholer et al., 1989). However, the exact mechanisms for this transfer are not clear for mammalian embryogenesis.

Early embryonic development in many species is primarily supported by maternal RNAs and proteins synthesized during oogenesis. As development proceeds these proteins degrade, and embryogenesis becomes solely dependent on embryonic derived genes. Transcriptional activation of the embryonic genes is the result of a gradual degradation of the maternal RNAs and proteins due to the reprogramming of the cell nucleus (Memili and First, 2000). This transition period sets the stage for later stages of embryonic development and is normally initiated at the 8- to 16-cell stage in the cow (Kopency et al., 1989). However, recent reports indicate that the transition may occur slightly earlier in development.

_in vitro_ embryo development is dependent on oocyte maturation, fertilization and subsequent culture. Although cleavage of the embryo is an indicator of development, the very early stages of development are largely supported by the cytoplasm of the oocyte. In particular,
the 5 to 6 day post fertilization period of *in vitro* production (IVP) of embryos is the time when various developmentally important events occur including the first cleavage division, which is critical in determining the subsequent development of the embryo (Lonergan *et al.*, 1999), activation of the embryonic genome (Memili and First, 2000) or the maternal to zygotic transition, compaction of the morula, which involves the establishment of the first cell-to-cell contacts within the embryo, and blastocyst formation which is the time point of the first differentiation into two cell types the trophectoderm and the inner cell mass (ICM). This time period is generally referred to as the pre-implantation period. This period is characterized by distinct morphological steps that must include a well-orchestrated expression of genes derived from both the maternal and embryonic genomes to allow for compaction, cavitation, and blastocoel expansion (Kidder, 1992). After the pre-implantation period, the trophectoderm in ruminant animals will elongate exponentially reaching > 150 mm before the embryo will eventually implant in the uterine wall (Degrelle *et al.*, 2005). Clearly any modifications to the *in vitro* culture environment can have profound effects on the quality of the resulting embryos measured in terms of cryotolerance and relative transcript abundance (Lonergan *et al.*, 2003).

IVP of bovine embryos has become a reliable alternative method to conventional superovulation induction techniques and has been used as a tool to study pre-implantation embryo development (Bavister, 1995). However, little thought is often given to what implications could arise from using artificial technologies in terms of its effects on gene expression.

**Abnormalities Observed in Pre-implantation Development**

**IVP versus NT Embryos**

Somatic cell nuclear transfer (SCNT) or cloning allows researchers to precisely manipulate the genome of livestock. The resulting embryos can be transferred to female recipients and the resulting progeny will harbor the predetermined genetic modification (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998). Cloned offspring have been reported in several
species, including the sheep (Wilmut et al., 1997), mouse (Wakayama et al., 1998), cow (Kato et al., 1998; Wells et al., 1999), goat (Baguisi et al., 1999; Keefer et al., 2001), pig (Onishi et al., 2000; Polejaeva et al., 2000), cat (Shin et al., 2002), and horse (Galli et al., 2003). The rate of success in development to term for these cloned offspring are extremely low (0.2%-3.4% of reconstructed oocytes) with a few exceptions (Boiani et al., 2002; Yanagimachi, 2002), particularly in the mouse with less than 3% of reconstructed oocytes surviving to term (Wakayama and Yanagimachi, 1999). Approximately 80 to 90% of immature bovine oocytes undergo successful nuclear maturation in vitro, of which 80% undergo fertilization, 30 to 40% develop to the blastocyst stage, and around 40% of the transferred embryos establish and successfully maintain a pregnancy (Hasler et al., 1995). Therefore, IVP is more efficient than NT, but substantial losses are observed. Most of the losses in IVP seem to be during the in vitro culture stages (between the 2-cell and blastocyst stages) suggesting that the post-fertilization environment is the most critical period of development in terms of determining blastocyst yield (Lonergan et al., 2006).

Offspring produced by in vitro means and somatic cell nuclear transfer (SCNT) have been affected by numerous genetic abnormalities in particular, “large offspring syndrome” (LOS). These offspring are derived from in vitro produced embryos, including IVF and NT, which are subsequently transferred into recipient animals. It is thought that an extended in vitro culture may contribute to LOS. This syndrome is characterized by a variety of phenotypes in the newborn including significant increases in birth weight, polyhydramnios, hydrops fetalis, breathing difficulties, reluctance to suckle, altered organ growth, various skeletal and placental defects, immunological defects, and increased perinatal death (reviewed by Young et al., 1998). Some of these defects, such as increased organ growth, can persist into adulthood (Lonergan et al., 2006). LOS has also been attributed to an increase in embryonic/conceptus losses, particularly during the first trimester of pregnancy (Zhang et al., 2004).
Recent studies of NT embryos during pre-implantation development have revealed striking defects indicating that cloned embryos fail to recapitulate many of the events essential to normal development (Mann et al., 2003). Blastocyst stage cloned embryos from several species tend to have fewer cells than normal blastocysts (Koo et al., 2000, 2002; Chesne et al., 2002; Chung et al., 2002). Even though mammalian blastocysts have some plasticity in their ability to compensate for this variation in cell number, they do not develop at a normal frequency when the cell number is drastically reduced (Boaini et al., 2003). The oocyte enucleation and donor cell transfer (either somatic or stem cells) procedures themselves may result in the production of cloned embryos with inappropriate gene expression levels. Such alterations are probably the primary cause of embryonic loss in NT (Ushizawa et al., 2004). However, most studies have been limited to the few, rare, surviving clones that reach fetal, neonatal, or adult stages of development (Mann et al., 2003). Studies of gene expression levels have been conducted in fetuses, live young, placenta, and of course in pre-implantation embryos (Boiani et al., 2002; Mann et al., 2003; Ecker et al., 2004). Successful cloning can only be expected when proper recapitulation of the normal embryonic pattern of gene expression is established (Mann et al., 2003).

Differences in gene expression patterns between IVP and NT derived embryos when compared to in vivo embryos may originate from all steps of the manipulation process: in vitro maturation (IVM), in vitro fertilization (IVF), in vitro culture (IVC), and for NT embryos from different NT protocols (Wrenzycki et al., 1996, 1998, 2001 and 2004). Specifically, cloned embryos have a disadvantage in survival rate compared to IVP embryos because of the multiple potential causes for abnormal gene expression due to the inability of cloned embryos to recapitulate many of the essential events of normal development (see Table 2.1). When compared to NT, IVP embryo gene expression levels appear to be more analogous to their in vivo equivalents.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Normal Embryos</th>
<th>Cloned Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gametogenesis</td>
<td>Genome “competent” for activation of “early” genes, establishment of imprints</td>
<td>None</td>
</tr>
<tr>
<td>Cleavage</td>
<td>Global demethylation Activation of embryonic (“oct-4-like”) genes</td>
<td>Abnormal methylation Random/faulty activation of “oct-4-like” genes</td>
</tr>
<tr>
<td>Post-implantation</td>
<td>Global de novo methylation, X-inactivation, telomere length adjustment (post-zygotic events)</td>
<td>Normal imprinting and gene expression Abnormal imprinting, global gene dysregulation</td>
</tr>
<tr>
<td>Postnatal</td>
<td>Normal animal</td>
<td>Large offspring syndrome, premature death</td>
</tr>
</tbody>
</table>

(reviewed by Jaenisch et al., 2004)
Aberrant gene expression in NT embryos/offspring may be caused by abnormal expression of transcription factors (Memili and First, 2000), epigenetic effects such as DNA methylation (Cezar et al., 2003), incomplete reprogramming of the somatic cell nucleus (Boiani et al., 2003), chromatin remodeling (Kim et al., 2002), and a particular hypothesis of our laboratory which focuses on the embryo culture environment. NT embryos tend to prefer culture medium conditions more like somatic cell characteristics indicating a lack of nuclear reprogramming which affects basic physiology and metabolism (Mann et al., 2003). However, these are just some of the speculations. As mentioned before, the mechanisms are still not completely understood.

Possible Origins of Abnormal Gene Expression Levels

Transcription factors are proteins that bind to the DNA and either activate or repress transcription of specific genes (Chau et al., 2002). Thus, transcription factors are the key regulators of gene expression in cells. Transcriptional control in the various differentiated cell types allows each type of cell (e.g., epithelial, muscle, neuron) to express different amounts of the possible proteins. The transcription factors are regulated by signal transduction pathways that relay signals from outside of cells to the cell nucleus (Gilbert, 2003). As stated before, embryonic transcription is initiated at the 8- to 16-cell stage in cattle. However, recent reports indicate that embryonic transcription may be as early as the 2-cell stage (Memili and First, 2000). Nevertheless, embryos can survive without embryonic transcription until a certain stage of development. The embryo relies on the maternal mRNA source for genetic information until the point of the maternal to zygotic transition. This generally occurs around the 8-cell stage in cattle.

This transition is sometimes referred to as the “block to development” because under suboptimal culture conditions, embryos often do not develop past this point. This block suggests that embryonic transcription is particularly sensitive to the culture conditions presented to the embryo (Memili and First, 2000). It has also been suggested that the developmental block could
be alleviated by co-culture systems, addition of fetal calf serum to the medium, or a reduction of glucose in the medium (Gandolfi and Moor, 1987). After this transition point, the embryo relies on its own transcription factors necessary for the production of RNA. Memili and First (2000) and others reported that there is a low level of transcription activation termed the ‘minor gene activation’ between the 1- and 4-cell stages and a high level of gene activation termed the ‘major gene activation’ at the 8-cell stage in bovine embryos (See Figure 2.1). Figure 2.1 illustrates the importance and necessity of precise timing of the activation of gene expression via the presence of transcription factors. Under or over-expression of these transcription factors in NT embryos prevents the binding of the promoter to the DNA allowing the start of transcription. For example, the POU domain proteins include a family of transcription factors crucial to development and cell differentiation, particularly in murine embryo development (Kirchhof et al., 2000). This domain is characterized by the presence of DNA-binding sites of 81 and 60 conserved amino acids and includes mammalian transcription factors Pit-1, Oct-1, Oct-2, Oct-3, and oct-4 in various species (Yeom et al., 1991). Abnormal embryonic development has been linked to both upregulations and downregulations of these transcription factors.

Epigenetics is the alteration in gene function and expression that cannot be explained by the DNA sequence, but rather are explained by structural modifications of the chromatin (reviewed by Jaenisch et al., 2004). It is likely that epigenetic changes occurring in differentiated somatic cells contribute to incomplete or faulty reprogramming during nuclear transfer. DNA methylation, a primary epigenetic modification, involves the addition of methyl groups to the cytosine residues found in CpG motifs. DNA methylation plays a critical role in controlling the expression of a particular gene. Increased methylation inhibits transcription, a situation commonly found in differentiated somatic cells while embryonic cells are usually under-methylated. For example, elevated insulin-like growth factor-2 (IGF-2) concentrations are associated with large offspring syndrome in sheep and mice. This altered gene expression is
Figure 2.1. Bovine embryonic cell cycles and zygotic/embryonic gene expression (mRNA synthesis) in cattle. The cell cycle was adapted from Barnes & Eyestone (1990). Data suggests that there is a ‘minor gene activation’ between the 1- and 4-cell stages. Changes in the transcriptional machinery and chromatin structure play an important role in the control of early gene expression. Several references reviewed suggest there is a ‘major gene activation’ starting at the 8-cell stage (Memili and First, 2000).
linked with decreased DNA methylation in these species (Eggenschwiler et al., 1997; Young et al., 2001). Methylation patterns in NT embryos differ from those of IVP controls (Cezar et al., 2003). Cezar and associates (2003) were the first to find that NT-generated fetuses have overall decreased levels of methylated cytosines, suggesting a hypomethylated genome when compared with in vivo produced fetuses. Conversely, there was no significant difference in methylation patterns between in vitro and in vivo produced embryos in this same study. The mechanism behind these methylation patterns is unclear. It is unknown to what extent that DNA methylation, which occurs in normal development, needs to be mimicked for NT to be successful (Cezar et al., 2003).

A favored hypothesis for the developmental incompetence of clones is inadequate reprogramming of the transplanted somatic cell nucleus to an equivalent state of an early nucleus in the developmental process (Boiani et al., 2002). Reprogramming, in the context of SCNT, can be defined as the transformation of a somatic cell nucleus into a functional embryonic nucleus capable of forming a viable organism. For the successful development of a reconstructed NT embryo, a transferred nucleus must undergo reprogramming to establish the temporal and spatial gene expression patterns associated with normal development (Cezar et al., 2003). “Correct” expression of embryonic genes is essential for development and is indicative of nuclear reprogramming (Boiani et al., 2003). Several studies have shown that changes in the nuclear material prior to transcription include: nuclear swelling, dispersal of nucleoli, nuclear envelope breakdown, and premature chromosome condensation.

Comprehensive changes in transcriptional activity of these transplanted nuclei also have been detected (Boiani et al., 2002). One way to address the ability of cloned embryos to undergo genetic reprogramming is to determine the fate of imprinted gene modifications during clonal development. Mann et al. (2003) examined gene imprinting during the earliest stages of clonal development to determine how epigenetic information in cloned embryos may be affected. They analyzed allele-specific expression of imprinted genes in cloned mouse
blastocysts and found widespread defects in imprinted gene regulation at all three levels of expression: total transcript abundance, allele specificity of expression, and allelic DNA methylation. The results indicated that only ~ 4% of the NT embryos had normal expression patterns of the imprinted genes analyzed which may help explain the low efficiency of mouse cloning. From these results, it is clear that several of the questions regarding nuclear reprogramming of the somatic cell have yet to be answered.

Nuclear reprogramming is generally preceded by chromatin remodeling. One component of chromatin remodeling is the breakdown of the nucleosome consequently permitting access to the chromatin and allowing for transcription and gene expression (reviewed by Becker, 2004). Silencing of transcription versus the accumulation of TATA box binding protein and the loosening of chromatin structure are important events in chromatin remodeling. The failure of oocytes to successfully accomplish these events results in inadequate somatic nuclei remodeling in NT embryos, thus leading to abnormal development in NT offspring (Kim et al., 2002). As a result, nuclear reprogramming is much more successful when the chromatin structure is unwrapped allowing more accessibility to the chromosomes (Piedrahita et al., 2004). The direct exposure of chromosomes to the cytoplasm is thought to be important in somatic nuclei remodeling to support further development. High levels of maturation promoting factor (MPF) in enucleated Metaphase II (MII) or mature oocytes induces the breakdown of the germinal vesicle and chromosome condensation (actually premature chromosome condensation) within the oocyte. When activation is stimulated in the oocyte, the MPF levels decrease allowing chromosome decondensation and formation of the pronucleus. The timing of these events is critical for optimum chromatin remodeling. Altered timing is primarily due to the origin of the host cytoplast in NT-produced embryos (Campbell et al., 1996; Choi et al., 2004).

In addition to the mechanisms of transcription described above, other factors that may be involved in the observed abnormal development of NT clones could include: pre-implantation
and post-implantation development, effects of micromanipulation, oocyte activation, and *in vitro* culture (Boiani *et al.*, 2002).

**In Vitro Culture Conditions and Abnormal Gene Expression Levels**

While it is the intrinsic quality of the oocyte that will determine whether or not that oocyte will develop to a blastocyst, it is the post fertilization culture environment that has the biggest influence on blastocyst quality regardless of oocyte origin (Rizos *et al.*, 2003). The post-fertilization culture environment encompasses not only the culture medium itself but also the number of embryos cultured together, the embryo: medium ratio, any supplementation used, the temperature, and the gas atmosphere. The relative abundance of numerous transcripts varies through the early pre-implantation period and is strongly influenced by the culture environment (Wrenzycki *et al.*, 1996, 1999, 2001, 2004; Eckert and Niemann, 1998; Lazzari *et al.*, 2002; Rizos *et al.*, 2002, 2003; Lonergan *et al.*, 2003). The effects of a suboptimal culture environment on the embryo are mediated through modifications of the gene expression levels in the resulting embryos. Differences in gene expression patterns are sometimes apparent at very early cleavage stages, in some cases only after one day of *in vitro* culture implicating the temporal association between the culture environment and expression patterns (Rizos *et al.*, 2002, 2003). Most of the time, the effects are apparent at later stages of development resulting in a lack of blastocyst formation or abnormal development (Tefaye *et al.*, 2004). These changes in the transcript abundance measured in blastocyst stage embryos are likely the result of disturbed expression earlier in embryo development (Lonergan *et al.*, 2003).

Suboptimal culture conditions may result in abnormal embryonic, fetal, perinatal, and postnatal development, collectively referred to as LOS described above (Young *et al.*, 1998; reviewed by Wrenzycki *et al.*, 2004). Current culture systems can also lead to either persistent silencing or enhanced expression of a particular gene throughout the critical phases of fetal development (Wrenzycki *et al.*, 2001). A higher expression of a particular gene under *in vitro*
culture conditions when compared to *in vivo* embryos can be considered as a response of the embryo to the suboptimal culture condition or that the gene was induced as a result of the *in vitro* culture condition. An extended *in vitro* culture of bovine pre-implantation embryos has been shown to perturb the well organized and executed gene expression pattern essential for normal development (Wrenzycki *et al.*, 1996, 1998). A prolonged *in vitro* culture of early sheep embryos resulted in several abnormalities including: cytoplasmic fragmentation, premature blastocoel formation, and a reduced number of nuclei per blastocyst (Walker *et al.*, 1992). Rizos *et al.* (2002) found clear differences in the level of expression of several developmentally important gene transcripts when bovine embryos were cultured in different media. More specifically, blastocysts produced following culture in synthetic oviductal fluid (SOF), a medium designed for the demands of the pre-implantation embryo, have significantly altered patterns of genes involved in gap junction formation, apoptosis, oxidative stress and differentiation compared to their *in vivo* counterparts. Ho *et al.* (1995) reported that in the mouse, the optimized cell culture potassium simplex optimized medium (KSOM) supplemented with amino acids or serum provided very similar expression profiles (almost identical) as those found in *in vivo* embryos. Enright *et al.* (2000) reported that the culture of *in vitro* produced bovine zygotes in the well established ewe oviduct model can dramatically increase their cryotolerance to a level similar to that of totally *in vivo* produced embryos. The ligated ewe oviduct has been reported to support the growth and development of both bovine (Eyestone *et al.*, 1987) and porcine embryos (Prather *et al.*, 1991). This ability to support embryonic development utilizing a “semi-*in vivo*” model has been used extensively in IVP systems where embryos are allowed to co-culture with oviduct epithelial cells (Eyestone and First, 1989; Moore and Bondioli, 1993).

Typically, most culture media used in embryo culture systems are supplemented with either serum or bovine serum albumin (BSA) as a protein source. However, these undefined complex mixtures result in highly variable developmental rates and/or mRNA expression levels and are often contaminated with various small peptides, energy substrates, or growth factors.
Different protein sources in culture medium can greatly influence development of IVP embryos, ranging from either a marked stimulation in some aspects to a significant inhibition of other processes. In fact, the quality of serum and BSA can vary greatly depending on the source and specifically for serum, the lot number (Bavister, 1995). Serum has been shown to have a biphasic influence on the developmental capacity of bovine embryos by inhibiting the first cleavage division but enhancing later blastocyst development (Wrenzycki et al., 1998). Serum can affect the speed of embryo development and the resulting quality of the embryos produced (Rizos et al., 2003). Serum supplementation between 5% and 20% was shown to increase blastocyst production rates when compared to BSA supplementation (Lonergan, 1999). Serum supplementation has also been linked to a high incidence of apoptosis in IVP blastocysts (Gjorret et al., 2001). The presence of serum in tissue culture medium (TCM) 199, which is a complex media designed for cell culture, over a prolonged period of time has also been shown to alter the expression pattern of various genes (Wrenzycki et al., 1999), embryo morphology, and biochemistry (Rizos et al., 2003). In contrast, Lonergan et al. (2003) showed that simply omitting serum from the culture medium during the post-fertilization period changed mRNA abundance for several developmentally important genes. Most report that the majority of genes are upregulated in the presence of serum. In contrast, Wrenzycki et al. (1999) found that the majority of the genes they studied were upregulated in polyvinyl alcohol (PVA), a synthetic macromolecule, when compared to their serum-generated counterparts. In addition, Barcelo-Fimbres and Seidel (2007) reported that a serum-free culture did not affect blastocyst yield. Therefore, differences are only detectable at the transcript level. Omission of serum can also significantly improve the cryotolerance of the blastocysts to a level intermediate between serum supplemented blastocysts and in vivo derived blastocysts (Rizos et al., 2003). This observation also supports that hypothesis that serum affects developmental competence at the transcript level. There also is a positive correlation between high serum content, in particular with fetal calf serum (FCS), added to a medium with LOS in the resulting offspring (Thompson
et al., 1995). FCS is a good source of nutrients and anti-oxidants for the embryo. On the other hand, because of its association with LOS, altered metabolism, excess lipid accumulation, abnormalities of organelles, premature blastulation, and altered gene expression levels, the efficacy of FCS is under investigation (Barcelo-Fimbres and Seidel, 2007). Serum is often replaced by BSA, a more defined protein source when compared to serum (Wrenzycki et al., 1998). Often these less defined components are replaced by a synthetic macromolecule known as PVA resulting in a defined media composition. However, PVA is not a true protein source but rather just reduces the surface tension of the media. PVA is believed to be metabolically inert to the embryo and is less able to support embryo development (Kane and Bavister, 1988; Wrenzycki et al., 2001).

Wrenzycki and coworkers (2001) were the first to report of the effects of two different culture systems TCM199 or SOF containing either serum, BSA, or PVA on the relative levels of several gene transcripts in bovine morulae and blastocysts involved in cellular compaction, metabolism, RNA processing, stress, and maternal recognition. No significant difference was found in the embryonic developmental rate for the different treatments, but significant differences were observed at the transcript level. The basic culture system itself whether in vivo or in vitro had profound effects on transcript levels, while the individual protein source had only weak effects on abundance levels. It was also determined that the morula stage was more susceptible to aberrations in gene expression than the blastocyst stage indicating that the morula stage could be an indicator when testing culture media for suitability (Wrenzycki et al., 2001).

Another key component of culture media that has been shown to affect post fertilization events is the energy substrate chosen. Generally simple energy sources such as pyruvate are preferred early in development, while more complex energy sources such as glucose or fructose are preferred substrates after the morula stage. It is a well accepted fact that glucose plays an important role in embryonic metabolism and is frequently used in most in vitro embryo systems despite its putative toxic effects to the early embryo (Barcelo-Fimbres and Seidel, 2007).
Glucose was found to be detrimental to *in vitro* cultured bovine embryos from the time of fertilization through the morula stage (Takahashi and First, 1992); however, bovine embryos cultured after the morula stage preferred glucose as an energy source (Reiger, 1992). The use of fructose during *in vitro* culture increased total cell number in hamster embryos (Ludwig *et al.*, 2001), reduced the amount of fragmented DNA found in nuclei of porcine blastocysts, and enhanced embryo quality by reducing lipid granule accumulation in bovine embryos (Barcelo-Fimbres and Seidel, 2007). The addition of either fructose or glucose to bovine embryo culture media improved blastocyst rates per oocyte matured; however, the beneficial effects of fructose were only apparent after the 8-cell stage (Barcelo-Fimbres and Seidel, 2007). Sucrose addition to early culture medium lowered the frequency of apoptosis and increased nuclei number in blastocysts, therefore, improving overall development of pre-implantation porcine NT and IVP embryos (Hwang *et al.*, 2006).

Pre-implantation embryos are able to develop *in vitro* in a wide array of culture conditions. They are able to develop in media ranging in composition from simple balanced salt solutions to complex systems supplemented with serum and somatic cells (Lonergan *et al.*, 2006). However, this ability to adapt to the environment presented to the embryo especially under suboptimal or even nonphysiological conditions comes at a cost to the embryo in terms of embryo quality (Rizos *et al.*, 2002). Wrenzycki *et al.* (2004) indicated that when the capacity of an embryo to compensate for its environment is overloaded, development arrests. To date, no one culture medium is truly optimal in regards to producing embryos with ideal developmental rate, cell/nuclei number, temperature sensitivity, freezability, viability, pregnancy rates after transfer, and gene expression patterns. Aberrant gene expression will continue to arise as long as embryos continue to be cultured in a suboptimal medium. Rizos *et al.* (2002) found that modifications to the *in vitro* culture system can somewhat improve blastocyst quality on both a morphological and molecular level. For pre-implantation mouse development, it was found that an optimized *in vitro* culture system is correlated with an mRNA expression pattern identical to
in vivo derived embryos (Ho et al., 1995). Therefore, future research needs to be conducted to improve current embryo culture protocols.

While the majority of the research focusing on in vitro culture conditions in relation to altered gene expression levels is conducted in pre-implantation embryos, some work focuses on fetuses post implantation, placental tissues, and neonates or young shortly after birth. Kwong and colleagues (2000) reported that the Barker hypothesis could be traced as far back as pre-implantation development. The Barker hypothesis is fetal adaptations in utero to maternal undernutrition or malnutrition resulting in specific diseases in the adult (Barker et al., 1993). To test Barker’s hypothesis and to determine whether early perturbations in metabolism and gene expression in response to in vitro culture have long term effects exhibited in the resulting offspring, Ecker et al. (2004) examined the effects of in vitro culture of mouse pre-implantation embryos to the blastocyst stage on development and behavior of the resulting offspring. This group found no effect of the culture environment on development to term; however, small but significant long term alterations in behavior specifically anxiety, locomotor activity, and spatial memory were observed. Rodents are often apprehensive in both open and elevated areas. The culture-derived animals, however, spent more time in the open quadrant of a maze used to test anxiety levels than did the in vivo derived animals. This suggested that alterations in anxiety were the direct result of artificial embryo culture. Spatial memory was impaired in in vitro culture-derived animals when compared with normal mice suggesting that the culture environment could actually alter brain function, specifically memory storage. They also concluded that the differences could not be attributed to delays in normal development since there was no variation in physical, neurological, and behavioral development. Behavior among treatment groups was also conserved, thus, differences were not due to a few aberrant mice. Mann et al. (2004) tested the effects of in vitro culture conditions on post implantation embryos/fetuses and placental tissue. Results indicated a loss of imprinted expression in these later stage embryos and some placental tissues. These data indicated that the effects of alterations in pre-implantation
embryos can be seen long after they have been isolated from the culture medium itself. Therefore, the findings in later stage embryos, placental tissue, and offspring reveal long term behavioral and developmental consequences of in vitro culture environments. Similar experiments have not been conducted with human embryos. Due to the increase in assisted reproductive technologies being applied to humans today, it would be beneficial to determine what could be accomplished to minimize the effect of the culture environment on the human pre-implantation embryo.

**Genes of Interest**

Some of the genes shown to be important to normal mammalian embryonic development include: oct-4, nanog, connexin 43, and glucose transporter-1 (GLUT-1). Oct-4 and nanog are ICM specific pluripotency factors. ICM cells can be isolated to form embryonic stem cells that are pluripotent since they are capable of giving rise to all different cell types found within an organism (Martin, 1981). Thus, oct-4 and nanog are not only important to embryonic development but also to the field of stem cell research. Connexin 43 is a cell adhesion factor, and GLUT-1 is a gene important in cellular metabolism.

The POU transcription factor family regulates gene expression via binding to the octamer sequence ‘ATGCAAT’ resulting in two domains: a homeodomain distantly related to the prototype Antennapedia homeodomain and a N-terminal domain designated the POU-specific domain, both required for the activation of transcription (Palmieri et al., 1994). The POU domain proteins consist of a family of structurally related transcription factors including oct-4. Most POU proteins play a role in development and cell differentiation as they are expressed throughout embryogenesis. Oct-4 is a member of Class V of the POU transcription factor family (Kirchhof et al., 2000). Oct-4 is the earliest known transcription factor to be developmentally expressed (Chau et al., 2002). Oct-4 is a maternally and zygoticly expressed gene product found primarily in developing embryos during pre-implantation development and thus regulates
initial events of development. Zygotic expression does not achieve peak abundance until the 16-cell stage, therefore, the maternal oct-4 message diminishes sometime after fertilization and zygotic expression rises rapidly thereafter. In addition to oct-4 itself, other oct-4-related genes are found within the early developing embryo (Yeom et al., 1991). Oct-4 is predominantly localized to the nuclei in all cleavage stages of embryonic development (Palmieri et al., 1994). Oct-4 is also found in the germ line including primary germ cells of both sexes and in unfertilized oocytes (Scholer et al., 1989). Oct-4 is expressed at very low levels in the unfertilized oocyte and is subsequently localized to the female pronuclei upon fertilization (Palmieri et al., 1994). Unlike in the female gametes, oct-4 is not found in spermatozoa (Scholer et al., 1989). Oct-4 is a transcription factor that is specifically expressed in pluripotent cells, such as embryonic stem cells and embryonal carcinoma cells, associating the cells with a state of totipotency (Scholer et al., 1989; Prusa et al., 2003). When embryonic stem cells are triggered to differentiate, oct-4 is downregulated (Prusa et al., 2003). Therefore, since oct-4 is expressed in embryonic stem cells, they are more compatible with early embryonic development allowing for less reprogramming for NT purposes (Rideout et al., 2000). Oct-4 encodes an early transcription factor that is required for embryonic mouse development and differentiation past the blastocyst stage (Ovitt and Scholer, 1998). After gastrulation of the murine embryo around day 8.5 of gestation, oct-4 is restricted to the germ cell lineage. Oct-4 is silent in somatic cells but active in embryonic cells of embryos (Boiani et al., 2002). Somatic cells can be induced to express oct-4 via contact with embryonic blastomeres mediated by signaling through gap junctions (Burnside and Collas, 2002). In the mouse, oct-4 expression begins at the 4-8 cell stage and becomes restricted to the ICM cells (Palmieri et al., 1994). Yeom and colleagues (1991) stated that oct-4 abundance appears to coincide with the timing of compaction of the embryo. Oct-4 mRNA in pig and cow embryos can be detected in both the ICM and the trophectoderm of a blastocyst; however, oct-4 is strictly limited to the ICM in mouse embryos correlating with the undifferentiated cell type, suggesting that it may be a marker for pluripotency (Kirchhof et al., 2000). Oct-4 is
downregulated during the formation of the blastocyst and is later restricted to the ICM of expanded and hatched blastocysts (Palmieri et al., 1994). Oct-4 can be found in bovine pre-implantation embryos until day 10 of development (Ponsuksili et al., 2002). In mice, the restriction of oct-4 to the ICM or primitive endoderm (hypoblast) suggests that oct-4 regulates both genes involved in determining cell commitment and genes involved in the regulation of proliferation in specific cell lineages (Palmieri et al., 1994). Subtle changes in oct-4 expression levels (as small as 30%) have serious consequences for the early post-implantation embryo. These small changes regulate the differentiation of embryonic stem cells into putative endoderm or trophectoderm. Increased oct-4 expression triggers differentiation into endoderm or mesoderm while decreased oct-4 expression triggers dedifferentiation into trophectoderm (Chau et al., 2002). Bovine IVP blastocysts generally express oct-4 variably. IVP embryos can exhibit either upregulation or downregulation of the oct-4 gene (Li et al., 2005). This may be in part due to the variation among embryos in development or due to varying levels of atresia, but the exact cause is not known (Chau et al., 2002). NT embryos also display marked differences in oct-4 expression when compared to in vivo embryos. A large portion of morula stage clones do not form blastocysts; thus, oct-4 is an essential marker for pre-implantation development and beyond. Mammalian clones that do not survive to term tend to either lack oct-4 expression or the expression is random (Boiani et al., 2002). The abnormalities in oct-4 expression in these cloned embryos suggest that the state of pluripotency is compromised in many cloned blastocysts. The reactivation of oct-4 in clones is random and faulty. The abnormal pattern of oct-4 expression in clones suggests that the developmental competence of the clones is already compromised at the blastocyst stage to be later reflected in subsequent development (Boiani et al., 2002). However, Boiani et al. (2003) found that the reduced expression of oct-4 observed in mice may only be due to a lack in cell number in the cloned embryos. The lack in cell number could be attributed to apoptotic cell death, a delayed cell cycle progression, or inhibition of cell proliferation due to metabolic restraints. They found that increasing the cell number in clones by
aggregation of clones with one another at the 4-cell stage to precede the onset of oct-4 expression dramatically improved expression of oct-4 in blastocysts to levels found in in vivo embryos. Embryos completely lacking oct-4 generally are lacking ICM cells (Chau et al., 2002), and thus arrest in development (reviewed by Jaenisch et al., 2004). The failure of this reactivation may be the cause of embryonic loss seen in NT embryos post-implantation (reviewed by Jaenisch et al., 2004). Oct-4 acts as a transcription factor in early embryos and its pattern of expression may be a marker for the extent of nuclear reprogramming that has occurred in NT embryos. The high degree of sequence similarity, genomic organization, and chromosomal localization are highly conserved suggesting that oct-4 plays a similar role in all mammalian species. It has been reported that the murine oct-4 protein shows a widespread sequence similarity with the bovine protein with a conserved identity of 81.7% (Kirchhof et al., 2000). It has also been shown that the oct-4 amino acid sequence found in humans is 87% identical to that of the mouse (Takeda et al., 1992). The high degree of similarities found among most animals suggests that oct-4 has a universal role among mammalian species.

Nanog is a newly identified transcription factor that contains a homeodomain (like oct-4) which is specific to maintaining the pluripotency state of embryonic stem, germ, and carcinoma cells and in pre-implantation embryos in a dose dependent manner (Yamaguchi et al., 2005). For example, low levels of nanog results in instability in the pluripotency of embryonic stem cells (Hatano et al., 2005). Nanog has two important characteristics: (1) it has the capacity to maintain embryonic stem cell self-renewal capabilities independent of other renewal factors, (2) it is critical for pluripotency status of both the ICM and the trophectoderm. This second characteristic was confirmed by the deletion of nanog, resulting in the loss of pluripotency in embryonic stem cells (Li et al., 2005) and the loss of the epiblast in nanog-null murine embryos (Mitsui et al., 2003). Nanog is generally silent in somatic cells, but can be reactivated during reprogramming of cells via nuclear transplantation in SCNT (Hatano et al., 2005). Nanog transcription in stem cells appears to be controlled by the synergistic actions of oct-4 and the
Sox binding proteins. However, the function of nanog in germ cell development remains largely unknown. Nanog is detectable in both male and female primary germ cells (Yamaguchi et al., 2005), and at very low levels in mouse ovaries (Li et al., 2005), but there is no report of the presence of nanog in the mature germ cells themselves. Degrelle et al. (2005) found nanog to be expressed in all embryonic and extra-embryonic tissues in later stages of development (12-17 days post insemination), but was restricted to the ICM of earlier stage blastocysts. The onset of nanog embryonic transcription is generally at the 8-cell stage. Therefore, nanog is generally not detected in very early stage embryos; however, this is not the case when embryonic stem cells were used as a donor cell for SCNT or in parthenotes. Nanog was found to be expressed in lower levels of most cloned blastocysts when compared to in vitro blastocysts (Li et al., 2005).

Gap junctions are aggregations of intercellular membrane channels that coordinate metabolic and electrical activities by controlling the exchange of small metabolites and ions among adjacent cells (Loewenstein, 1981). These gap junction channels in each cell are composed of two hemichannels termed connexons. Each connexon is provided by each of the two communicating cells. Each connexon is subdivided into 6 identical subunits or connexins (Itahana et al., 1996). The gap junction protein connexin 43 is necessary for the maintenance of compaction (gap junction formation) and thus, for subsequent blastocyst formation in mice (Lee et al., 1987). Gap junction formation and communication is essential for growth, cellular differentiation, and embryonic development (Wrenzycki et al., 1996). Gap junction formation is also essential for the transport of cryoprotectants and fluids during the cryopreservation and thawing processes (Rizos et al., 2002, 2003). Connexin 43 is one of the most abundant connexins and is expressed in numerous tissues including ovaries, placenta, and deciduas in several species (Wrenzycki et al., 1996). Connexin 43 shares 97% amino acid identity among mammals (Itahana et al., 1996). Functional gap junctions are first observed during compaction at the 8-cell stage in murine pre-implantation development, and mRNAs encoding connexin 43 are detected from the 4-cell stage onwards (Wrenzycki et al., 1998), but expression decreases
slightly thereafter. A peak in connexin 43 expression in mice occurs at the compacted morula stage and is expressed in both the trophectoderm and ICM at the blastocyst stage (Rizos et al., 2002). The null (non-lethal) mutant mice for connexin 43 have small gonads in both sexes because of, at least in part, a deficiency in germ cells traced back to day 11.5 of gestation. From this finding, Lonergan et al. (2003) concluded that connexin 43 is also required for the earliest stages of folliculogenesis. During *in vitro* culture, the cumulus cells of bovine cumulus oocyte complexes (COCs) are connected to each other via connexin 43 gap junctions; however, these connections will disappear within 6 to 9 h of *in vitro* culture (Wrenzycki et al., 1996). The connexin 43 gene is transcribed only up to the morula stage in IVP bovine embryos whereas the transcript is present in *in vivo* derived, cocultured, and ewe oviduct cultured bovine morulae and blastocysts (Wrenzycki et al., 1996; Rizos et al., 2002, 2003). This result indicates that connexin 43 could be of both maternal and embryonic origin since the morula stage is just after the maternal to zygotic transition *in vivo*. In cattle, the maternal to zygotic transition normally occurs at the 8- to 16-cell stage in *in vivo* embryos, whereas *in vitro* produced embryos tend to initiate embryonic transcription slightly earlier at the 4-cell stage (Wrenzycki et al., 1996).

McLachlin et al., (1983) found that the gap junctions required for blastocyst formation in mice could be constructed from precursor pools of connexin 43, and this may be lacking in *in vitro* produced embryos. Lonergan et al. (2003) found that the relative transcript abundance of connexin 43 was significantly increased in the presence of serum compared with when serum was omitted. Connexin 43 was altered *in vitro* by the presence of serum, disappearing at the 8- to 16-cell stage and reappearing at the hatched blastocyst stage (Lonergan et al., 2003).

However, Wrenzycki et al. (1998) reported that connexin 43 was not affected by the addition of either serum or BSA, so it is therefore a sensitive candidate gene to be studied as markers for determining the quality of an *in vitro* culture system. The difference in expression is probably due to the extended *in vitro* culture of these bovine embryos (Wrenzycki et al., 1998). Due to the
varying differences in connexin 43 observed between IVP and in vivo bovine embryos, this gene could possibly be a factor for aberrant gene expression in NT embryos as well.

In the mouse, most of the glucose taken up into the system is mediated by facilitative carriers, otherwise known as glucose transporters (Glut's) (Gardner and Leese, 1988). Expression of GLUT-1 is frequently used when testing different culture systems (Wrenzycki et al., 1999, 2001; Lazzari et al., 2002; de Oliveira et al., 2006). GLUT-1 expression is responsible for incorporating glucose into embryonic cells throughout the period of pre-implantation development suggesting that the mRNA is derived from both maternal and embryonic origin (Lequarre et al., 1997; de Oliveira et al., 2006). Glucose is the critical energy source required during compaction and blastulation in bovine embryos. GLUT-1 is also expressed in bovine oocytes aspirated from adult cows (Oropeza et al., 2004). GLUT-1 is distributed in both the trophectoderm and the inner cell mass in mouse embryos transporting glucose from the trophectoderm to the ICM, but is predominantly expressed in the bovine trophectoderm (Wrenzycki et al., 2003). Specifically, GLUT-1 has been shown to increase drastically between the 8 to 16 cell stage in bovine embryos (Wrenzycki et al., 1998). This upregulation coordinates with the maternal to zygotic transition and subsequent activation of the embryonic genome. The morula stage increase in GLUT-1 also indicates that this time period is the point in development in which the embryos become predominantly dependent on glucose as an energy source (Wrenzycki et al., 1999). Simply, this is the transition point from the utilization of simple energy sources such as pyruvate to more complex sources such as glucose or fructose. Hogan and colleagues (1991) found that GLUT-1 was expressed in a similar pattern in both mouse and bovine embryos. In addition, both growth hormone (GH) and insulin growth factor 1 (IGF-1) have been shown to affect GLUT-1 expression in embryos (Orpeza et al., 2004). IGF-1 was found to have a beneficial effect on the developmental rate of bovine embryos by stimulating the transport of glucose into the cells during compaction and blastulation. This supports the hypothesis that the positive effect of IGF-1 is attributed to an enhanced glucose uptake by the
embryos (Wrenzycki et al., 1998). GLUT-1 expression is altered in bovine in vitro embryos in response to changes in oxygen concentrations (Lonergan et al., 2006). GLUT-1 may be a candidate gene for LOS, and could have profound effects on future in vitro culture protocols. Niemann and Wrenzycki (2000) and Wrenzycki et al. (2001) both found that the relative levels of the GLUT-1 transcript were higher in in vivo embryos than IVP embryos. This is in agreement with Morita et al. (1994), who stated that expression of the GLUT-1 gene and glucose uptake itself are reduced in murine blastocysts produced in vitro when compared to their in vivo counterparts. Conversely, when either serum or PVA was added to culture media, GLUT-1 was significantly upregulated in IVP embryos (Wrenzycki et al., 1999). In contrast to other reports, de Oliveira et al. (2006) did not detect any significant differences among in vivo and IVP embryos supplemented with different protein sources and serum concentrations. They suggested that it was not the protein source that was the critical factor contributing to irregular expression of GLUT-1 between in vitro and in vivo embryos. Therefore, variable results have been found when analyzing GLUT-1. Due to these contrasting results, GLUT-1 should be further investigated.

Several genes have been analyzed thus far in connection with abnormal gene expression in manipulated embryos; however, numerous growth factors could be attributed to this potential block to normal development. Further research must be done to determine the exact mechanism(s) responsible for the abnormal development of these fetuses and/or offspring.

**Measuring Gene Expression (mRNA Transcripts)**

*In Situ Hybridization*

Northern blots and microarrays only give an approximate analysis of gene expression at best. *In situ* hybridization gives a more detailed report of gene expression by using a labeled antisense mRNA probe (either DNA or RNA) to hybridize with the mRNA in the sample. The
labeled probe can be visualized, thus allowing the mRNA sequence of concern to also be visualized (Gilbert, 2003). However, *in situ* hybridization can only produce qualitative results establishing whether or not the gene is being transcribed in the sample, but the level of gene expression can not be directly determined.

**Microarray Analysis**

Unlike traditional molecular biology methods that tend to focus only on a single gene at a particular time, a complementary DNA (cDNA) microarray is an efficient tool for analyzing thousands of genes in a single experiment to identify potential genes that are critical during a particular time point or responsible for a certain response (Ushizawa *et al.*, 2004). Microarrays are commonly used to examine global changes in mRNA abundance across different settings (Cox *et al.*, 2005). Microarray data can suggest candidate genes for embryonic development even if they do not have a known function (Ushizawa *et al.*, 2004).

**Real-Time Reverse Transcriptase Polymerase Chain Reaction**

Quantitative polymerase chain reaction (Q-PCR) is a powerful method to quantify mRNA levels at very low abundance levels even in single embryos. It is currently the method of choice in biological studies of gene expression. Several factors have contributed to the transformation of Q-PCR into mainstream science: (1) since the assay is homogeneous it avoids the need for post-PCR processing as in traditional reverse transcription PCR (RT-PCR), (2) a wide range allows straightforward comparisons between RNAs differing in abundance, and (3) the assay is both quantitative as well as qualitative (Bustin *et al.*, 2005). The transcript of interest can be measured in a ratio to an endogenous reference gene (or housekeeping gene) to correct for variation in the quality of RNA preparations, the efficiency of the reverse transcription (RT) and PCR steps, and in cell number. Thus, Q-PCR measures relative levels of mRNA. Q-PCR is rapid and robust and the labor to perform the procedure is modest (Bjarnadottir and Jonsson, 2005). A brief review of Reverse Transcriptase PCR (RT-PCR) is necessary to understand how Real Time RT-PCR (same as Q-PCR) measures the level of gene expression. mRNA is copied
to cDNA by the reverse transcriptase enzyme. PCR allows the logarithmic copying of small strands of DNA via a heat tolerant DNA polymerase enzyme. Heat tolerance is important since RT-PCR is done in cycles thus avoiding the need to add enzyme after the completion of every cycle. A PCR mix containing a heat-stable polymerase (such as Taq polymerase), specific primers for the target gene of interest, deoxynucleotides, and a suitable buffer is formulated. The primers flank the region of DNA to be amplified. It is very important to select primers that will be optimal for the experimental conditions as to avoid primer-dimer formation, mismatches, or cross-reactions. It is usually necessary to design, synthesize, and validate several primer pairs to obtain a set that generates no primer-dimers and results in an amplification efficiency near 100% (Bustin et al., 2005). The cDNA reaction mix is then heated and cooled allowing the DNA to denature, primers to anneal and the polymers to extend the product, amplifying the product after every cycle. A thermocycler is used to precisely control the temperature in each step. Ethidium bromide (the stain for gel electrophoresis in RT-PCR) is insensitive leading to the inability to detect small differences between genes. Therefore, SYBR Green, which is generally used in Q-PCR, will fluoresce brighter than ethidium bromide and is able to detect very small differences between genes (http://pathmicro.med.sc.edu/pcr/realtime-home.htm).

The basis behind Q-PCR is the more copies of a target gene present at the beginning of an assay, the fewer cycles of amplification are required to generate the number of amplicons (the cDNA produced) that can be detected and reach the threshold cycle (Bustin et al., 2005). The more cDNA of interest (selected for by primers) produced, the more fluorescent will bind and be detected (http://pathmicro.med.sc.edu/pcr/realtime-home.htm). The fluorescence level is measured after every Q-PCR cycle and is considered substantial when it crosses a threshold or background level. The threshold cycle ($C_T$) is defined as the time point in which the sample fluorescence exceeds an amount above background fluorescence (the time when the threshold is crossed). The $C_T$ is calculated when the individual PCR reaction has synthesized approximately half of the amplicons. At this point in the reaction, the amount of accumulated
amplicons is linearly proportional to the amount of target present at the beginning of the assay. The CT is used for quantifying the amount of the gene of interest yet its value is completely subjective as the program selects a background value and the threshold can be altered at will (Bustin et al., 2005). Q-PCR works along the same basis as RT-PCR (some protocols vary in the reagent used) except the results are different. RT-PCR only gives qualitative results while Q-PCR gives quantitative results also. Therefore, Q-PCR can not only determine whether or not the gene is being transcribed in the sample, but it can also determine the level of transcript, thus measuring the level of gene expression. In particular, in serial dilution samples, it is possible to determine in which concentration of sample the product disappears. In addition, Q-PCR allows the comparison of amplicon products against one another via a standard curve. Q-PCR is advantageous when only a small portion of DNA is available to amplify. RT-PCR requires a larger starting sample size to enable accurate detection.

Q-PCR utilizes a reference gene to quantify the data. The reference gene will normalize the RNA levels and justify the observed variations (Wrenzycki et al., 2004). The amount of the gene of interest is calculated as a ratio to the amount of the reference gene. Selecting the correct reference gene is critical in properly designing a Q-PCR experiment. Numerous publications indicate that no one single gene is able to fulfill the criteria required for a universal reference gene. All genes are regulated to some extent and none are expressed at equal levels in all cell types irrespective of conditions presented by the experiment. Therefore, it remains up to the individual researchers to identify and validate the reference gene most appropriate for their experimental conditions (Bustin et al., 2005). It is very important to prove constant expression of a reference gene under different experimental conditions or in different tissues; otherwise data could be misinterpreted or invalid (Bjarnadottir and Jonsson, 2005). Poly (A) polymerase is often used as a reference gene in many gene expression experiments. The poly (A) tail is found at the 3’ end of nearly all eukaryotic mRNAs and regulates mRNA breakdown by
stabilizing the mRNA. Synthesis of the poly (A) tail is carried out by the enzyme poly (A) polymerase (Lodish et al., 2004). Thus, since the poly(A) tail is found in a ubiquitous manner, so should the enzyme. This concept makes poly(A) polymerase a valuable reference gene to use in gene expression analysis.

Because of its sensitivity, Q-PCR is currently the technique of choice to quantify gene expression. Care must be taken to optimize RNA isolation and PCR conditions prior to beginning a trial. It is absolutely essential to apply a validation and optimization strategy to control for the amount of starting material, variation of amplification efficiencies, and differences between samples in order to allow for reliable results. Nevertheless, this remains the most intractable problem for Q-PCR quantification (Bustin et al., 2005). If the primers are designed properly and the procedure is both validated and optimized, the process is relatively simple and produces accurate results. Q-PCR has replaced some of the older methods of measuring gene expression simply due to the benefit that Q-PCR lends itself to automation.

**Defining Developmental Competence**

At least a portion of IVP and NT blastocysts are developmentally competent because live offspring have resulted following transfer. SCNT derived morulae and blastocysts can be produced at a reasonable rate, but development to term of cloned mammalian offspring is extremely low so far (Boiani et al., 2002). Developmental abnormalities have been reported in IVP progeny, but are more pronounced and prevalent in embryos derived from NT. The ultimate test of an embryo is its ability to produce live and healthy offspring post-transfer into a recipient. Abnormalities of embryos are generally visualized as pregnancy losses or abnormal offspring. These data points are considered means of testing developmental competence; however, they are only visualized after transfer or require sacrificing the embryo. There is no specific “technique” to determine developmental competence of a manipulated embryo prior to transfer without sacrificing the embryo. In another words, we have no means to detect developmental
competence in a noninvasive manner. We can only rely on morphological characteristics, such as the visualization of an inner cell mass (ICM) and a blastocoel cavity at day 7 of in vitro culture to detect viable embryos. Pronuclear morphology has also been used to detect embryo developmental competence relying on the fact that zygotes with a normal pronuclear pattern cleave faster and have a greater chance of developing into blastocysts. However, the high lipid content of domestic animal embryos hinders the visualization of the pronuclei. Pronuclear morphological evaluations are limited to the more transparent human or murine zygotes. Nevertheless in most mammalian species, morphological evaluations with all its drawbacks remain the method of choice for selecting viable embryos prior to transfer in both cattle and humans (Lonergan et al., 2006). Generally, studies on the effects of various culture systems have only focused on the percentage of embryos that cleave and proceed to the blastocyst stage (Rizos et al., 2003) or hatched blastocyst stage (de Oliveira et al., 2006). Gene expression studies clearly demonstrate that embryo competence can be severely compromised without any obvious changes in morphology (Wrenzycki et al., 2004).

The effects of the suboptimal culture environment are mediated through modifications of the gene expression patterns in the resulting embryos (Tesfaye et al., 2004). Boiani et al. (2002) indicated that blastocyst formation is usually a good indicator of development preceding normally, but blastocysts can also be formed in the absence of genes essential for development including embryos with aberrant imprinting as is the case in parthenotes. Parthenogenesis is defined as the growth and development of an embryo without fertilization by a sperm cell. The oocyte is artificially activated and results in development through the pre-implantation period. Parthenotes generally have reduced cell numbers and an increased proportion of cells within the ICM (Van De Velde et al., 1999); however, look morphologically identical to normal embryos. Mammalian parthenotes normally do not survive far beyond the blastocyst stage and all will fail after implantation (Boiani et al., 2002).
A clear correlation between the time of first cleavage of the embryo and developmental competence, including those oocytes that cleave early are more likely to reach the blastocyst stage than their late cleaving counterparts, has been established (Dinnyes et al., 1999; Lonergan et al., 1999; Lequarre et al., 2003; Favetta et al., 2004). The end data points in most studies are limited by analyzing only blastocysts rates and possibly ICM:trophectoderm cell number ratios as indicators of developmental competence. It is likely that the effects of a given in vitro culture environment may not visibly manifest themselves during such a short period (generally 7 days for bovine embryos). Quantitative measurements of gene expression should give more detailed information on embryo developmental competence (de Oliveira et al., 2006). Relating expression profiles with noted phenotypic characteristics within definite developmental stages will elucidate the effect and function of a particular gene or group of genes necessary for normal development. Eventually, this may enable the establishment of molecular tests for embryonic developmental capacity (Ponsuksili et al., 2002).

What constitutes a normal embryo? We generally accept in vivo derived embryos as “normal”. However, not all in vivo embryos collected (generally on day 7) are “normal”. If embryos are nonsurgically collected from the cow at day 7 and transferred to recipient cows, not all embryos transferred will make it to term. In fact, pregnancy rates from transferred in vivo-derived embryos can range anywhere between ~50% and 75% (Farin et al., 1999). These data indicate that not all in vivo-derived embryos are viable in early stage embryonic development. Nevertheless, in vivo embryos are the accepted standard because they are as close to “normal” as can be obtained.

Comparison to Gene Expression in In Vivo Counterparts

In vivo-derived embryos are considered the “gold standard” to which IVP and NT produced embryos can be compared with. Studies tend to evaluate NT embryos versus IVP embryos, since IVP are much more analogous to in vivo embryos. However, no current
biotechnology application can reiterate the *in vivo* mechanism. *In vitro*-derived embryos display marked differences from their *in vivo* counterparts (Niemann and Wrenzycki, 2000). When comparing *in vivo* derived embryos to those produced *in vitro*, the embryos produced by artificial means display marked differences to embryos collected from the animal in gross morphology, ultrastructure, color, density, number of cells, size, developmental rate, temperature sensitivity, freezability, viability and pregnancy rates after transfer (Greve *et al.*, 1994). IVP bovine embryos have darker cytoplasm and lower buoyant density (Pollard and Leibo, 1994), a more fragile zona pellucida (Rizos *et al.*, 2002), increased proportional volume of cytoplasm (Crosier *et al.*, 2000), lower pregnancy rates and heavier fetuses after transfer (Hasler, 2001), more chromosomal abnormalities and a higher lipid accumulation rendering them less tolerant to cryopreservation (Viuff *et al.*, 1999; Barcelo-Fimbres and Seidel, 2007), and altered gene expression (Wrenzycki *et al.*, 1996, 1999, 2001; Rizos *et al.*, 2002, 2003). Simply, IVP embryos are inferior to those derived *in vivo*. Thus, the *in vitro* culture effect has a profound effect on the transcriptional level of the resulting embryo at different stages of development (Lonergan *et al.*, 2003). Gjorret *et al.* (2001) reported that apoptosis was more frequently detected in *in vitro*-produced blastocysts when compared to those derived *in vivo*. It is hypothesized that this high incidence of apoptosis is linked to the presence of serum. Differences have been reported between IVP and *in vivo* embryos, but also when compared to NT embryos. It is virtually impossible to copy “Mother Nature”. This inability is not due to the lack of or unavailability of technology, but rather due to the lack of understanding of the precise and extensive system of mammalian embryonic development.

**A Suitable Model**

The outcomes of many experiments conducted using animals not only improve efficiencies in embryo biotechnologies in animals but also possibly in human infertility treatment. In humans, the number of patients undergoing assisted reproductive technologies (ART) has
increased significantly over the past few years. IVP human embryos are now a widely accepted treatment for unexplained infertility. However, with estimated live-birth rates per cycle varying between 13% and 28%, its effectiveness has not been rigorously evaluated in comparison with other ART treatments. A typical human IVF cycle can cost more than $10,000, and insurance coverage may range anywhere from zero to 100%. Multiple gestations occur in 25% of IVF human pregnancies and carry an increased risk of premature delivery, low birth weight, spontaneous abortion, and congenital abnormalities (Harrison and Taylor, 2006). Due to the effects contributed to the extended culture period in mice or farm animals in terms of aberrant gene expression, there has been a recent push for a shorter culture environment in the human ART field. However, the transfer of blastocyst stage embryos increases pregnancy rates and avoids multiple pregnancies as fewer embryos are needed for transfer per patient (Gardner and Lane, 1997). Therefore, a prolonged culture is necessary even under potentially deleterious conditions. Little controlled research has been done on the human embryo, and most of the experiments have been conducted in the mouse. Most of the information available on transcription of certain genes in pre-implantation development is derived from studies involving mice (Wrenzycki et al., 1998). The timing of genomic activation in mice is different from those of larger mammals. The delay in genomic activation in larger animals may be the consequence of the lengthened period of pre-implantation development (Kirchhof et al., 2000). As a consequence, there continues to be growing evidence that the cow is a better model for the human embryo than the mouse in regards to microtubule patterns during fertilization, closer timing of genomic activation, intermediate metabolism, and interaction with the culture medium (Anderiesz et al., 2000; Menezo et al., 2000; Neuber and Powers, 2000; Niemann and Wrenzycki, 2000; Tesfaye et al., 2004). Furthermore, bovine in vivo embryos are easily generated and provide a benchmark against which all other embryos can be evaluated (Wrenzycki et al., 2004).
Possible Solutions

Consequences of alterations in gene expression levels, epigenetic status, or chromatin structure may only be visible in the offspring. Understanding how the alternations in gene expression can be normalized will enhance developmental success and may reduce the long term affects to the resulting organism (Boiani et al., 2002). Some methods than could increase the efficiency of NT fetuses and offspring include: aggregation of embryos to normalize gene expression as was done with oct-4 (Boiani et al., 2003), addition of methyl groups to cytosine residues of the NT embryo’s DNA, and as hypothesized by our laboratory defining a more optimal culture environment for manipulated embryos. Improving the culture of embryos during the pre-implantation period can contribute to the production of qualitatively superior embryos for transfer resulting in an increased efficiency and healthier offspring.

Various technologies such as artificial insemination, embryo transfer (mostly in bovine), and cloning have been applied to mammalian reproduction. The effects of the short term in vitro culture environment raise questions about long term consequences and the safety of ART (Lonergan et al., 2006). A precise knowledge of the control of gene expression during the entire pre-implantation period is necessary to improve these technologies (Ushizawa et al., 2004). Detailed analyses of gene expression profiles during bovine embryogenesis will be a valuable tool to better understand basic cellular and molecular mechanisms that control very early development, thus allowing for the refinement of culture systems and better strategies for transgenic and cloning studies. Analysis of differences in mRNA expression could possibly allow the opportunity to alter gene expression by modifying the culture environment thus improving post-thaw viability of IVP embryos (Rizos et al., 2003). SCNT will also continue to play a role in biotechnology. Therefore, many of the factors controlling transcription and gene expression of these NT fetuses and offspring remain to be studied and therefore understood.

Some cloned offspring have been successful. As a result, it is imperative to evaluate why only a small percentage of NT embryos develop into healthy offspring. We believe it would
be beneficial to try to compose a list of developmentally important genes that could better
determine developmental competence of manipulated embryos prior to transfer. By analyzing
this potential list of genes on a select few embryos from a donor or IVP/NT pool, one could
better predict the viability of the embryo once transferred into a recipient. In may be possible in
the future to even determine developmental competence by either taking a biopsy or bisection
of an embryo to analyze the potential list of candidate genes (Li et al., 2005). Combining
traditional measurements of developmental competence (cleavage and blastocyst rates) with
qualitative measures, such as PCR should give a more complex picture of the consequences of
modifying a culture environment on the embryo (Rizos et al., 2003). Therefore, our goal was to
increase the efficiency of embryo biotechnologies through a better understanding of the
transcriptional mechanisms controlling gene expression. Specifically, the objective of this thesis
research is to determine if alterations to current in vitro culture conditions will affect gene
expression and/or make embryo biotechnologies more efficient.
CHAPTER III

EFFECTS OF CULTURE MEDIUM AND PROTEIN SUPPLEMENTATION ON mRNA EXPRESSION OF IN VITRO PRODUCED PREIMPLANTATION BOVINE EMBRYOS

Introduction

*In vitro* production (IVP) of bovine embryos has become a reliable alternative to conventional superovulation induction techniques and has been used as a tool to study pre-implantation embryo development (reviewed by Bavister, 1995). In particular, the 5-6 day post fertilization period of IVP embryos is the time when various developmentally important events occur including the first cleavage division which is critical in determining the subsequent development of the embryo (Lonergan *et al*., 1999), activation of the embryonic genome (Memili and First, 2000) also known as the maternal to zygotic transition, compaction of the morula, which involves the establishment of the first cell-to-cell contacts within the embryo, and blastocyst formation, which is the time point of the first differentiation into two cell types the trophectoderm and the inner cell mass (ICM). This time period is generally referred to as the pre-implantation period. This period is characterized by distinct morphological steps that must include a well-orchestrated expression of genes derived from both the maternal and embryonic genomes to allow for compaction, cavitation, and blastocoel expansion (Kidder, 1992).

IVP is more efficient in terms of producing live offspring than is nuclear transfer (NT), but substantial losses have been noted (reviewed by Farin *et al*., 2006). Most of the losses in IVP seem to be during the *in vitro* culture stages (between the 2-cell and blastocyst stages) suggesting that the post-fertilization environment is the most critical period of development in terms of determining blastocyst yield (Lonergan *et al*., 2006). The relative abundance of numerous transcripts varies through the early pre-implantation period and is strongly influenced by the culture environment (Wrenzycki *et al*., 1996, 1999, 2001, 2004; Eckert and Niemann, 1998; Lazzari *et al*., 2002; Rizos *et al*., 2002, 2003; Lonergan *et al*., 2003). Current culture systems can also lead to either persistent silencing or enhanced expression of a particular gene.
throughout the critical phases of fetal development (Wrenzycki et al., 2001). Therefore, any modifications to the in vitro culture environment can have profound effects on the quality of the resulting embryos measured in terms of cryotolerance, relative transcript abundance (Lonergan et al., 2003), and development to term. The culture environment is a common aspect of both IVP and NT; consequently, research focusing on the in vitro culture system will not only better IVP but NT also. However, little effort is often given to what implications could arise from using these artificial technologies in terms of its effects on gene expression.

It is evident that pre-implantation embryos are able to develop in vitro in a wide array of culture conditions. They are able to develop in media ranging in composition from simple balanced salt solutions to complex systems supplemented with serum and somatic cells (Lonergan et al., 2006). However, this ability to adapt to the environment, especially under suboptimal or even nonphysiological conditions, comes at a cost to the embryo in terms of embryo quality (Rizos et al., 2002). Blastocysts produced following culture in synthetic oviductal fluid [SOF (Tervit et al., 1972)], a medium designed for pre-implantation embryos, have significantly altered patterns of genes involved in gap junction formation, apoptosis, oxidative stress and differentiation compared to their in vivo counterparts (Rizos et al., 2002). Ho et al. (1995) reported that in the mouse, the potassium simplex optimized medium [KSOM (Lawitts and Biggers, 1991a, b)] supplemented with amino acids or serum provided very similar expression profiles (almost identical) as those found in in vivo embryos.

Most commonly used culture media are supplemented with either calf serum (CS) or bovine serum albumin (BSA) as a protein source. Different protein sources in culture media can greatly influence development of IVP embryos, ranging from either a marked stimulation in some aspects to a significant inhibition of other processes. In fact, the quality of serum and BSA can vary greatly depending on the source and specifically for serum, the lot number (Bavister, 1995). Serum has been shown to have a biphasic influence on the developmental capacity of bovine embryos by inhibiting the first cleavage division but enhancing later blastocyst
development (Wrenzycki et al., 1998). Serum can rate the speed of embryo development and the resulting quality of the embryos produced (Rizos et al., 2003). Serum supplementation has also been linked to a high incidence of apoptosis in IVP blastocysts (Gjorret et al., 2001). However, Barcelo-Fimbres and Seidel (2007) reported that a serum free culture yielded a blastocyst rate not significantly different from the blastocyst rate achieved with serum supplementation. The presence of serum over a prolonged period of time has been shown to alter the expression pattern of various genes (Wrenzycki et al., 1999). Lonergan et al. (2003) reported that simply omitting serum from the culture medium during the post-fertilization period changed mRNA abundance for several developmentally important genes.

Embryonic anomalies are generally visualized as pregnancy losses or abnormal offspring. There is no specific “technique” to determine developmental competence of an embryo prior to transfer without sacrificing the embryo. We can only rely on morphological characteristics such as the visualization of an ICM and a blastocoel cavity at day 7 of in vitro culture to detect viable embryos. Boiani et al. (2002) indicated that blastocyst formation is usually a good indicator of development preceding normally, but blastocysts can also be formed in the absence of genes essential for development including embryos with aberrant imprinting as is the case in parthenotes. Gene expression studies clearly demonstrate that embryo competence can be severely compromised without any obvious changes in morphology (Wrenzycki et al., 2004). It is likely that the effects of a given in vitro culture environment may not visibly manifest themselves during such a short period (generally 7 days for bovine embryos). Quantitative measurements of gene expression should give more detailed information on embryo developmental competence (de Oliveira et al., 2006). Specifically, we believe that PCR analysis of suitable candidate genes for developmental competence would be a valuable tool to test a select group of embryos prior to transfer.

Some of the genes shown to be important to normal mammalian embryonic development include: oct-4, nanog, connexin 43, and glucose transporter-1 (GLUT-1). Oct-4 is
the earliest known transcription factor to be developmentally expressed (Chau et al., 2002) and thus regulates initial events of development. Oct-4 mRNA in pig and cow embryos can be detected in both the ICM and the trophectoderm of a blastocyst; however, oct-4 is strictly limited to the ICM in mouse embryos (Kirchhof et al., 2000). Nanog is a newly identified transcription factor that contains a homeodomain (like oct-4) which is specific to maintaining the pluripotency state of embryonic stem, germ, and carcinoma cells and in pre-implantation embryos in a dose dependent manner (Yamaguchi et al., 2005). The gap junction protein connexin 43 is necessary for the maintenance of compaction (gap junction formation) and thus, for subsequent blastocyst formation in mice (Lee et al., 1987). Gap junction formation and communication is essential for growth, cellular differentiation, and embryonic development (Wrenzycki et al., 1996). Expression of GLUT-1 is frequently measured when comparing different culture systems (Wrenzycki et al., 1999, 2001; Lazzari et al., 2002; de Oliveira et al., 2006). GLUT-1 is responsible for incorporating glucose into embryonic cells throughout the period of pre-implantation development (Lequarre et al., 1997; de Oliveira et al., 2006). GLUT-1 is expressed in both the trophectoderm and the inner cell mass in mouse embryos transporting glucose from the trophectoderm to the ICM, but is predominantly expressed in the trophectoderm of bovine embryos (Wrenzycki et al., 2003).

Since the culture environment itself may alter gene expression levels of various developmentally important genes, this study examined the development potential of in vitro produced bovine embryos cultured in two commonly used culture systems, SOF or KSOM supplemented with amino acids (SOFaa or KSOMaa, respectively) to determine if differences in media composition would effect gene expression levels. A second experiment assessing the same two media with or without CS (BSA supplemented) was also conducted to determine if the protein source utilized would affect gene expression levels. Additionally, we investigated the expression of oct-4, nanog, connexin 43, and GLUT-1 in IVP and in vivo embryos on day 7 of pre-implantation development.
Materials and Methods

Experimental Design

Experiment 1

Ten pools of *in vitro* produced blastocysts (10 embryos per pool) were generated for each treatment. *In vivo* embryos were obtained by nonsurgical embryo collection on day 7 from mature cows and allotted into pools of 10 (10 pools, n=10). IVP embryos were synthesized as follows. IVF was performed and embryos were cultured according to treatment in either SOFaa or KSOMaa. Blastocysts were recovered after 7 days of culture (either *in vivo* or *in vitro*) and morphology was recorded. RNA was isolated, reverse transcribed to cDNA, and gene-specific amplified via Quantitative (Real-Time) PCR (Q-PCR).

Experiment 2

Eleven pools of *in vitro* produced blastocysts (5 embryos per pool) were generated for each treatment. As in Experiment 1, transcriptional expression levels were compared with the levels present *in vivo* embryos.

*In vivo* embryos were obtained as in Experiment 1 and were allotted into pools of 10 (10 pools, n=10). IVP embryos were synthesized as in Experiment 1 with the exception of the culture environment. Embryos were cultured according to treatment in either SOFaa, SOFaa with CS, KSOMaa, or KSOM with CS medium (4 treatments total). Blastocysts were recovered after 7 days of culture (either *in vivo* or *in vitro*) and morphology was recorded. RNA was again isolated, reverse transcribed to cDNA, and gene-specific amplified via Q-PCR.

*In Vitro* Production of Embryos

Cumulus oocyte complexes (COCs) were obtained from a commercial supplier (Conco Valley Genetics, TX, USA; Bomed, Inc., WI, USA). COCs arrived in vials containing *in vitro* maturation (IVM) medium (TCM-199, 10% fetal bovine serum (FBS), 0.1% Penicillin/ Streptomycin (P/S), Na pyruvate, L-glutamine, 50 ng/ml of epidermal growth factor, FSH, LH, and E₂) held at 39°C, and stabilized with CO₂. At 22 h post-maturation COCs were subjected to
in vitro fertilization (IVF). Briefly, COCs were aspirated from the IVM vials and washed twice in HEPES-TALP (Tyrodes’s, albumin, lactate, pyruvate) containing 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.34 mM NaH₂PO₄, 10 mM HEPES, 10 mM lactic acid, 2 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 0.2 mM Na pyruvate, 3 mg/ml of Fraction V BSA, 1% P/S, and 5 μg/ml of phenol red.

COCs were inseminated for 17 to 18 h in 50 μl droplets of IVF medium under mineral oil at 39°C in a humidified atmosphere using frozen-thawed semen from one Holstein bull with a history of proven fertility in our IVF program (post-thaw motility as high as 90-95%). After semen was thawed for 30 sec in a 37°C water bath, motile spermatozoa were separated using a Percoll discontinuous gradient by centrifugation for 20 min at 750 X g. Motile spermatozoa were also washed in Sperm-TALP containing 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.29 mM NaH₂PO₄, 10 mM HEPES, 21.6 mM lactic acid, 2.1 mM CaCl₂·2H₂O, 1.5 mM MgCl₂·6H₂O, 1mM Na pyruvate, 6 mg/ml of Fraction V BSA, 1% P/S, and 5 μg/ml of phenol red by centrifugation for an additional 10 min at 400 X g to remove any excess cryoprotectant. A sperm concentration of 1 X 10⁶ motile sperm per ml was used for IVF based on hemacytometer counts. The IVF medium consisted of IVF-TALP (114 mM NaCl, 3.2 mM KCl, 25 mM NaHCO₃, 0.34 mM NaH₂PO₄, 10 mM lactic acid, 2 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 0.2 mM Na pyruvate, 6 mg/ml of essentially fatty acid free BSA, 1% P/S, and 5 μg/ml of phenol red) supplemented with 1 mM epinephrine, 10 μM hypotaurine, 20 μM penicillamine, and 2 μg/ml of heparin. After IVF, the presumptive zygotes with cumulus cells were denuded in a hyaluronidase solution (1 mg/ml) via vortexing for 2 min. To ensure that the transcripts analyzed did not originate from residual cumulus cells, “cumulus-free” zygotes were washed twice in HEPES-TALP prior to initiating culture. Presumptive zygotes were cultured in 30 μl drops of the appropriate culture medium held at 39°C under embryo-tested, sterile-filtered mineral oil (Sigma-Aldrich, Inc., St. Louis, MO, USA) in a 5% CO₂, 5% O₂, and 90% N₂ humidified atmosphere up to 8 days. On day 3, all
embryos were transferred to fresh medium and cleavage rate was determined. In Experiment 2, half the embryos per culture treatment (SOFaa or KSOMaa) were transferred to fresh medium containing 5% calf serum on day 3 of culture. Embryos reaching the blastocyst stage by day 7 were used for gene expression analysis or cell counting. Embryos not reaching the blastocyst stage until day 8 were used as a separate treatment for cell counting.

**Culture Media**

KSOM medium (Lawitts and Biggers, 1991a, b) consisted of 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH$_2$PO$_4$, 0.20 mM MgSO$_4$·7H$_2$O, 20.49 mM DL-lactic acid, 0.20 mM D-glucose, 25 mM NaHCO$_3$, 0.20 mM pyruvic acid, and 5 μg/ml of phenol red. KSOMaa was supplemented with 1 mM L-glutamine, essential amino acids (50X Basal Medium Eagle [BME]), non-essential amino acids (100X Minimum Essential Media [MEM]), 3 mg/ml of Fraction V BSA, and 1% P/S. SOF medium (Tervit et al., 1972) consisted of 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH$_2$PO$_4$, 25.07 mM NaHCO$_3$, 3.3 mM DL-lactic acid, 0.49 mM MgCl$_2$·6H$_2$O, 1.71 mM CaCl$_2$·2H$_2$O, and 5 μg/ml of phenol red. SOFaa was supplemented with 1 mM L-glutamine, 0.33 mM Na pyruvate, 1.5 mM D-glucose, 50X BME, 100X MEM, 3 mg/ml of Fraction V BSA, and 1% P/S. In Experiment 2, half the embryos per culture medium were supplemented with 5% CS on day 3 of *in vitro* culture. All culture conditions were stabilized by embryo-tested, sterile-filtered mineral oil and held at 39°C in a 5% CO$_2$, 5% O$_2$, and 90% N$_2$ humidified atmosphere.

**Collection of *In Vivo* Embryos**

Crossbred beef cows were synchronized and superovulation was induced using a standard protocol. Briefly, estrus synchronization was achieved by insertion of an Eazi-Breed CIDR™ (Controlled Intravaginal Releasing Device; Pharmacia & Upjohn Company, Kalamazoo, MI, USA) containing 1.38 g of progesterone. An injection of 15 mg progesterone and 2.5 mg estradiol-17β was given at CIDR insertion (day 0). Stimulation was achieved by twice daily (A.M. and P.M.) Follitropin V (Bioniche Animal Health, Canada) FSH injections on days 4, 5, and 6 of
the treatment. On day 6 (P.M.), the CIDR was removed and 5 ml of Lutalyse (5 mg/ml) was given. On day 7, a final FSH injection was given in the morning. The total FSH dose per donor was 236 mg. Donors exhibiting estrus behavior were artificially inseminated at 12 and 24 h after the onset of standing estrus. Estradiol was given on the morning of artificial insemination, followed by a nonsurgical embryo collection 7 days later. Collected embryos were held in a commercial embryo holding medium (Holding Plus®, BIONICHE, Pullman, WA, USA) for a period of 1 to 2 h at 37°C until long term storage at -80°C for further gene expression analysis.

**Differential Staining**

Day 7 and day 8 blastocysts were incubated in dPBS containing 1 mg/ml of Hoechst 33342 for 10 min. Trophoderm cells were permeabilized with 0.4% Triton X-100 for 1 min and counterstained with 25 mg/ml of propidium iodide (PI) for 45 sec. Stained embryos were mounted onto a glass microscope slide in a drop of 25% glycerol. Embryos were gently flattened with a coverslip and visualized for cell counting. The number of trophoblast (nucleus stained with PI) and inner cell mass (ICM, nucleus stained with Hoechst 33342) cells was determined using an epifluorescent microscope equipped with a UV filter cube.

**Isolation of mRNA**

mRNA (or poly(A)+ RNA) was isolated from pools of bovine blastocysts (*in vitro* and *in vivo*) using the Dynabeads® mRNA Direct™ Micro Kit (Dynal Biotech, Inc., Lake Success, NY, USA) within a PCR isolation hood. The protocol used is described by Wrenzycki et al. (1998) and consists of the manufacturer’s instructions with minor modifications. Briefly, pools of embryos were first stored at -80°C in approximately 3 μl of PBS plus 0.1% polyvinyl alcohol (PVA) in 1.5 ml siliconized tubes. Embryo pools were lysed in 150 μl lysis/binding buffer (100 mM Tris HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, and 5 mM dithiothreitol) followed by a 10 sec vortex to ensure complete lysis. Samples were centrifuged at 12,000 g for 15 sec and incubated for 10 min at room temperature. Pre-washed Dynabeads® (10 μl) were combined with the sample solute. The sample poly(A)+ RNAs were allowed to
anneal to the beads (dT25) while rotating on a hybridization mixer for 10 min. The beads were separated from the mix using a Dynal MPC-E-1 magnetic separator. The sample was washed once in 100 μl wash buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, and 0.1% lithium dodecylsulfate) and three times in 100 μl wash buffer B (10 mM Tris HCl (pH 8.0), 150 mM LiCl, and 1 mM EDTA). The mRNA was then eluted from the beads in 11 μl sterile water heated at 75°C for 2 min and was used immediately for reverse transcription.

Reverse Transcription

mRNA isolated from different pools of embryos or fibroblast cells (used as an internal calibrator) was reverse transcribed into cDNA in a total volume of 20 μl using both oligo (dT) and random hexamer primers to enable reverse transcription of a wide variety of targets <1kb in length. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The 20 μl iScript RT Reaction Mix consisted of: 4 μl of iScript reaction mix, 1 μl of reverse transcriptase, 4 μl of nuclease-free water, and 11 μl of sample mRNA. A reaction mix was formulated for the sample and for a no template negative control. The reaction was conducted at 25°C for 5 min, 42°C for 30 min, a denaturation step of 85°C for 5 min, and a final holding temperature of 4°C.

Validation and Optimization of Q-PCR

Reference genes that have been used in other gene expression experiments include: poly(A) polymerase (Wrenzycki et al., 1998), Histone H2a and glucose 6-phosphate dehydrogenase (Lonergan et al., 2003). All three primer sets for the reference genes were validated first using fibroblasts and then with embryos using RT-PCR and gel electrophoresis. From these results, poly(A) polymerase was chosen as the reference gene due to its ability to produce more consistent results as compared with the other two primer sets.

PCR products for each gene of interest were also obtained via RT-PCR and were electrophoresed on an agarose gel. Each amplicon was sequenced to confirm the amplification of the proper product. To assure that the primer sets did not amplify genomic DNA, 1 ng of
genomic DNA was used as a template for the amplification of the target genes. No amplicons were recovered after RT-PCR of genomic DNA (data not shown).

An annealing temperature gradient and primer concentration matrix were performed for each target gene to determine the optimal annealing temperature and primer concentration (both sense and antisense primers) to ensure maximum amplification. To ensure that the primers amplified a single product in a quantitative manner, amplification efficiency and a correlation coefficient from a standard curve of cDNA at four different 10-fold dilutions were determined for each gene utilizing Q-PCR (Figure 3.1). All of the target genes had acceptable efficiencies (80 to 120%) and correlation coefficients (close to 1.0).

For optimization purposes, fibroblast cDNA was used in optimizing poly(A) and connexin 43. For optimization of oct-4 and nanog, plasmids containing the PCR amplicons were constructed using the pCR-XL-TOPO Cloning® Kit (Invitrogen Corporation, Carlsbad, CA, USA). All Q-PCR for optimization purposes was performed following the same RT and Q-PCR protocols discussed elsewhere with the exception of the mRNA isolation from the fibroblasts. Briefly, mRNA was isolated using the same protocol discussed above with alternative volumes. Fibroblast cells were lysed in 1250 μl lysis buffer. The sample was sheared with a 21 g needle 3 to 5 times using a 1 to 2 ml syringe to ensure complete lysis and vortexed for 10 sec. Pre-washed Dynabeads® (250 μl) were combined with the sample solute. As before, the sample poly(A) tails were allowed to anneal to the beads (dT25) while rotating on a hybridization mixer for 10 min. The beads were washed twice in 1 ml of wash buffer A and three times in 1 ml of wash buffer B. The mRNA was eluted from the beads as before.

Q-PCR

PCR primers were designed to amplify poly(A) polymerase, oct-4, nanog, connexin 43, and GLUT-1 from the coding regions in bovine gene sequences using the Beacon Designer 4.0 (PREMIER Biosoft International) (Table 3.1). cDNA from 2 embryo equivalents for the first experiment and 1 embryo equivalent for the second experiment were amplified for each gene.
Figure 3.1. Dilution Ct values, melting curves, and standard curve obtained using primers for the amplification of connexin 43. Four 10-fold dilutions (100,000, 10,000, 1,000, and 100 cells) were used to generate dilution curves (A), melting curve data (B), and standard curve (C).
Table 3.1 Primers used for Q-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers</th>
<th>Amplicon Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A)</td>
<td>X63436</td>
<td>Sense AAGCAACTCCATCAACTACTG</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense ACGGACTGGTCTTCATAGC</td>
<td></td>
</tr>
<tr>
<td>Oct-4</td>
<td>NM_174580</td>
<td>Sense CCACCAGCAGGCAAACAC</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense GAGAAGGCGAAGTCAGAAGC</td>
<td></td>
</tr>
<tr>
<td>Nanog</td>
<td>DQ069776</td>
<td>Sense AATTCCCAGCAGCAAAATCAC</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense CCCTTCCCTCAAATTGACAC</td>
<td></td>
</tr>
<tr>
<td>Connexin 43</td>
<td>BC105464</td>
<td>Sense CGTGTCATTGGTGTTCTTTG</td>
<td>386</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense AATTCTGGTGTTGGTCGTCTGG</td>
<td></td>
</tr>
<tr>
<td>GLUT-1</td>
<td>M60448</td>
<td>Sense TGGGAAAGTCTTTTGGAGATG</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense TCGTTGCAGTTAATGAGC</td>
<td></td>
</tr>
</tbody>
</table>
using the iQ™SYBR Green Supermix in the MyiQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Q-PCR Reaction Mix consisted of 12.5 μl of iQ SYBR™ Green 2X Supermix, 4 μl of cDNA or H2O, 6.5 μl of nuclease-free water, and 1 μl of each primer (sense and antisense at 20 pmol concentrations). For each sample (embryo pool), reactions were performed for the reference gene, each gene of interest, and a no template negative control. Within each Q-PCR assay or plate setup, reactions for the reference gene and each gene of interest were performed using the calibrator cDNA. The calibrator consisted of a mixture of cDNA from fibroblasts, plasmids, and embryos. The PCR program used for the amplification of all genes consisted of a denaturing cycle of 3 min at 95°C; 40 cycles of PCR (95°C for 10 sec and 55°C for 45 sec); a melting curve analysis which consisted of 95°C for 1 min followed by 55°C for 1 min, a step cycle with 80 repeats starting at 55°C for 10 sec with a +0.5°C/sec transition rate; and a final holding temperature of 4°C.

Data was quantified using the method for relative quantification in Q-PCR described by Pfaffl (2001). Values are reported as relative transcription or the n-fold difference relative to a calibrator. A mixture of cDNA from fibroblast cells, plasmids, and embryos was used as a calibrator for all of the target genes. poly(A) polymerase was used as the internal reference gene. The threshold cycle (C_T) value of the reference gene was used to normalize the target gene signals in each sample. The amount of target transcripts relative to the calibrator was calculated using the following equation: n-fold difference = Efficiency Target Gene^{ΔC_T}/ Efficiency Reference Gene^{ΔC_R}. The ΔC_T (for the target gene) value was calculated by subtracting the sample C_T value of the target gene from the calibrator C_T value of the target gene. The ΔC_R (for the reference gene) value was calculated by subtracting the sample C_T value of the reference gene [poly(A)] from the calibrator C_T value of the reference gene. Therefore, all target abundance levels were expressed as n-fold differences relative to a calibrator.
and normalized to the reference gene in order to compensate for PCR variations between runs.

Statistical Analysis

Data were analyzed using SigmaStat Statistical Software Version 3.5 (Systat Software, Richmond, CA, USA). Cleavage and blastocyst rates between treatment groups were analyzed using Chi-Square analysis. ICM cell number, trophectoderm cell number, total cell number, ICM:Trophectoderm ratio, and gene expression levels were tested for normality and equal variance using Kolmogorov-Smirnov and Levene’s Median test, respectively. One-way ANOVA, followed by multiple pair-wise comparisons using Tukey’s test when applicable, was used to detect differences in abundance levels between treatments and in vivo embryos. One-way ANOVA was also used to detect differences in ICM cell number, trophectoderm cell number, total cell number, and cell ratio of treatment embryos when compared to in vivo counterparts and treatment embryos produced after different times in culture. Differences of $P \leq 0.05$ were considered to be significant.

A 95% confidence interval for the gene expression levels of in vivo embryos for each gene of interest was formulated using descriptive statistics. If the n-fold difference relative to the calibrator for each treatment embryo equivalent did not fall within the confidence interval for the in vivo embryos, they were considered abnormal, either upregulated or downregulated.

It should be noted that different commercial oocyte suppliers were used in each of the experiments. As a consequence, no comparisons were made between experiments and embryos were produced at separate times for each experiment. Additionally, in Experiment 2, SOF treatment (SOFaa and SOFaa with CS) embryos were generated from separate pools of oocytes than KSOM treatment (KSOMaa and KSOMaa with CS) embryos. Therefore, to ensure that differences were attributed to the
treatment itself and not variation between oocyte pools, all comparisons were only made
between the two related treatment groups (SOFaa or KSOMaa with and without CS) and
in vivo embryos for the second experiment.

Results

Experiment 1: Effect of Media Composition on Gene Expression Levels

Embryo Development and Cell Number

KSOMaa embryos produced did not differ in cleavage rate or in the number of 8
to 16-cell embryos present 72 h post-insemination (hpi) when compared with SOFaa
embryo production rates. The percentage of blastocysts, calculated from the total
number of cleaved embryos, was also not significantly different among the two
treatments (Table 3.2).

The mean total cell number and mean trophectoderm cell number in blastocysts
at day 7 cultured in either KSOMaa or SOFaa (143 ± 9.2 and 99 ± 7.7 versus 156 ± 9.5
and 117 ± 8.2, respectively) was significantly higher than the mean total cell number and
mean trophectoderm cell number of day 7 in vivo embryos (100.8 ± 11.9 and 69.3 ± 8.6,
respectively). Additionally, the mean ICM cell number in day 7 in vivo embryos (32 ± 3.4)
was significantly lower than the KSOMaa cultured blastocysts (44 ± 2.8), and the
ICM:TE ratio was significantly greater in day 7 SOFaa cultured blastocysts (1:3.0 ± 0.3)
when compared to both KSOMaa cultured blastocysts (1:2.3 ± 0.2) and in vivo
blastocysts (1:2.2 ± 0.1) (Table 3.3). Mean cell number data for day 7 blastocysts was
also compared with data collected from day 8 blastocysts (Table 3.4). Significant
differences for mean ICM number, trophectoderm number, and total cell number were
detected among day 7 and day 8 IVP blastocysts. No significant difference in ICM:TE
ratio was detected among day 7 and day 8 IVP blastocysts (Figure 3.2).
Table 3.2 Embryonic development of IVP embryos cultured in either KSOMaa or SOFaa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Clvd (%)</th>
<th>8-16 cell* (%)</th>
<th>Blastocysts † (%)</th>
<th>From Clvd</th>
<th>From 8-16 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>909</td>
<td>675 (74)</td>
<td>174 (19)</td>
<td>125 (19)</td>
<td>125 (72)</td>
<td></td>
</tr>
<tr>
<td>SOFaa</td>
<td>932</td>
<td>611 (74)</td>
<td>158 (17)</td>
<td>119 (20)</td>
<td>119 (75)</td>
<td></td>
</tr>
</tbody>
</table>

*8-16 cell embryos 72 h post-insemination
†Total blastocysts at day 8.
No significant differences between treatments.
Table 3.3  Cell number of blastocysts after 7 days of *in vivo* or *in vitro* culture in either KSOMaa or SOFaa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>14</td>
<td>44 ± 2.8a</td>
<td>99 ± 7.7a</td>
<td>1:2.3 ± 0.2a</td>
<td>143 ± 9.2a</td>
</tr>
<tr>
<td>SOFaa</td>
<td>17</td>
<td>42 ± 3.0a,b</td>
<td>117 ± 8.2a</td>
<td>1:3.0 ± 0.3b</td>
<td>156 ± 9.5a</td>
</tr>
<tr>
<td>In Vivo</td>
<td>10</td>
<td>32 ± 3.4b</td>
<td>69.3 ± 8.6b</td>
<td>1:2.2 ± 0.1a</td>
<td>100.8 ± 11.9b</td>
</tr>
</tbody>
</table>

*TE = Trophoectoderm
Data presented as mean ± SEM.
Statistical differences were determined by One-Way ANOVA (P<0.05).

\(^a,b\) Different letters within columns indicate significant difference between treatments.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>21</td>
<td>28 ± 2.7</td>
<td>59 ± 4.0</td>
<td>1:2.7 ± 0.4</td>
<td>86 ± 5.8</td>
</tr>
<tr>
<td>SOFaa</td>
<td>16</td>
<td>24 ± 2.9</td>
<td>53 ± 5.8</td>
<td>1:2.5 ± 0.3</td>
<td>77 ± 8.0</td>
</tr>
</tbody>
</table>

*TE = Trophectoderm
Data presented as mean ± SEM.
Statistical differences were determined by t-test (P<0.05).
No significant differences between treatments.
Figure 3.2 Differential staining cell numbers for in vivo embryos compared to IVP blastocysts cultured for 7 days (dark gray bars) and 8 days (light gray bars). Mean ICM cell number (A), mean trophectoderm (TE) cells (B), mean ICM:TE ratio (C), and mean total cell number (D). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

a,bSignificant differences within treatments between days.
1,2Significant differences between treatments within days.

*Mean values are given as 1:TE cell ratio.
Transcription Levels in Pre-implantation Embryos

Oct-4 transcript levels in blastocysts cultured in KSOMaa were significantly different from both blastocysts cultured in SOFaa and day 7 in vivo embryos. Connexin 43 transcript levels were below detectable levels in KSOMaa, SOFaa, and in vivo cultured embryos. In addition, the mRNA expression levels for nanog and GLUT-1 were not significantly different among treatment groups (Figure 3.3).

Mean relative levels may not be the best method of analysis of gene expression data in particular when using smaller sample sizes (number of embryo pools). Therefore, confidence levels for in vivo embryos were calculated to better interpret the data. A high proportion of IVP blastocysts cultured in KSOMaa displayed abnormal levels of nanog. Only 40% of KSOMaa cultured blastocysts had normal expression levels of nanog, whereas, 63% of SOFaa blastocysts had normal transcript levels on day 7. Irrespective of the treatment, the majority of the IVP blastocysts analyzed for GLUT-1 fell within the normal confidence interval for in vivo embryos with a few having significantly upregulated expression (Figure 3.4 and Table 3.5).

Experiment 2: Effect of Protein Source Utilized on Gene Expression Levels

Embryo Development and Cell Number

There was no significant difference in cleavage rate or in the number of 8-16 cell embryos present 72 hpi in any of the four treatments (KSOMaa or SOFaa with and without CS) analyzed. The percentage of blastocysts, calculated from the total number of cleaved embryos, was also not significantly different among the treatments (Tables 3.6 and 3.7).

The mean total cell number and mean ICM cell number in blastocysts at day 7 cultured in KSOMaa with CS (151 ± 9.3 and 48 ± 3.1, respectively) was significantly higher than the mean total cell number and mean ICM cell number of day 7 in vivo
Figure 3.3 Average relative transcript levels for *in vivo* embryos (black bars) compared to IVP blastocysts cultured for 7 days in SOFaa (light gray bars) or KSOMaa (dark gray bars). Relative abundance levels for Oct-4 (A), Nanog (B), and GLUT-1 (C). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

\(^{a,b}\)Significant differences between treatments.
Figure 3.4 Relative transcription levels of IVP blastocysts on day 7 of culture in either SOFaa or KSOMaa. Relative levels of Oct-4 (A), Nanog (B), and GLUT-1 (C) on day 7. Dashed lines represent the 95% confidence intervals for in vivo embryos.
Table 3.5  Number of IVP blastocyst pools cultured in either KSOMaa or SOFaa that fall within or out of the 95% CI range for in vivo embryo pools.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Comparison to In Vivo CI</th>
<th>KSOMaa</th>
<th>SOFaa*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>Upregulated</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Nanog</td>
<td>Upregulated</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Upregulated</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note: 10 pools of SOFaa blastocysts were analyzed, but 2 of the blastocyst pools were rejected due to lack of the poly(A) transcript.
### Table 3.6  Embryonic development of IVP embryos cultured in KSOMaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Clvd (%)</th>
<th>8-16 cell* (%)</th>
<th>Blastocysts† (%)</th>
<th>From Clvd</th>
<th>From 8-16 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>527</td>
<td>401 (76)</td>
<td>129 (25)</td>
<td>76 (19)</td>
<td>76 (59)a</td>
<td></td>
</tr>
<tr>
<td>KSOMaa + CS</td>
<td>429</td>
<td>336 (78)</td>
<td>84 (20)</td>
<td>67 (20)</td>
<td>67 (80)b</td>
<td></td>
</tr>
</tbody>
</table>

*8-16 cell embryo 72 h post insemination.
†Total blastocysts at day 8.

a,b Different letters within columns indicate significant difference between treatments.
Table 3.7  Embryonic development of IVP embryos cultured in SOFaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Clvd (%)</th>
<th>8-16 cell* (%)</th>
<th>Blastocysts† (%)</th>
<th>From Clvd</th>
<th>From 8-16 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFaa</td>
<td>381</td>
<td>302 (79)</td>
<td>116 (31)</td>
<td>53 (18)</td>
<td>53 (46)</td>
<td></td>
</tr>
<tr>
<td>SOFaa + CS</td>
<td>372</td>
<td>283 (76)</td>
<td>100 (27)</td>
<td>57 (20)</td>
<td>57 (57)</td>
<td></td>
</tr>
</tbody>
</table>

*8-16 cell embryo 72 h post insemination.
†Total blastocysts at day 8.
No significant differences between treatments.
embryos (100.8 ± 11.9 and 69.3 ± 8.6, respectively). There was no significant difference in mean trophectoderm cell number or ICM:TE ratio for any treatment (Tables 3.8 and 3.9). Mean cell numbers for day 7 blastocysts was also compared with data collected from day 8 blastocysts (Tables 3.10 and 3.11). There was a significant difference in mean ICM number among day 7 and day 8 KSOMaa with CS blastocysts when compared with all other IVP treatments. There was a also a significant difference in mean total cell number and mean trophectoderm number among day 7 and 8 SOFaa blastocysts when compared to all other IVP treatments. Additionally, there was a significant difference in mean ICM:TE ratio among day 7 and day 8 SOFaa with CS blastocysts when compared with all other IVP treatments (Figures 3.5 and 3.6).

**Transcription Levels in Pre-implantation Embryos**

Transcript levels for oct-4 were detected at significantly higher levels in day 7 KSOMaa with CS cultured IVP blastocysts when compared with KSOMaa cultured and in vivo embryos. There was no significant difference in relative nanog abundance levels across treatment groups. Connexin 43 was detectable in SOFaa, SOFaa with CS, and KSOMaa cultured blastocysts in contrast to in vivo embryos. However, blastocysts cultured in either SOFaa with CS or KSOMaa exhibited significantly higher GLUT-1 transcript levels than their treatment counterparts or in vivo embryos (Figures 3.7 and 3.8).

As in Experiment 1, confidence levels for in vivo embryos were calculated to better interpret the data. A significant amount of KSOMaa and KSOMaa with CS cultured blastocysts displayed upregulation of the oct-4 transcript (88% and 90%, respectively) when compared with in vivo levels. Only 33% of the SOFaa cultured blastocysts and 30% of the SOFaa with CS blastocysts displayed normal levels of nanog. Additionally, 60% of the IVP blastocysts cultured in KSOMaa with CS displayed abnormal levels of nanog. All of the blastocysts cultured in KSOMaa and 60% of those cultured in KSOMaa
Table 3.8  Cell number of blastocysts after 7 days of *in vivo* or *in vitro* culture in KSOMaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>14</td>
<td>36 ± 3.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>88 ± 8.7</td>
<td>1:2.6 ± 0.2</td>
<td>123 ± 11&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KSOMaa + CS</td>
<td>12</td>
<td>48 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102 ± 6.7</td>
<td>1:2.2 ± 0.1</td>
<td>151 ± 9.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>In vivo</td>
<td>10</td>
<td>32 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.3 ± 8.6</td>
<td>1:2.2 ± 0.1</td>
<td>100.8 ± 11.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Statistical differences were determined by One-Way ANOVA (P<0.05).
*TE = Trophectoderm
Data presented as mean ± SEM.
<sup>a,b</sup>Different letters within columns indicate significant difference between treatments.
Table 3.9  Cell number of blastocysts after 7 days of *in vivo* or *in vitro* culture in SOFaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFaa</td>
<td>7</td>
<td>41 ± 5.8</td>
<td>79 ± 6.0</td>
<td>1:2.1 ± 0.3</td>
<td>120 ± 10.2</td>
</tr>
<tr>
<td>SOFaa + CS</td>
<td>7</td>
<td>39 ± 4.1</td>
<td>100 ± 16</td>
<td>1:2.5 ± 0.3</td>
<td>142 ± 19.8</td>
</tr>
<tr>
<td>In vivo</td>
<td>10</td>
<td>32 ± 3.4</td>
<td>69.3 ± 8.6</td>
<td>1:2.2 ± 0.1</td>
<td>100.8 ± 11.9</td>
</tr>
</tbody>
</table>

Statistical differences were determined by One-Way ANOVA (P<0.05)
*TE = Trophoderm
Data presented as mean ± SEM.
No significant differences between treatments.
Table 3.10  Cell number of IVP blastocysts after 8 days of culture in KSOMaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>8</td>
<td>28 ± 2.7</td>
<td>59 ± 5.0</td>
<td>1:2.2 ± 0.2</td>
<td>87 ± 6.8</td>
</tr>
<tr>
<td>KSOMaa + CS</td>
<td>11</td>
<td>25 ± 2.3</td>
<td>45 ± 4.8</td>
<td>1:1.8 ± 0.1</td>
<td>70 ± 6.6</td>
</tr>
</tbody>
</table>

Statistical differences were determined by t-test (P<0.05).
*TE = Trophectoderm
Data presented as mean ± SEM.
No significant differences between KSOMaa treatments.
Table 3.11  Cell number of IVP blastocysts after 8 days of culture in SOFaa with or without CS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ICM</th>
<th>TE*</th>
<th>ICM:TE</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFaa</td>
<td>10</td>
<td>37 ± 2.4</td>
<td>65 ± 4.9</td>
<td>1:2.0 ± 0.2</td>
<td>101 ± 6.1</td>
</tr>
<tr>
<td>SOFaa + CS</td>
<td>11</td>
<td>31 ± 2.5</td>
<td>59 ± 7.0</td>
<td>1:1.9 ± 0.2</td>
<td>90 ± 9.0</td>
</tr>
</tbody>
</table>

Statistical differences were determined by t-test (P<0.05).

*TE = Trophectoderm
Data presented as mean ± SEM.
No significant differences between SOFaa treatments.
Figure 3.5 Differential staining cell numbers for in vivo embryos compared to KSOMaa blastocysts cultured with or without CS for 7 days (dark gray bars) and 8 days (light gray bars). Mean ICM cell number (A), mean trophectoderm (TE) cells (B), mean ICM:TE ratio (C), and mean total cell number (D). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

a,bSignificant differences within treatments between days.
1,2Significant differences between treatments within days.

*Mean values are given as 1:TE cell ratio.
Figure 3.6 Differential staining cell numbers for in vivo embryos compared to SOFaa blastocysts cultured with or without CS for 7 days (dark gray bars) and 8 days (light gray bars). Mean ICM cell number (A), mean trophectoderm (TE) cells (B), mean ICM:TE ratio (C), and mean total cell number (D). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

a,bSignificant differences within treatments between days.

*Mean values are given as 1:TE cell ratio.
Figure 3.7 Average relative transcript levels for *in vivo* embryos (dark gray bars) compared to KSOMaa (light gray bars) blastocysts cultured with or without CS for 7 days. Relative abundance levels for Oct-4 (A), Nanog (B), Connexin 43 (C), and GLUT-1 (D). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

\textit{a,b} Significant differences between treatments.
Figure 3.8 Average relative transcript levels for *in vivo* embryos (dark gray bars) compared to SOFaa (light gray bars) blastocysts cultured with or without CS for 7 days. Relative abundance levels for Oct-4 (A), Nanog (B), Connexin 43 (C), and GLUT-1 (D). Bars indicate SEM. Statistical differences were determined by One-Way ANOVA (P<0.05).

*a,b* Significant differences between treatments.
with CS displayed higher expression levels of GLUT-1 than the in vivo controls. In addition, 91% of the SOFaa with CS blastocysts were significantly upregulated for GLUT-1. Overall, dysregulation of these transcripts was more commonly detected in the KSOMaa treatments than in the SOFaa treated blastocysts (Figure 3.9 and Table 3.12).

**Discussion**

Numerous reports have demonstrated that the in vitro production environment affects the gene expression pattern in the resulting embryos (Wrenzycki *et al.*, 1996, 1999, 2001, 2004; Eckert and Niemann, 1998; Lazzari *et al.*, 2002; Rizos *et al.*, 2002, 2003; Lonergan *et al.*, 2003). This is observed not only when comparing IVP embryos to in vivo embryos but also between IVP systems. Studies examining the effects of various medium components or additives generally focus on the blastocyst rate and possibly on average cell number. While these data points indicate successful development, they fail to accurately imply developmental competence post-transfer of the blastocyst into a recipient. By combining traditional measures of developmental capacity (cleavage and blastocyst rates) with more quantitative measures such as relative mRNA abundance levels, a more detailed description of the consequences of a given culture environment may be apparent. The present study focused on the effects of different culture systems and protein sources on the development of IVP embryos measured by cleavage and blastocyst rates, cell number, and relative abundance levels of oct-4, connexin 43, nanog, and GLUT-1.

It has been stated that embryos have a tremendous capability to compensate for suboptimal culture conditions. Morphologically the blastocysts may appear to develop normally and at the same rate; however, differences may be detected at the transcript level. This was apparent in both experiments. Despite similar cleavage and blastocyst rates among treatments, significant differences were observed at the mRNA level. This
Figure 3.9 Relative transcription levels of IVP blastocysts on day 7 of culture in either SOFaa or KSOMaa with or without CS. Relative differences of Oct-4 (A), Connexin 43 (B), Nanog (C), and GLUT-1 (D) on day 7. Dashed lines represent the 95% confidence intervals of in vivo embryos.
Table 3.12 Number of IVP blastocyst pools cultured in either KSOMaa or SOFaa with or without CS that fall within or out of the 95% CI range for *in vivo* embryo pools.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Comparison to <em>in vivo</em> CI</th>
<th>KSOMaa*</th>
<th>KSOMaa + CS*</th>
<th>SOFaa*</th>
<th>SOFaa + CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>Upregulated</td>
<td>8</td>
<td>9</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Nanog</td>
<td>Upregulated</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Connexin 43</td>
<td>Upregulated</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>4</td>
<td>10</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Upregulated</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Downregulated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note: 11 pools of blastocysts were analyzed for each treatment. However, 2 of the KSOMaa, 1 of the KSOMaa with CS, and 2 of the SOFaa blastocyst pools were rejected due to lack of the poly(A) transcript. Only 10 *in vivo* blastocyst pools were analyzed.*
was in agreement with data collected by Wrenzycki et al. (2001) and others. Cleavage rates also did not differ between media supplemented with BSA or CS, which is in agreement with previous reports (Eckert and Niemann, 1995; Lonergan et al., 1999; Rizos et al., 2003). Differences were evident when comparing cell numbers between treatments in Experiment 1. Differential staining revealed differences among treatments in Experiment 2 for only ICM and total cell number data. Differences in cell number data were observed among treatments in both experiments when comparing day 7 and day 8 blastocysts.

KSOMaa has a significantly higher concentration of potassium and lactic acid than SOFaa. SOFaa has a significantly higher concentration of glucose and an overall higher osmolarity than KSOMaa. These differences in media composition attribute to the differences observed in gene expression levels.

In the first experiment, oct-4 was the only gene found to have a mean transcript level significantly higher in KSOMaa cultured blastocysts when compared to both SOFaa cultured blastocysts and in vivo embryos. Blastocysts cultured in SOFaa either expressed oct-4 at normal levels or were downregulated as related to in vivo embryos. The same pattern of upregulation of oct-4 in KSOMaa or KSOMaa with CS cultured blastocysts was observed in the second experiment. In Experiment 1, KSOMaa cultured blastocysts had a significant increase in ICM cells when compared to in vivo blastocysts, but were not significantly different to ICM cells present in SOFaa cultured blastocysts. Therefore, this upregulation is not exclusively attributed to the additional ICM cells present in KSOMaa and KSOMaa with CS treated blastocysts when compared to both SOFaa and in vivo embryos. The difference in ICM number may be a contributing factor to the increase in oct-4 expression, but it is not the only causative agent. We hypothesize that this increase in oct-4 expression may be due to the differences in media composition. The lower osmolarity level and higher potassium and lactic acid
concentrations present in KSOMaa when compared with SOFaa could be increasing the pluripotency status of the ICM cells through increased transcription rates, thus stimulating oct-4 expression. High osmolarity has been shown to induce osmotic stress in embryos that is thought to alter the expression of various genes. The lower osmolarity level in KSOMaa may relieve osmotic stress in the ICM cells allowing for the increase in oct-4 expression. Additionally, numerous reports have indicated that bovine IVP blastocysts generally express oct-4 variably. The contrasting results we observed between the KSOMaa and SOFaa treatments are in accordance with reports of Li et al. (2005) who stated that IVP embryos can exhibit either upregulation or downregulation of the oct-4 gene, suggesting that the developmental competence of the embryos is already compromised at the blastocyst stage.

Nanog transcription in stem cells appears to be controlled by the synergistic actions of oct-4 and the Sox binding proteins (Yamaguchi et al., 2005). Therefore, we would expect nanog to be expressed in a similar pattern to oct-4. Our results indicate otherwise. Unlike the differences detected between treatment groups in oct-4, there was no significant upregulations or downregulations of nanog in either experiment. This was unexpected since nanog is thought to be ICM specific and differences were observed in oct-4 expression. Due to the lack of variation in nanog expression, we hypothesize that oct-4 would be the better ICM specific candidate gene to analyze responses to in vitro culture environments. Degrelle et al. (2005) found nanog to be expressed in all embryonic and extra-embryonic tissues in later stages of development (12 to 17 days post-insemination), but was restricted to the ICM of earlier stage blastocysts.

mRNAs encoding connexin 43 are first detectable from the 4-cell stage onwards (Wrenzycki et al., 1998) and expression decreases slightly thereafter. Connexin 43 peaks in expression at the compacted morula stage and is somewhat downregulated at the blastocyst stage (Rizos et al., 2002). Previous work has shown that the connexin 43
gene is transcribed only up to the morula stage in IVP bovine embryos whereas the transcript is present in in vivo derived, cocultured, and ewe oviduct cultured bovine morulae and blastocysts (Wrenzycki et al., 1996; Rizos et al., 2002, 2003). However, connexin 43 was not expressed at detectable levels in in vivo embryos analyzed in our studies. It is well accepted that in vivo derived embryos collected at a particular time can vary in development as much as 12-24 h or even greater. In vivo embryos collected in our studies varied in development from the early morula stage to the late blastocyst stage. Due to the variability in development, we propose that connexin 43 expression would also be variable or possibly faulty. As expected, connexin 43 was not detected in IVP blastocysts used in Experiment 1. Conversely, connexin 43 was detected in KSOMaa, SOFaa, and SOFaa with CS cultured blastocysts in Experiment 2. These variable results and the lack of connexin 43 in our in vivo embryos are likely due to differences in relation to time of development. Q-PCR measures relative abundance levels at a specific time point. Different results could be expected if embryos were collected as little as a few hours earlier or later. Our results indicate that the presence of serum in IVP embryos significantly downregulated the connexin 43 gene. This concurs with Lonergan et al. (2003) who reported that connexin 43 was altered in vitro by the presence of serum, disappearing at the 8- to 16-cell stage and reappearing at the hatched blastocyst stage. Conversely, Wrenzycki et al. (1998) reported that connexin 43 was not affected by the addition of either serum or BSA. The variable reports indicate that connexin 43 is a sensitive candidate gene to be studied as a marker for determining the quality of an in vitro culture system.

GLUT-1 increases in expression from the 8-16-cell stage onward coinciding with the point in development in which embryos become dependent on glucose as an energy source. Niemann and Wrenzycki (2000) and Wrenzycki et al. (2001) found that the relative levels of the GLUT-1 transcript were higher in in vivo embryos than IVP
embryos. Our data does not agree with these reports as in vivo embryos analyzed expressed low levels of the transcript. This discrepancy is likely due to the variation in embryonic development observed in the in vivo embryos collected in our experiments. The low in vivo cell numbers are possibly responsible for the low GLUT-1 abundance levels observed in the in vivo embryos. The addition of serum to culture media significantly upregulates GLUT-1 in IVP embryos (Wrenzycki et al., 1999). In contrast, de Oliveira et al. (2006) did not detect any significant differences among in vivo and IVP embryos supplemented with different protein sources and serum concentrations. Data presented in this study demonstrates that blastocysts cultured in SOFaa with CS have a significant upregulation of GLUT-1 when compared to other treatments and in vivo embryos. As stated before, GLUT-1 transports glucose into embryonic cells and SOFaa medium has a higher concentration of glucose than KSOMaa medium. Therefore, we hypothesize that the upregulation of GLUT-1 in SOFaa with CS is attributed to both the high glucose content present in SOFaa and the stimulatory effects of CS. KSOMaa cultured IVP blastocysts also expressed GLUT-1 at significantly higher levels. In all treatments analyzed in Experiment 2, the majority of blastocysts expressed GLUT-1 at higher levels than the in vivo control range. Therefore, we hypothesize that under certain in vitro conditions embryos can be produced with high levels of GLUT-1 transcript. It should also be noted that we observed variation in the expression of GLUT-1 both between treatments and between replicates within treatments. This suggests that the lack of statistical significance in some treatments (specifically KSOMaa in the first experiment) could be due to greater variation between embryo pools. Due to variable results reported by our group and others when analyzing GLUT-1, this transcript should be further investigated.

In most experiments measuring the effects of the in vitro culture environment on development a control, usually a “normal” embryo, is used. We generally accept in vivo
derived embryos as “normal.” However, not all in vivo embryos collected (generally on day 7) are “normal”. If embryos are flushed from the cow at day 7 and transferred to recipient cows not all embryos transferred will make it to term. In fact, pregnancy rates from transferred in vivo derived embryos can range anywhere between 50 to 75% (Farin et al., 1999). As stated before, in vivo derived embryos collected at a particular time can vary in development as much as 12 to 24 h or greater. IVP embryos are precisely timed off IVF. Our cell number data demonstrates that IVP blastocysts have a greater number of total cells than in vivo blastocysts. This is likely due to differences in development between day 7 IVP and in vivo embryos. This difference exposes the problem in comparing IVP with in vivo embryos in regards to the effects of culture conditions. Our data indicates that not all in vivo derived embryos are equal in developmental status and some may not be viable in early stage embryonic development. Nevertheless, in vivo embryos are the accepted standard because they are as close to “normal” as we can get. It is also of common practice to report IVP blastocyst rates after 8 days of in vitro culture. We clearly demonstrate that blastocysts not developing until day 8 of culture are not equal to blastocysts developing after only 7 days of culture. In both experiments, differences were observed in cell numbers between day 7 and day 8 IVP blastocysts irrespective of culture treatment. Day 8 blastocysts had significantly lower cell numbers than day 7 blastocysts. It is likely that these day 8 blastocysts are not viable. Therefore, by reporting blastocyst rates after 8 days of in vitro culture, a higher rate is achieved; however, this rate does not accurately reflect viable blastocysts.

Our results indicate that the transcript levels of the majority of the genes analyzed were significantly altered by the in vitro culture condition. We and others (Wrenzycki et al., 2001; Lonergan et al., 2006) hypothesize that bovine embryos respond to alterations in their environment by modifying the expression levels of several developmentally important transcripts. The findings of this study not only have
applications to the field of IVP in animals, but also to human assisted reproduction technologies. To ensure the delivery of normal and healthy offspring, research must be conducted to improve the *in vitro* culture environment. The basic mechanisms that take place during the pre-implantation period and the effects that culture components may have on the resulting embryo are still unknown. Differences continue to be found between *in vitro* cultured and *in vivo* embryos, and until these differences are minimized, aberrations in *in vitro* development will continue to be observed. Further research will possibly modify the current culture conditions during this critical period of development allowing for the production of *in vitro* embryos of higher developmental potential similar to that noted in *in vivo* derived embryos.
Numerous studies have reported aberrant gene expression levels attributed to suboptimal or even nonphysiological in vitro culture conditions presented to embryos (Wrenzycki et al., 1996, 1999, 2001, 2004; Eckert and Niemann, 1998; Lazzari et al., 2002; Rizos et al., 2002, 2003; Lonergan et al., 2003). Current culture systems can also lead to either persistent silencing or enhanced expression of a particular gene throughout preimplantation development (Wrenzycki et al., 2001). Lonergan et al. (2003) indicated that any modifications to the in vitro culture environment can have profound effects on the quality of the resulting embryos measured in terms of cryotolerance and relative transcript abundance. Since the culture environment is the common aspect of both in vitro production (IVP) and nuclear transfer (NT), research focusing on the in vitro culture system will not only better IVP but NT also. However, little thought is often given to what implications could arise from using these artificial technologies in terms of its effects on gene expression.

The present studies focused on the effects of different culture systems and protein sources on the developmental competence of IVP embryos measured by cleavage and blastocyst rates, cell number, and relative abundance levels of oct-4, nanog, connexin 43, and GLUT-1. All data points were compared with those found in in vivo embryos. Experiment 1 compared IVP embryos cultured in either synthetic oviductal fluid (SOFaa) or potassium simplex optimized medium (KSOMaa) supplemented with amino acids. Experiment 2 compared the same two culture systems with and without the addition of calf serum (CS). Results from both experiments indicated that despite similar cleavage and blastocyst rates among treatments, significant differences were found at the mRNA level. In addition, differences were evident when contrasting cell number data between treatments in Experiment 1. Differential staining revealed differences among treatments in Experiment 2 for only ICM and total cell number data.
In the first experiment, oct-4 was the only transcript found to have a mean abundance mRNA level significantly higher in KSOMaa cultured blastocysts when compared to both SOFaa cultured blastocysts and \textit{in vivo} embryos. As expected, the same pattern of upregulation of oct-4 in KSOMaa or KSOMaa with CS cultured blastocysts was observed in the second experiment. Unlike the differences detected between treatment groups in oct-4 expression, there was no significant upregulations or downregulations of the ICM specific transcript nanog in either experiment. In contrast to reports by others (Wrenzykci \textit{et al.}, 1996; Rizos \textit{et al.}, 2002, 2003), connexin 43 was not expressed at detectable levels in \textit{in vivo} embryos analyzed in our studies. As expected, connexin 43 was not detected in IVP blastocysts used in Experiment 1. Conversely, connexin 43 was detected in KSOMaa, SOFaa, and SOFaa with CS cultured blastocysts in Experiment 2. These variable results indicate that connexin 43 is a sensitive candidate gene to be studied as a marker for determining the quality of an \textit{in vitro} culture system. \textit{In vivo} embryos analyzed expressed low levels of GLUT-1 contrary to other reports. Data presented in this study also demonstrated that blastocysts cultured in SOFaa with CS had a significant upregulation of GLUT-1 when compared with other treatments and \textit{in vivo} embryos. Additionally, KSOMaa cultured IVP blastocysts also expressed GLUT-1 at significantly higher levels. Due to variable results observed by our group and others when analyzing GLUT-1, this transcript should also be further investigated. The variable results obtained in these studies are likely the effects of the inconsistency in expression levels observed with relation to time of development. Q-PCR measures relative abundance levels at a specific time point. Different results could be expected if embryos were collected as little as a few hours earlier or later.

Our cell number data demonstrates that IVP blastocysts have a greater number of total cells than \textit{in vivo} blastocysts, in contrast to other reports. This is likely due to differences in development between day 7 IVP and \textit{in vivo} embryos. This difference exposes the problem in comparing IVP with \textit{in vivo} embryos in regards to the effects of culture conditions. We also clearly demonstrate that blastocysts not developing until day 8 of culture are not equal to
blastocysts developing after only 7 days of culture. In both experiments, day 8 blastocysts had significantly lower cell numbers than day 7 blastocysts. It is likely that these day 8 blastocysts are not viable. Therefore, by reporting blastocyst rates after 8 days of *in vitro* culture, a higher rate is achieved; however, this rate does not accurately reflect viable blastocysts.

By combining traditional measures of developmental capacity (cleavage and blastocyst rates) with more quantitative measures such as relative mRNA abundance levels, a more detailed description of developmental competency is achievable. Our results indicate that the transcript levels of the majority of the genes analyzed were significantly altered by an *in vitro* culture condition. The findings of this study not only have applications to the field of IVP in animals but also to human assisted reproduction technologies. The basic mechanisms that take place during the preimplantation period and the effects that culture components may have on the resulting embryo are still unknown. Differences continue to be observed between *in vitro* cultured and *in vivo* embryos, and until these differences are minimized, aberrations in *in vitro* development will continue to be observed. Further research will possibly modify the current culture conditions during this critical period of development allowing for the production of *in vitro* embryos of higher developmental potential similar to that observed in *in vivo* derived embryos.
REFERENCES


http://pathmicro.med.sc.edu/pcr/realtime-home.htm


APPENDIX A: PROTOCOLS

BOVINE IVF PROTOCOL

Preparations:

1. Move PHE (100 μl) (Appendix B) and Heparin (100 μl) (Appendix B) from freezer to oven (39°C). PHE should be covered with aluminum foil (light sensitive).

2. Move two centrifuge carriers to oven (39°C).

3. Make fertilization microdrops
   a. Make five 44 μl drops of IVF-TALP in each 35mm dish. Cover with pre-warmed and pre-gassed mineral oil.
   b. Equilibrate in CO₂ incubator (39°C) at least 2 h.


5. Fill 1 conical tube with ~20 ml HEPES-TALP (Appendix B). Label tube.


7. Transfer tube of HEPES-TALP (cap tight) and SP-TALP (cap tight) to the 39°C oven.

8. Transfer IVF-TALP (cap loose) to CO₂ incubator.

9. Prepare Percoll gradient:
   a. Label 1 conical tube “Percoll gradient” and fill the tube with 100 μl of Sperm-TL and 900 μl of ENHANCE-S Plus.

10. Carefully, transfer Percoll gradient to the pre-warmed centrifuge carrier within the oven.

Procedures:

1. At 22-24 h post-maturation thaw 1 straw of semen in water at 39°C for 30 seconds. When getting semen straws out of the liquid nitrogen tank, make sure not to raise anything above the frost line. Use special semen forceps.
2. Dry a straw, hold it in a kimwipe to keep it warm and dark, cut the sealed end off and slowly layer thawed semen on top of the Percoll gradient by gently pushing the plug in the straw with a metal rod. Place the conical tube back into the centrifuge carrier and centrifuge at 1200 rpm for 20 min.

3. Check viability of the thawed semen by placing a drop remaining in the straw on a slide. View at 40X magnification to assure that motile sperm are present.

4. While centrifuge is running, pour 2 ml of HEPES-TALP (from conical tubes in CO₂ oven) into Petri dish (35 mm). Remove oocytes from each well of OMM plate/vial and transfer to a separate corner in the HEPES-TALP. Thoroughly wash oocytes through 2 dishes of HEPES-TALP to remove OMM, which is detrimental to fertilization.

5. Transfer up to 10 oocytes into each 44 μl fertilization drop or 425 μl in 4-well dish (IVF-TALP, previously located in the incubator). Return IVF plate back to incubator when finished. *You only have 15 min to wash and transfer all oocytes to IVF 4-well plates. Set a timer and ask for help if necessary.

6. After centrifuge stops, carefully remove carrier with the Percoll gradient from centrifuge. There should now be a sperm pellet, if not you must start completely over with new gradient and semen.

7. Within the laminar flow hood, aspirate the Percoll down to the sperm pellet. Slowly add the 5 ml of pre-warmed Sperm-TALP to the conical tube containing the sperm pellet. Transfer the tube to the second pre-warmed centrifuge carrier and centrifuge at 1200 rpm for an additional 10 min.

8. After the centrifuge stops, aspirate the Sperm-TALP down to the sperm pellet. Return the conical tube with the sperm pellet to the oven.

9. Determine Sperm Pellet Concentration
A. Gently swirl the sperm pellet to mix the sperm with any remaining medium. Use a clean pipette tip to transfer 5 μl of sperm into 95 μl of water, pipetting gently to mix.

B. Clean the hemocytometer and coverslip by washing with water followed by 70% EtOH; dry with a Kimwipe.

C. Using a new pipette tip, transfer 10 μl of diluted sperm into each chamber (each side) of the hemocytometer.

D. Use 40X magnification to count sperm cells in the 5 squares arranged diagonally across the central square on one side of the hemocytometer. Use an event counter to keep track of how many cells are counted. Record the count.

E. Continue counting on the second side of the hemocytometer counting 5 diagonally arranged squares to obtain the total hemocytometer count. If the count of one side varies more than 10% from the other side, then the diluted sample was not properly mixed. Repeat procedure starting at step 1. When the count is consistent, record the total count and continue procedure.

F. Clean hemocytometer and coverslip with water followed by EtOH.

10. Preparing sperm suspension for insemination (See “Explanation of Sperm Suspension”)

Note: The final sperm suspension used to IVF is composed of fertilization medium and sperm pellet produced by Percoll separation. A worksheet is attached and can be duplicated and used to assist in calculating sperm suspensions. The calculations on the worksheet are modified from those listed
below, although the equations may appear different, they will yield identical results. Following the worksheet will simplify this procedure.

A. Calculations are based on the following parameters:
   a. 300 μl of final sperm suspension will be prepared
   b. 1 x 10^6 sperm/ml is desired in the final fertilization medium
      (this concentration can be adjusted if needed using Step 3 below)

B. Calculate the volume of sperm pellet needed per 300 μl of final sperm suspension using the formula:
   \[ \frac{7,500}{X} = \mu l \text{ of sperm pellet to make 300 } \mu l \text{ of final sperm suspension} \]
   when inseminating with 1 x 10^6 sperm/ml
   When X is the average hemocytometer count (total hemocytometer count divided by 2)

C. Adjust for desired sperm concentration: If a concentration other than 1 x 10^6 sperm/ml is desired, then the volume of the sperm pellet must be adjusted to accommodate that difference. To adjust this volume perform the following calculation:
   \[ \frac{\text{Sperm concentration desired}}{1 \times 10^6 \text{ sperm/ml}} = \text{sperm concentration adjustment factor} \]
   Multiply the volume of the sperm pellet calculated in Step 2 by this adjustment factor to yield the volume sperm pellet needed to prepare 300 μl of final sperm suspension at the desired concentration.
   Example: If a bull requires are 2 x 10^6 sperm/ml rather than 1 x 10^6 sperm/ml
   \[ \frac{2 \times 10^6 \text{ sperm/ml}}{1 \times 10^6 \text{ sperm/ml}} = \text{adjustment factor of 2} \]
(Adjustment factor) \times (\mu l \text{ of pellet needed for } 1 \times 10^6 \text{ sperm/ml}) = \mu l \text{ of sperm pellet needed to yield } 2 \times 10^6 \text{ sperm/ml in } 300 \mu l \text{ of final sperm suspension providing } 2 \times 10^6 \text{ sperm/ml in the fertilization drop.}

D. Calculate volume of fertilization medium needed in the final sperm suspension: Subtract the volume found in Step 3 from 300 \mu l from Step 1.

E. Place the calculated amount of fertilization medium (Step 4) into and Eppendorf microcentrifuge tube. Then add the calculated amount of sperm pellet (Step 3) to the tube. Sperm stick to plastic, so add the fertilization medium to the tube first. Mix gently by pipetting up and down several times within the tube. Immediately begin fertilizing the drops since the pH of this solution will change rapidly.

11. Fertilization

A. Add 2 \mu l heparin (for a final concentration of 2 \mu g/ml of heparin in the fertilization medium), 2 \mu l of PHE and 2 \mu l of final sperm suspension to each drop. If 4-well dish was used, add 20 \mu l heparin, 20 \mu l PHE and 20 \mu l sperm.

B. Record time and date on each fertilization dish.

C. Incubate for 17 to 18 h at 39°C in a humidified atmosphere of 5% CO₂ in air.
EXPLANATION OF SPERM SUSPENSION CALCULATIONS

Sperm concentrations recommended for IVF refer to the final concentration of sperm present in the fertilization well or drop after the sperm suspension has been added. Calculation of sperm concentrations made using the assumption that a concentration yielding $1 \times 10^6$ sperm/ml is desired in the final sperm suspension. Once the calculations are performed, the values can be adjusted for the actual concentration prescribed for a particular bull.

To make a $1 \times 10^6$ sperm/ml concentration, you first must find out how many sperm are needed for a 500 µl well ($425 \mu l$ Fertilization Medium + $20 \mu l$ heparin + $20 \mu l$ PHE + $20 \mu l$ of sperm suspension + $15 \mu l$ to move eggs into well).

$$\frac{1 \times 10^6 \text{ sperm}}{1000 \mu l} = \frac{X}{500 \mu l \text{ well}}$$

$$X = 500,000 \text{ sperm needed in each 500 } \mu l \text{ well}$$

You want to deliver 500,000 sperm into your well using a volume of $20 \mu l$ of sperm suspension. The concentration of sperm needed in the final sperm suspension is:

$$\frac{500,000 \text{ sperm}}{20 \mu l} = 25,000 \text{ sperm/} \mu l \text{ of final sperm suspension}$$

To make $300 \mu l$ of final sperm suspension, the total number of sperm cells needed is:

$$\frac{25,000 \text{ sperm}}{\mu l} \times 300 \mu l = 7.5 \times 10^6 \text{ sperm cells}$$

The value derived from the hemacytometer count and calculations (Step H of SOP: Hema) results in a value of Average Hemacytometer Count (AHC) $\times 10^6$ sperm/ml of sperm pellet which is the same as $\text{AHC} \times 10^3$ sperm/µl of sperm pellet.

To determine the volume of pellet needed to make the final sperm suspension the following equation is solved:

$$(X \text{ } \mu l \text{ of sperm pellet}) \times (\text{AHC} \times 10^3 \text{ sperm/} \mu l) = 7.5 \times 10^6 \text{ sperm cells}$$

Solve for X:

$$X = \frac{7.5 \times 10^6 \text{ sperm}}{\text{AHC} \times 10^3 \text{ sperm/} \mu l} = 7.5 \times 10^3 \mu l = 7,500 \mu l \text{ of sperm pellet}$$
SPERM DILUTION WORKSHEET

Date: __________________ Sire: ____________________ Dam: __________________

Step 1. Sperm concentration required: __________________________

Step 2. Total Sperm count = Chamber 1 _____ & Chamber 2 _____ = ________

Step 3. Total Sperm count (Step 2) ______ + 2 = Ave. hemacytometer count______

Step 4. Determine volume of sperm pellet required using one of the following equations (depending upon Step 1).
   
   If $2 \times 10^6$ sperm/ml desired: $15,000 + _____$ (from Step 3) = ________µl
   
   If $1 \times 10^6$ sperm/ml desired: $7,500 + _____$ (from Step 3) = ________µl
   
   If $.75 \times 10^6$ sperm/ml desired: $5,625 + _____$ (from Step 3) = ________µl
   
   If $.5 \times 10^6$ sperm/ml desired: $3,750 + _____$ (from Step 3) = ________µl

Step 5. $300 - _____$ (from Step 4) = ________ µl of fertilization medium required.

Pipette (from Step 5) ______ µl fertilization medium into an Eppendorf tube and add (from Step 4) ______µl sperm pellet. Total volume will equal 300µl.
DIFFERENTIAL STAINING PROTOCOL

1. Wash day 7 or day 8 embryos in dPBS with Ca and Mg.

2. Incubate the embryos with 10 µl of Hoechst stock solution (Appendix B) plus 500 µl dPBS with Ca and Mg for 10 min.

3. Permeabilize the membranes with 500 µl of 0.04% Triton X-100 (Appendix B) solution for 1 min.

4. Incubate the embryos with 20 µl of PI stock solution (Appendix B) plus 500 µl of dPBS with Ca²⁺ and Mg²⁺ for 45 sec.

5. Wash embryos in 500 µl of dPBS with Ca²⁺ and Mg²⁺.

   Note: Steps 2-5 can be performed in a 4-well dish.

6. Mount the embryos onto a glass microscope slide in a drop of 25% glycerol.

7. Gently flatten the embryos with a coverslip.

8. Count number of cells using an epifluorescent microscope equipped with an UV filter cube.
**SOMATIC CELLS RNA ISOLATION PROTOCOL**

1. Store cells in the minimum possible amount of PBS plus 0.1% polyvinyl alcohol in 1.5 ml siliconized tubes.

2. Bring Dynabeads mRNA® DIRECT™ Kit (Dynal Biotech, Inc., Lake Success, NY, USA, Cat No. 610.11) to room temperature.

3. Lyse the embryos in 1250 μl of lysis/binding buffer (100 mM Tris HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, 5 mM dithiothreitol).

4. Shear RNA with 21 g needle 3 to 5 times using a 1 to 2 ml syringe and vortex for 10 sec.

5. Add 250 μl of pre-washed oligo dT Dynabeads (dT25) to the sample. Pre-wash beads in lysis/binding buffer.

6. Incubate the Dynabeads and sample by rotating on a mixer or roller for 10 min at room temperature.

7. Place the tubes in a magnetic separator for 2 min.

8. After removal of the supernatant, wash the beads two times with 1 ml of buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% lithium dodecylsulfate) and three times with 1 ml of buffer B (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA).

9. Elute the RNA from the beads by adding 11 μl of sterile water and heating the sample at 75°C for 2 min.

10. Use the sample directly for reverse transcription.
EMBRYO RNA ISOLATION PROTOCOL

1. Store pools of embryos (10 embryos per pool for Experiment 1 and 5 embryos per pool for Experiment 2) in approximately 3 μl of PBS plus 0.1% polyvinyl alcohol in 1.5 ml siliconized tubes.

2. Bring Dynabeads mRNA® DIRECT™ Kit (Dynal Biotech, Inc., Lake Success, NY, USA, Cat No. 610.11) to room temperature.

3. Lyse the embryos in 150 μl of lysis/binding buffer (100 mM Tris HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, 5 mM dithiothreitol) and vortex for 10 sec.

4. Centrifuge the samples at maximum speed for 15 sec and incubate at room temperature for 10 min.

5. Add 10 μl of pre-washed oligo dT Dynabeads (dT25) to the sample. Pre-wash beads in lysis/binding buffer.

6. Incubate the Dynabeads and sample by rotating on a mixer or roller for 10 min at room temperature.

7. Place the tubes in a Dynal MPC-E-1 magnetic separator for 2 min.

8. After removal of the supernatant, wash the beads once with 100 μl of buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% lithium dodecylsulfate) and three times with 100 μl of buffer B (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA).

9. Elute the RNA from the beads by adding 11 μl of sterile water and heating the sample at 75°C for 2 min.

10. Use the sample directly for reverse transcription.

cDNA SYNTHESIS PROTOCOL

1. Mix 4 μl of iScript reaction mix, 1 μl of reverse transcriptase, 4 μl of nuclease-free water (Appendix B) (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat No. 170-8891).

2. Make master mixers when possible.

3. Add 11 μl of mRNA sample.

4. Extra mix should be prepared for the no mRNA template negative control.

5. Total volume mix should be 20 μl.

6. Place the mix in the thermocycler.

7. Run the thermocycler at 25°C for 5 min, 42°C for 30 min, denaturation at 85°C for 5 min, and a final hold at 4°C.
RT-PCR PROTOCOL

1. Mix 5 µl of 10X PCR buffer, 1 µl dNTP mix (Appendix B), 2 µl Jump Start™ REDTaq™ DNA Polymerase (Sigma-Aldrich, Inc., St. Louis, MO, USA, Cat. No. D-8187) and 35 µl of water. Make a bigger volume for multiple samples when necessary (Master Mix-MMM; Appendix B).

2. Add 1 µl of each primer (20 pmol) to the MMM. Make a bigger volume for multiple samples when necessary (Master Mix-MM; Appendix B).

3. Add 5 µl of the cDNA sample to the MM (RT-PCR Mix; Appendix B).

4. Save enough MMM and MM for the negative control (no cDNA).

5. Place the samples in the thermocycler.

6. Run one cycle of 1 min at 95°C; 35 cycles of PCR (95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec); followed by 72°C for 4 min; with a final hold at 4°C.
Q-PCR PROTOCOL

1. Mix 12.5 μl of iQ SYBR™ Green 2X Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat. No. 170-8882), 4 μl of cDNA/Calibrator/H₂O for NT control, 6.5 μl of water (Reaction Mix; Appendix B) for each well.

2. A master mix per cDNA sample/Calibrator/H₂O for NT control enough for all 5 genes should be formulated (Appendix B).

3. Prepare enough master mix to run negative controls (no cDNA) for each gene analyzed.

4. Add 1 μl of each primer (20 pmol) (Appendix B) to appropriate wells.

5. Place the sample in the thermocycler.

6. Run one cycle of 3 min at 95°C; 40 cycles of PCR (95°C for 10 sec and 55°C for 45 sec); a melting curve consisting of 95°C for 1 min followed by 55°C for 1 min, a step cycle with 80 repeats starting at 55°C for 10 sec with a +0.5°C/sec transition rate; and a final hold at 4°C.
GENE EXPRESSION QUANTIFICATION PROTOCOL

1. Use the method described by Pfaffl (2001).

2. Use a mix of cDNA from fibroblast cells, plasmids (Appendix A) and embryos as the calibrator for the target genes. The same calibrator mix should be used throughout all the experiments and plates.

3. Use Poly A as the endogenous control gene.

4. Use the signal of the reference gene Poly A to normalize the target gene signals of each sample.

5. Calibrate the $\Delta C_T$ for gene transcription against the sample used as calibrator.

6. Report gene quantification as relative transcription or the n-fold difference relative to a calibrator.

7. Calculate the relative linear amount of target molecules relative to the calibrator by using the following equation.

$$n\text{-fold difference} = \frac{\text{Efficiency Target Gene}^{\Delta C_T}}{\text{Efficiency Reference Gene}^{\Delta C_R}}$$

8. Efficiencies are obtained via a 10-fold dilution standard curve performed prior to analysis.

9. Calculate the $\Delta C_T$ value by subtracting the sample $C_T$ value of the target gene from the calibrator $C_T$ value of the target gene.

10. Calculate the $\Delta C_T$ value by subtracting the sample $C_T$ value of the reference gene (Poly A) from the calibrator $C_T$ value of the reference gene.
PLASMID FORMULATIONS

1. cDNA from PCR analysis was run on a 2% agarose gel. The PureLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA, Cat. No. K2100-12) was used to isolate and obtain the cDNA from the gel. A portion of the purified cDNA was used for sequence analysis.

   a. Cut the area of the gel containing the desired cDNA fragment using a clean, sharp blade while minimizing the amount of agarose collected around the sample.

   b. Weigh the gel slice and add 30 μl of Gel Solubilization Buffer for every 10 mg of gel.

   c. Incubate the tube at 50°C for 15 min. Pipette the mixture every 3 min to ensure gel dissolution. After the gel slice has disappeared, incubate for an additional 5 min.

   d. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from step c onto the column.

   e. Centrifuge at >12,000 X g for 1 min. Discard the flow-through.

   f. Add 500 μl Gel Solubilization Buffer to the column. Incubate at room temperature for 1 min. Centrifuge at >12,000 X g for 1 min. Discard the flow-through.

   g. Add 700 μl Wash Buffer with ethanol to the column and incubate at room temperature for 5 min. Centrifuge at >12,000 X g for 1 min. Discard the flow-through.

   h. Centrifuge the column at >12,000 X g for 1 min to remove any residual buffer. Place the column into a 1.5 ml recovery tube.
i. Add 50 μl warm (65-70°C) TE Buffer to center of cartridge. Incubate at room temperature for 1 min and centrifuge at >12,000 X g for 2 min. Store cDNA at -20°C.

2. The TOPO® XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA, Cat. No. K4750-20) was used to clone the cDNA by generating a plasmid vector.

**TOPO® Cloning Reaction:**

a. Set up a cloning reaction in a sterile microcentrifuge tube containing 4 μl gel-purified long PCR product and 1 μl pCR®-XL- TOPO® vector.

b. Mix gently and incubate for 5 min at room temperature (~25°C). Do not exceed 5 min!

c. After the 5 min incubation, add 1 μl of the 6X TOPO® Cloning Stop Solution and mix for several seconds at room temperature.

d. Briefly centrifuge the tube and immediately place on ice.

**One Shot® Chemical Transformation:**

a. Add 2 μl of the TOPO® Cloning reaction into a vial of One Shot® cells and mix gently. Do not pipette!

b. Incubate on ice for 30 min and then heat shock cells for 30 sec at 42°C without shaking.

c. Immediately transfer the tubes to ice and incubate for 2 min.

d. Add 250 μl of room temperature S.O.C. medium.

e. Cap the tube tightly and shake horizontally at 37°C for 1 h.

f. Spread 50 to 150 μl from each transformation onto prewarmed LB agar plates containing 50 μg/ml kanamycin.

g. Incubate the plates overnight at 37°C.

h. An efficient reaction should produce several hundred colonies.
3. The plasmid was isolated from a cell culture using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA, Cat. No. K2100-11). Before starting, preheat an aliquot of TE Buffer to 65-70°C for elution step.

   a. Pellet 1 to 5 ml of an overnight culture and thoroughly remove all medium from the cell pellet.

   b. Completely resuspend the pellet in 250 μl Resuspension Buffer with RNase A. No cell clumps should remain.

   c. Add 250 μl Lysis Buffer to cells. Mix gently by inverting the capped tube 5 times. Do not vortex.

   d. Incubate the tube for 5 min at room temperature. Do not exceed 5 min.

   e. Add 350 μl Precipitation Buffer and mix immediately by inverting the tube until the solution is homogeneous. Do not vortex.

   f. Centrifuge the mixture at ~12,000 X g for 10 min at room temperature to clarify the lysate from the lysis debris.

   g. Load the supernatant from step f onto a spin column.

   h. Place the spin column with supernatant into a 2 ml Wash Tube.

   i. Centrifuge at ~12,000 X g for 1 min and discard the flow-through.

   j. Add 500 μl Wash Buffer with ethanol to the column. Incubate for 1 min at room temperature, centrifuge at ~12,000 X g for 1 min, and discard the flow-through.

   k. Add 700 μl Wash Buffer with ethanol to the column and centrifuge the column at ~12,000 X g for 1 min discarding the flow-through.

   l. Centrifuge the column at ~12,000 X g for 1 min to remove any residual Wash Buffer. Discard the Wash Tube with the flow-through.

   m. Place the spin column in a clean 1.5 ml recovery tube.

   n. Add 75 μl preheated TE Buffer to the center of the column and incubate the column for 1 min at room temperature.
o. Centrifuge at \(12,000 \times g\) for 2 min

p. The recovery tube will contain the purified plasmid.

4. A Qiagen QIAfilter Plasmid MidiKit (QIAGEN, Inc., Valencia, CA, USA, Cat. No. 12243) was used to isolate a higher yield of plasmid.

a. Pellet 100 ml of an overnight culture by centrifugation at 6000 \(X\) g for 15 min at 4°C and thoroughly remove all medium from the cell pellet.

b. Resuspend the bacterial pellet in 4 ml Buffer P1 supplemented with RNase A and LyseBlue. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

c. Add 4 ml of Buffer P2 and mix thoroughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature (15 to 25°C) for 5 min. Do not vortex and do not allow the reaction to exceed 5 min! If LyseBlue has been added to Buffer P1, the reaction will turn blue after the addition of Buffer P2.

d. Add 4 ml of chilled Buffer P3 to the lysate and mix immediately and thoroughly by vigorously inverting 4 to 6 times. The solution will precipitate and the precipitated material will contain genomic DNA, proteins, cell debris, and SDS. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated. Do not incubate the lysate on ice, proceed immediately!

e. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature (15 to 25°C) for 10 min. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer at the top of the solution. Do not insert the plunger!

f. Equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT, and allow the column to empty by gravity flow.
g. Remove the cap from the QIAfilter Cartridge outlet nozzle and gently insert the plunger into the QIAfilter Midi Cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip.

h. Allow the cleared lysate to enter the resin by gravity flow.

i. Wash the QIAGEN-tip with 10 ml Buffer QC twice.

j. Elute DNA with 5 ml of Buffer QF into a 15 ml tube.

k. Precipitate DNA by adding 3.5 ml room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 5000 X g for 60 min at 4°C. Carefully decant the supernatant.

l. Wash the DNA pellet with 2 ml room temperature 70% ethanol, and centrifuge at 5000 X g for 60 min at 4°C. Carefully decant the supernatant without disturbing the pellet.

k. Air dry the pellet for 5 to 10 min, and redissolve the DNA in a suitable volume of TE buffer (pH 8.0).
IN VIVO EMBRYO PRODUCTION – SYNCHRONIZATION AND SUPEROVULATION

1. Administer an injection of 15 mg progesterone and 2.5 mg estradiol-17β intramuscular (IM) at CIDR insertion (day 0), or start of treatment to each donor.

2. Administer IM Follitropin V FSH injections (20 mg/ml) to each donor as follows:

   Day 4:
   - A.M. 2.2 ml
   - P.M. 2.0 ml

   Day 5:
   - A.M. 2.0 ml
   - P.M. 1.8 ml

   Day 6:
   - A.M. 1.6 ml
   - P.M. 1.2 ml

   Day 7:
   - A.M. 1.0 ml

   **Total FSH Dose: 11.8 ml**

3. Administer Lutalyse PGF$_{2α}$ injections (5 mg/ml) IM to each donor on day 6 of treatment.

4. On the P.M. of day 6 of treatment remove the CIDR inserts.

5. Check for estrus and give estradiol-17β at time of AI (AI is performed 12 and 24 hours post standing estrus).

6. Nonsurgically collect embryos from donors on day 7 post AI.
## APPENDIX B: MEDIA FORMULATIONS AND STOCK SOLUTIONS

### PHE STOCK\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM Hypotaurine</td>
<td>H-1384</td>
<td>Sigma</td>
<td>5 ml</td>
</tr>
<tr>
<td>2 mM Penicillamine</td>
<td>P-4875</td>
<td>Sigma</td>
<td>5 ml</td>
</tr>
<tr>
<td>250 mM Epinephrine</td>
<td>E-1635</td>
<td>Sigma</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.9% Saline</td>
<td></td>
<td></td>
<td>8 ml</td>
</tr>
</tbody>
</table>

\(^1\)Prepare primary stocks of 1 mM hypotaurine (Sigma H-1384) (1.09 mg/10 ml saline), 2 mM penicillamine (Sigma P-4875) (3 mg/10 ml saline) and 250 mM epinephrine (Sigma E-1635) [1.83 mg/ 40 ml of the following solution (165 mg 60% Na lactate syrup, 50 mg Na metabisulfite (Sigma S-9000) and 50 ml water]. Epinephrine is easily oxidized by direct light so take precautions to avoid this problem (wrap in aluminum foil or place in dark container). Sterile filter and aliquot 100 \(\mu\)l into sterile 0.5 ml microcentrifuge tubes. Store in a light resistant container at -20°C indefinitely.

### HEPARIN STOCK\(^1\)

<table>
<thead>
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<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>H-3149</td>
<td>Sigma</td>
<td>1 mg</td>
</tr>
<tr>
<td>0.9% Saline</td>
<td></td>
<td></td>
<td>20 ml</td>
</tr>
</tbody>
</table>

\(^1\)Sterile filter and aliquot 100 \(\mu\)l into sterile 0.5 ml microcentrifuge tubes. Store at -20°C indefinitely.
### SPERM – TL

<table>
<thead>
<tr>
<th>Component</th>
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<th>Company</th>
<th>Final (mM)</th>
<th>mg/100 ml</th>
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</thead>
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<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>100</td>
<td>582</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
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<td>23</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-5761</td>
<td>Sigma</td>
<td>25</td>
<td>209</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>S-5011</td>
<td>Sigma</td>
<td>0.29</td>
<td>3.48</td>
</tr>
<tr>
<td>HEPES</td>
<td>H-3375</td>
<td>Sigma</td>
<td>10</td>
<td>238</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>L-7900</td>
<td>Sigma</td>
<td>21.6</td>
<td>183.4 μl</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>1 μl/ml</td>
<td>100 μl</td>
</tr>
<tr>
<td>*CaCl₂·2H₂O</td>
<td>C-7902</td>
<td>Sigma</td>
<td>2.1</td>
<td>29</td>
</tr>
<tr>
<td>*MgCl₂·6H₂O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>1.5</td>
<td>31</td>
</tr>
</tbody>
</table>

1 Add NaCl, KCl, NaHCO₃, NaH₂PO₄, HEPES, lactic acid, and phenol red into a beaker. Bring volume to 90ml with ddH₂O and dissolve completely.
*CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 100ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label “SP-TL”, and store at 4°C for one month.
<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>114</td>
<td>666</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>3.2</td>
<td>23.5</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-5761</td>
<td>Sigma</td>
<td>25</td>
<td>210.4</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>S-5011</td>
<td>Sigma</td>
<td>0.34</td>
<td>4.08</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>L-7900</td>
<td>Sigma</td>
<td>10</td>
<td>84.92 μl</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>1 μl/ml</td>
<td>100 μl</td>
</tr>
<tr>
<td>*CaCl₂·2H₂O</td>
<td>C-7902</td>
<td>Sigma</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>*MgCl₂·6H₂O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>0.5</td>
<td>10</td>
</tr>
</tbody>
</table>

¹Add NaCl, KCl, NaHCO₃, NaH₂PO₄, lactic acid, and phenol red into a beaker. Bring volume to 90ml with ddH₂O and dissolve completely. *CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 100ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label “IVF-TL”, and store at 4°C for one month.
<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>mg/500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>114</td>
<td>3330</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>3.2</td>
<td>120</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-5761</td>
<td>Sigma</td>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>S-5011</td>
<td>Sigma</td>
<td>0.34</td>
<td>20.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>H-4034</td>
<td>Sigma</td>
<td>10</td>
<td>1200</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>L-7900</td>
<td>Sigma</td>
<td>10</td>
<td>424.6 μl</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>1 μl/ml</td>
<td>500 μl</td>
</tr>
<tr>
<td>*CaCl₂·2H₂O</td>
<td>C-7902</td>
<td>Sigma</td>
<td>2</td>
<td>150</td>
</tr>
<tr>
<td>*MgCl₂·6H₂O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>0.5</td>
<td>50</td>
</tr>
</tbody>
</table>

1Add NaCl, KCl, NaHCO₃, NaH₂PO₄, HEPES, lactic acid, and phenol red into a beaker. Bring volume to 480 ml with ddH₂O and dissolve completely.

*CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 500 ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label “HEPES-TL”, and store at 4°C for one month.
### IVF – TALP\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA, EFAF</td>
<td>A-6003</td>
<td>Sigma</td>
<td>60 mg</td>
</tr>
<tr>
<td>IVF-TL</td>
<td>-</td>
<td>-</td>
<td>9.8 ml</td>
</tr>
<tr>
<td>Na pyruvate (20 mM stock)</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

\(^{1}\)pH should be ~7.4 – do not check! Sterile-filter. Date, label “IVF-TALP”, and store at 4°C for one week.

### HEPES – TALP\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA, Fraction V</td>
<td>A-4503</td>
<td>Sigma</td>
<td>60 mg</td>
</tr>
<tr>
<td>HEPES-TL</td>
<td>-</td>
<td>-</td>
<td>20 ml</td>
</tr>
<tr>
<td>Na pyruvate (20 mM stock)</td>
<td>-</td>
<td>-</td>
<td>200 µl</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

\(^{1}\)pH should be ~7.4 – do not check! Sterile-filter. Date, label “HEPES-TALP”, and store at 4°C for one week.

### SPERM – TALP\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA, Fraction V</td>
<td>A-4503</td>
<td>Sigma</td>
<td>60 mg</td>
</tr>
<tr>
<td>SPERM-TL</td>
<td>-</td>
<td>-</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>Na pyruvate (20 mM stock)</td>
<td>-</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

\(^{1}\)pH should be ~7.4 – do not check! Sterile-filter. Date, label “SP-TALP”, and store at 4°C for one week.
**HYALURONIDASE SOLUTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-TALP</td>
<td>-</td>
<td>-</td>
<td>10 ml</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>H-3506</td>
<td>Sigma</td>
<td>10 mg</td>
</tr>
</tbody>
</table>

1 Filter and aliquot 1 ml into 1.5 ml tubes. Solution may be stored at -80°C indefinitely.

**KSOM STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (add first)</td>
<td>E-5134</td>
<td>Sigma</td>
<td>0.01</td>
<td>100 μl stock</td>
</tr>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>95</td>
<td>555.18</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>2.5</td>
<td>18.5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>P-5655</td>
<td>Sigma</td>
<td>0.35</td>
<td>4.75</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>M-1880</td>
<td>Sigma</td>
<td>0.20</td>
<td>4.95</td>
</tr>
<tr>
<td>DL-lactic acid</td>
<td>L-7900</td>
<td>Sigma</td>
<td>20.49</td>
<td>174 μl</td>
</tr>
<tr>
<td>D-glucose</td>
<td>G-7021</td>
<td>Sigma</td>
<td>0.20</td>
<td>3.6</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-5761</td>
<td>Sigma</td>
<td>25</td>
<td>210</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>P-4562</td>
<td>Sigma</td>
<td>0.20</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>1 μl/ml</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

1 Add all components to 90 ml ddH₂O and dissolve completely. Dissolve 25 mg CaCl₂ (anhydrous; 1.71 mM) (Sigma C-7902) in about 10 ml of double distilled water. Add the dissolved CaCl₂ to the above ingredients and bring the total to 100 ml with ddH₂O. Vacuum filter stock solution into a plastic bottle; store at 4°C for up to one month.
### KSOMaa MEDIUM\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOM Stock</td>
<td>-</td>
<td>-</td>
<td>4.77 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G-8540</td>
<td>Sigma</td>
<td>25 μl stock</td>
</tr>
<tr>
<td>BSA Fraction V</td>
<td>A-9647</td>
<td>Sigma</td>
<td>15 mg</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>50 μl</td>
</tr>
<tr>
<td>BME Essential Amino Acids, 50X</td>
<td>B-6766</td>
<td>Sigma</td>
<td>100 μl</td>
</tr>
<tr>
<td>MEM Nonessential Amino Acids, 100X</td>
<td>M-7145</td>
<td>Sigma</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

\(^1\)Made media on day of use; medium should be salmon color. Sterilize the solution by filtration and store at 4°C for one week.

### SOF STOCK\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (add first)</td>
<td>E-5134</td>
<td>Sigma</td>
<td>0.01</td>
<td>100 μl stock</td>
</tr>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>107.7</td>
<td>629.4</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>7.16</td>
<td>53.38</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>P-5655</td>
<td>Sigma</td>
<td>1.19</td>
<td>16.2</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>S-5761</td>
<td>Sigma</td>
<td>25.07</td>
<td>210.6</td>
</tr>
<tr>
<td>DL-lactic acid</td>
<td>L-7900</td>
<td>Sigma</td>
<td>3.3</td>
<td>47.33 μl</td>
</tr>
<tr>
<td>*MgCl(_2)·6H(_2)O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>0.49</td>
<td>9.96</td>
</tr>
<tr>
<td>*CaCl(_2)·2H(_2)O</td>
<td>C-7902</td>
<td>Sigma</td>
<td>1.71</td>
<td>25.14</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>1 μl/ml</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

\(^1\)Add all components except CaCl\(_2\)·2H\(_2\)O and MgCl\(_2\)·6H\(_2\)O to 90 ml ddH\(_2\)O and dissolve completely. *Separately, dissolve CaCl\(_2\)·2H\(_2\)O into ~5ml ddH\(_2\)O and then combine with other ingredients. Separately, dissolve MgCl\(_2\)·6H\(_2\)O into ~5ml ddH\(_2\)O and then combine with other ingredients. Bring pH to 7.3 (Osmolarity of ~270 mOsm) and volume to 100ml. Vacuum filter stock solution into a plastic bottle; store at 4°C for up to one month.
### SOFaa MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF Stock</td>
<td>-</td>
<td>-</td>
<td>4.542 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G-8540</td>
<td>Sigma</td>
<td>25 μl stock</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>P-4562</td>
<td>Sigma</td>
<td>82.5 μl stock</td>
</tr>
<tr>
<td>Glucose</td>
<td>G-7021</td>
<td>Sigma</td>
<td>150 μl stock</td>
</tr>
<tr>
<td>BSA EFAF</td>
<td>A-6003</td>
<td>Sigma</td>
<td>15 mg</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>50 μl</td>
</tr>
<tr>
<td>BME Essential Amino acids, 50X</td>
<td>B-6766</td>
<td>Sigma</td>
<td>100 μl</td>
</tr>
<tr>
<td>MEM Non-essential Amino acids, 100X</td>
<td>M-7145</td>
<td>Sigma</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

1. Made media on day of use; medium should be pink color. Sterilize the solution by filtration and store at 4°C for one week.

### EDTA STOCK (0.1 mM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>E-5134</td>
<td>Sigma</td>
<td>3.8 mg</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

1. Do not filter. Make fresh each time.

### Na PYROVATE STOCK (20 mM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic acid</td>
<td>P-4562</td>
<td>Sigma</td>
<td>22 mg</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>-</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

1. Sterile filter into aluminum foil wrapped 15 ml tube. Store at 4°C for two months.
**L-GLUTAMINE STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>G-8540</td>
<td>Sigma</td>
<td>2.923 g</td>
</tr>
<tr>
<td>0.9% Saline</td>
<td>-</td>
<td>-</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

1Sterile filter into 100 µl aliquots in sterile 0.5 ml microcentrifuge tubes. Store at -20°C indefinitely.

**GLUCOSE STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>G-7021</td>
<td>Sigma</td>
<td>90.08 mg</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>-</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

1Sterile-filter into 15 ml tube. Store at 4°C for 2 months.

**HOECHST STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ H₂O</td>
<td>-</td>
<td>-</td>
<td>5 ml</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>H-2261</td>
<td>Sigma</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

1Make aliquots of 10 µl. Store at 4°C.

**0.04% TRITON SOLUTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>dPBS</td>
<td>14080-055</td>
<td>Gibco</td>
<td>10 ml</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>T-9284</td>
<td>Sigma</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

1Store at 4°C.
### PROPIDIUM IODIDE STOCK<sup>1</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ Water</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>P-4170</td>
<td>Sigma</td>
<td>25 mg</td>
</tr>
</tbody>
</table>

<sup>1</sup>Aliquot into Eppendorf tubes, 25μl per tube. Store at -80°C.

### iSCRIPT RT MIX

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>iScript Rxn Mix</td>
<td>170-8891</td>
<td>BioRad</td>
<td>4 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>170-8891</td>
<td>BioRad</td>
<td>1 μl</td>
</tr>
<tr>
<td>mRNA</td>
<td>-</td>
<td>-</td>
<td>11 μl (eluted from beads)</td>
</tr>
<tr>
<td>H₂O</td>
<td>170-8891</td>
<td>BioRad</td>
<td>4 μl</td>
</tr>
</tbody>
</table>

### dNTP MIX<sup>1</sup>

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>1-969-064</td>
<td>Roche</td>
<td>10 μl</td>
</tr>
<tr>
<td>dCTP</td>
<td>1-969-064</td>
<td>Roche</td>
<td>10 μl</td>
</tr>
<tr>
<td>dGTP</td>
<td>1-969-064</td>
<td>Roche</td>
<td>10 μl</td>
</tr>
<tr>
<td>dTTP</td>
<td>1-969-064</td>
<td>Roche</td>
<td>10 μl</td>
</tr>
<tr>
<td>H₂O&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>60 μl</td>
</tr>
</tbody>
</table>

<sup>1</sup>Store at -20°C for 2 to 3 months.

<sup>2</sup>Autoclave the water before use.
### RT-PCR MASTER MASTER MIX (MMM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O¹</td>
<td>-</td>
<td>-</td>
<td>35 µl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>-</td>
<td>-</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 X PCR Buffer</td>
<td>D-8187</td>
<td>Sigma</td>
<td>5 µl</td>
</tr>
<tr>
<td>Jump Start Taq Polymerase</td>
<td>D-8187</td>
<td>Sigma</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

¹Autoclave the water before use.

### RT-PCR MASTER MIX (MM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR MMM</td>
<td>-</td>
<td>-</td>
<td>43 µl</td>
</tr>
<tr>
<td>Primer 1</td>
<td>-</td>
<td>Invitrogen</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer 2</td>
<td>-</td>
<td>Invitrogen</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

### RT-PCR MIX

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR MM</td>
<td>-</td>
<td>-</td>
<td>45 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>-</td>
<td>-</td>
<td>5 µl</td>
</tr>
</tbody>
</table>
### Q-PCR REACTION MIX

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green 2X Supermix</td>
<td>170-8882</td>
<td>BioRad</td>
<td>12.5 μl</td>
</tr>
<tr>
<td>cDNA/Calibrator/ H₂O for NT control</td>
<td>-</td>
<td>-</td>
<td>4 μl</td>
</tr>
<tr>
<td>H₂O¹</td>
<td>-</td>
<td>-</td>
<td>6.5 μl</td>
</tr>
</tbody>
</table>

¹Autoclave the water before use.

### Q-PCR MASTER MIX (MM) FOR ALL 5 GENES

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Sample/Pool)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green 2X Supermix</td>
<td>170-8882</td>
<td>BioRad</td>
<td>65 μl</td>
</tr>
<tr>
<td>cDNA/Calibrator/ H₂O for NT control</td>
<td>-</td>
<td>-</td>
<td>20 μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>-</td>
<td>-</td>
<td>33.8 μl</td>
</tr>
</tbody>
</table>

¹Analyzing 5 genes; Multiplied per well values by 5.2 to ensure enough.

**Note:** To make a mix for all pools of embryos, leave out the cDNA/Calibrator/ H₂O for NT and multiply by the number of pools. Again overcompensate for loss in pipetting. For example, if analyzing 12 pools, multiply by 12.5.

### Q-PCR MIX

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Amount (Per Sample/Pool)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-PCR MM</td>
<td>-</td>
<td>-</td>
<td>23 μl</td>
</tr>
<tr>
<td>Primer 1</td>
<td>-</td>
<td>Invitrogen</td>
<td>1 μl</td>
</tr>
<tr>
<td>Primer 2</td>
<td>-</td>
<td>Invitrogen</td>
<td>1 μl</td>
</tr>
</tbody>
</table>
APPENDIX C: EFFECT OF EARLY NUTRITIONAL ENVIRONMENTS ON PORCINE FETAL AND PLACENTAL DEVELOPMENT

Pig Project Protocol

1. Donor gilts are synchronized and superovulated via Regumate (Intervet Inc., Millsboro, DE, USA) and eCG (Section 1) and artificially inseminated.

2. Gilts are sacrificed 2 to 3 days post-insemination and reproductive tracts are collected for subsequent embryo recovery. Embryos are flushed from the tracts via Beltsville Embryo Culture Medium (BECM) (Section 2). DNA is also taken from donors for later parentage microarray analysis. Serum and plasma samples are also obtained from donor gilts.

3. Collected embryos are allotted to either a high or low glucose North Carolina State University (NCSU) 23 Medium (Section 3) commonly used in porcine in vitro culture.

4. Embryos are cultured according to treatment for 4 days within the CO2 incubator.

5. Embryos are then surgically transferred ~ 4 to 5 mm below the utero-tubal junction into the tip of the uterine horn of a recipient(s) sow to allow gestation to proceed.

6. Fetuses should be collected via sacrificing the recipient sow at ~100 days of gestation. Note: To date, no recipient sow for treatment groups has successfully established a pregnancy. Fetuses from two control sows have been collected.

Section 1.

<table>
<thead>
<tr>
<th>Day of Treatment</th>
<th>250 μg/ml Estrumate</th>
<th>1500 IU eCG*</th>
<th>50 μg/ml Cystorelin†</th>
<th>Sacrifice gilts and collect embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>Regumate (days 1-14)</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>Check for estrus &amp; breed</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>~22</td>
</tr>
</tbody>
</table>

*Give 24 h post-Estrumate injection.
†Give 78 h post-eCG injection.
### Section 2.

#### BECM MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>g/1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na lactate syrup (60%)</td>
<td>L-4263</td>
<td>Sigma</td>
<td>19.3</td>
<td>3.6 ml</td>
</tr>
<tr>
<td>Ca lactate·H₂O</td>
<td>L-4388</td>
<td>Sigma</td>
<td>2.14</td>
<td>0.467</td>
</tr>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>90</td>
<td>5.26</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>8.43</td>
<td>0.36</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>M-2393</td>
<td>Sigma</td>
<td>0.54</td>
<td>0.11</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-5761</td>
<td>Sigma</td>
<td>2.14</td>
<td>0.18</td>
</tr>
<tr>
<td>HEPES</td>
<td>H-9136</td>
<td>Sigma</td>
<td>10.91</td>
<td>2.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>G-7021</td>
<td>Sigma</td>
<td>0.55</td>
<td>0.1</td>
</tr>
<tr>
<td>Mannitol</td>
<td>M-9647</td>
<td>Sigma</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G-5763</td>
<td>Sigma</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>Taurine</td>
<td>T-7146</td>
<td>Sigma</td>
<td>7</td>
<td>0.88</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>P-5280</td>
<td>Sigma</td>
<td>0.27</td>
<td>0.03</td>
</tr>
<tr>
<td>EDTA</td>
<td>E-6758</td>
<td>Sigma</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-5530</td>
<td>Sigma</td>
<td>0.001%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>1%</td>
<td>10 ml</td>
</tr>
<tr>
<td>MEM Nonessential Amino acids</td>
<td>M-7145</td>
<td>Sigma</td>
<td>1%</td>
<td>10 ml</td>
</tr>
<tr>
<td>BME Essential Amino acids</td>
<td>B-6766</td>
<td>Sigma</td>
<td>2%</td>
<td>20 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7906</td>
<td>Sigma</td>
<td>0.1 mg/ml</td>
<td>10</td>
</tr>
</tbody>
</table>

¹Dissolve each constituent completely in ddH₂O in order shown above before adding next component. Adjust pH to 7.2 with 1N NaOH. Osmotic pressure is about 280 mOsm. Sterile by vacuum filtration into a plastic bottle, date and initial bottle. Store at 4°C and use within 10 days.
### Section 3.

#### NCSU MEDIUM\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Product Number</th>
<th>Company</th>
<th>Final (mM)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>S-5886</td>
<td>Sigma</td>
<td>108.73 (↑G)</td>
<td>636 (↑G)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>113.73 (↓G)</td>
<td>665 (↓G)</td>
</tr>
<tr>
<td>KCl</td>
<td>P-5405</td>
<td>Sigma</td>
<td>4.78</td>
<td>35.6</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>S-5761</td>
<td>Sigma</td>
<td>25.07</td>
<td>210.6</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>P-5655</td>
<td>Sigma</td>
<td>1.19</td>
<td>16.2</td>
</tr>
<tr>
<td>glucose</td>
<td>G-7021</td>
<td>Sigma</td>
<td>5.55 (↑G)</td>
<td>100 (↑G)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.2 (↓G)</td>
<td>3.6 (↓G)</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>G-8540</td>
<td>Sigma</td>
<td>1</td>
<td>14.6</td>
</tr>
<tr>
<td>Taurine</td>
<td>T-8641</td>
<td>Sigma</td>
<td>7</td>
<td>87.6</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>H-1384</td>
<td>Sigma</td>
<td>5</td>
<td>54.6</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>15140-122</td>
<td>Gibco</td>
<td>1%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C-7352</td>
<td>Sigma</td>
<td>0.1 mg/ml</td>
<td>10</td>
</tr>
<tr>
<td><strong>MEM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonessential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td>M-7145</td>
<td>Sigma</td>
<td>1%</td>
<td>1 ml</td>
</tr>
<tr>
<td><strong>BME</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Essential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td>B-6766</td>
<td>Sigma</td>
<td>2%</td>
<td>2 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7906</td>
<td>Sigma</td>
<td>0.4%</td>
<td>400</td>
</tr>
<tr>
<td>Phenol red</td>
<td>P-0290</td>
<td>Sigma</td>
<td>0.001%</td>
<td>100 μl</td>
</tr>
<tr>
<td><em>CaCl(_2)·2H(_2)O</em></td>
<td>C-7902</td>
<td>Sigma</td>
<td>1.7</td>
<td>25</td>
</tr>
<tr>
<td><em>MgSO(_4)·7H(_2)O</em></td>
<td>M-1880</td>
<td>Sigma</td>
<td>1.19</td>
<td>29.3</td>
</tr>
</tbody>
</table>

\(^1\)Add all components except Ca\(^{2+}\) and Mg\(^{2+}\) to 90 ml of ddH\(_2\)O. *CaCl\(_2\)·2H\(_2\)O and MgSO\(_4\)·7H\(_2\)O should be dissolved in a small amount of ddH\(_2\)O before added to other ingredients. Adjust volume to 100 ml with ddH\(_2\)O. pH should be 7.4. Sterile by vacuum filtration into a plastic bottle, date and initial bottle. Store at 4°C and use within 10 days.

**NOTE:** BSA type is very important. Also, Pen/Strep is optional.
VITA

Megan Nicole Purpera was the first child born to Guy Patrick and Theresa Cline Purpera in Baton Rouge, Louisiana. Megan has a younger sister, Jena Ashley Purpera, who is currently attending Louisiana State University, Baton Rouge, to obtain a bachelor’s degree in kinesiology. Megan was raised in Erwinville, Louisiana. She attended False River Academy in New Roads, Louisiana, from kindergarten through high school.

After graduating in May 2001, she attended Louisiana State University where she earned her Bachelor of Science degree in animal, dairy, and poultry sciences with a minor in biological sciences in May 2005. Megan participated in undergraduate research projects under the supervision of Dr. Kenneth R. Bondioli and Dr. Robert A. Godke during her senior year. While pursuing her undergraduate degree, she worked at The Fertility Institute of New Orleans, a human infertility clinic, in Baton Rouge, Louisiana.

She entered Graduate School in August of 2005 under the direction of Dr. Kenneth Bondioli and is now a candidate for the degree of Master of Science in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.