REGULATION OF THE PREIMPLANTATION BOVINE EMBRYO BY COLONY STIMULATING FACTOR 2

By

BÁRBARA LOUREIRO

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To my husband, parents, family and friends

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LIST OF ABBREVIATIONS

AQP	Aquaporins
ATP	Adenosine triphosphate
βc	Beta common
BNC	Binucleated cell
CCCP	Carbonyl cyanide 3-chloro-phenylhydrazone
CpG	Cytosine-guanine dinucleotide
DNA	Deoxyribonucleic acid
EAG	Embryonic genome activation
ED	Embryonic disc
ES	Embryonic stem
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GTP	Guanosine triphosphate
H	hour
ICM	Inner cell mass
IFNT	Interferon tau
IVP	*In vitro* produced
JAK	Janus kinase
KSOM	Potassium simplex optimized medium
Min	minute
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NKT	Natural Killer T

PGF	Prostaglandin F2α
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
RNA	Ribonucleic acid
RNAse	Ribonuclease
STAT	Signal transduction and activation of transcription
Sec	Second
SOF	Synthetic oviduct fluid
TE	Trophectoderm
Tr	Trophoblast
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP
Vs	Versus

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REGULATION OF THE PREIMPLANTATION BOVINE EMBRYO BY COLONY STIMULATING FACTOR 2

By

Bárbara Loureiro

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Colony-stimulating factor 2 (CSF2) is a multifunctional cytokine originally recognized as a hematopoietic factor but now known to be expressed in several reproductive tract tissues including the bovine oviduct and endometrium. Colony-stimulating factor 2 may be an important intracellular regulator of endometrial, oviductal and embryonic functions during early pregnancy in the cow and other species. Addition of CSF2 to culture medium improved the proportion of *in vitro* produced embryos developing to the blastocyst stage in cow, mouse, human and pig and increased post-transfer embryonic survival in mice. A series of experiments was conducted to understand the role of CSF2 in preimplantation embryonic development including its effects on blastocyst formation, the embryonic transcriptome, and embryonic survival.

The first experiment was conducted to test whether addition of CSF2 to culture medium could enhance development and post-transfer survival of *in vitro* produced bovine embryos. Treatment of embryos with CSF2 at Day 1 after insemination increased the percentageage of embryos that became transferable morulae or blastocysts at Day 7 but had no significant effect on pregnancy rate at Day 30-35 or calving rate. When CSF2 was added at Day 5 after insemination, there was increase in

the percentageage of embryos that became transferable morulae or blastocysts at Day 7, an increase in the percentageage of recipient cows pregnant at Day 30-35, an increase in calving rate and a decrease in pregnancy loss after Days 30-35. Furthermore, blastocysts formed after treatment with CSF2 had an increase in the number of cells in the inner cell mass.

The second experiment was conducted to analyze the transcriptome of CSF2 treated embryos to identify genes involved in mediating CSF2 effects on development to the blastoctcyst stage and survival after transfer. The Agilent bovine microarray platform was used. Embryos were treated with CSF2 at Day 5 and selected at Day 6 after insemination. A total of 160 genes were differentially expressed, with 67 being higher in CSF2-treated embryos and 93 being lower. Analysis identified 13 biological process ontologies that were grouped into three major groups. The first group included genes functionally involved in developmental processes and differentiation. Actions of CSF2 would tend to inhibit genes involved in neurogenesis and stimulate genes involved in mesoderm or muscle formation. The second group were genes involved in cell communication, with the most characteristic effect caused by CSF2 being inhibition of β-catenin dependent WNT signaling. The third group were genes involved in apoptosis signaling, which was inhibited by CSF2. The antiapoptotic actions of CSF2 were confirmed in another experiment in which CSF2 decreased the percentageage of blastomeres in Day 6 embryos becoming apoptotic after heat-shock.

The third study evaluated possible mechanisms by which CSF2 acts during Day 5 to 7 of development to improve embryonic and fetal survival. Embryos were treated with CSF2 or served as controls and were transferred to recipient cows at Day 7 after

ovulation. Embryos were recovered at Day 15 and embryonic disc and trophoblast analyzed for gene expression by microarray analysis. Results suggest that higher pregnancy rates at Day 30-35 represent increased embryonic survival before Day 15 and a greater capacity of the embryo to elongate and secrete interferon tau (IFNT) at Day 15. This conclusion is based on greater recovery of embryos from cows receiving CSF2-treated embryos at Day 15 ($P<0.07$), a tendency for CSF2 treated embryos to be longer than control embryos, and greater antiviral activity (a measure of IFNT bioactivity) in uterine flushings of cows receiving CSF2 treated embryos ($P<0.07$, when considering those cows with detectable antiviral activity). Analysis of gene expression in filamentous embryos indicated no difference in transcription among this subset of embryos that survived to Day 15 and elongated successfully. Therefore, the reduction in embryonic and fetal loss after Day 30-35 caused by CSF2 is probably not a direct reflection of altered gene expression at Day 15.

Taken together these results indicate that CSF2 can regulate embryonic development, increase embryonic survival after transfer and decrease pregnancy loss. The ability of CSF2 to improve development is probably due to a set of effects that include an increase in the number of cells in the inner cell mass, a decrease in cell death and differentially regulation of the embryo transcriptome. Furthermore, before implantation CSF2 embryos tend to be longer and secret more IFNT. The increased pregnancy rates observed after Day 35 of pregnancy may be a combined result of the factors mentioned above. Moreover, CSF2 treatment can be inflicting epigenetic changes that persist latter in development or even morphological changes that are consequence of the differentially gene expression early before implantation.

CHAPTER 1
LITERATURE REVIEW

Introduction

The maternal microenvironment of the oviduct and uterus is a major determinant of the health and well being of the newly formed offspring during embryonic, fetal, neonatal and adult life. During development, the needs of the embryo constantly changes and, to cope with those changing requirements, the reproductive tract undergoes specific physiological and biochemical modifications throughout gestation (Buhi 2002; Spencer *et al.*, 2007; Hugentobler *et al.*, 2010).

The importance of the uterine environment for pregnancy has been shown in several experiments. By comparing the probability that twin embryos would survive pregnancy in embryo transfer studies, McMillam (1998) estimated that only about 50-70% of recipients are capable of maintaining a transferred embryo. In other embryo transfer experiments with cows, pregnancy rates were affected by a variety of physiological states of the recipient animal including heat stress (Chebel *et al.* 2008), circulating concentrations of urea (Tillard *et al.* 2007), treatment with somatotropin (Moreira *et al.* 2001), milk yield (Yániz *et al.* 2008), and asynchrony between recipient and embryo (Kubisch *et al.* 2004). Evidence that the uterine environment can affect adult life comes from many species including the sheep, in which a reduction in vitamin B12, folate and methionine content of the maternal diet around the time of conception caused high blood pressure, increased adiposity, insulin resistance and altered immune function in adult offspring (Sinclair *et al.* 2007). Males suffered greater effects than females. Long term effects in adult sheep are probably manifestation of epigenetic modifications of the genome.

The importance of the uterine environment for embryonic development means that *in vitro* systems for embryo production can be compromised unless critical features of the reproductive tract environment are replicated *in vitro*. In beef and dairy production systems, the *in vitro* produced (IVP) embryo is important for increasing genetic selection and as a required technical procedure for production of transgenic animals (Hansen and Block 2004). Embryo transfer can also be used to improve fertility in heat-stressed females (Block *et al.* 2003; Block and Hansen 2007). Embryo transfer is effective in this regard because embryos are transferred to the recipient at Day 7 of pregnancy when the embryo is resistant to heat stress (Ealy *et al.* 1993; Ealy *et al.* 1995).

Early embryonic development is not absolutely dependent upon regulatory molecules present in the reproductive tract because embryos produced *in vitro* in medium without growth factors can give rise to live calves after transfer to recipients (Block and Hansen 2007). However, culture conditions are suboptimal and result in alterations at the morphological (Fischer-Brown *et al.* 2002; Fischer-Brown *et al.* 2004), ultrastructural (Rizos *et al.* 2002a), physiological (Ushijima *et al.* 1999) and transcriptional levels (Bertolini *et al.* 2002; Sagirkaya *et al.* 2006; Smith *et al.* 2009) compared with embryos produced *in vivo*. Using microarray analysis, 200 genes were found to be differentially expressed between embryos produced *in vivo* and *in vitro* (Smith *et al.* 2009). In addition, several abnormalities have been reported in calves resulting from IVP embryos (Farin and Farin 1995; Lazzari *et al.* 2002; Miles *et al.* 2005). Thus, one or more components of the maternal environment are necessary for optimal embryonic development and birth of a healthy calf.

There are strong lines of evidence to implicate colony stimulating factor 2 (CSF2), otherwise called granulocyte–macrophage colony-stimulating factor, as a physiologically important regulator of early embryonic development. This cytokine is expressed in several reproductive tract tissues including the oviduct and endometrium of the cow (de Moraes *et al.* 1999) and human (Zhao and Chegini 1994; Chegini *et al.* 1999). Addition of CSF2 to culture medium improved the proportion of *in vitro* embryos developing to the blastocyst stage in cow (de Moraes and Hansen 1997a), mouse (Robertson *et al.* 2001), human (Sjöblom *et al.* 1999) and pig (Cui *et al.* 2004) and increased post-transfer embryonic survival in mice (Sjöblom *et al.* 2005).

The literature review here presented will focus on the events that take place during early embryonic development in the cow, unless stated otherwise, and the effects of CSF2 on preimplantation embryonic development.

Embryonic Development in the Bovine

Time Course of Early Development

After ovulation, the bovine oocyte is picked-up by the cilia-covered fimbria and guided through the infundibulum and ampulla of the oviduct (Oxenreider and Day 1965). It is in the ampullary-isthmic junction of the oviduct that the oocyte is fertilized (Bazer *et al.* 2009). Fusion of the two gametes and release of the second polar body represents the completion of meiosis (Marteil *et al.* 2009). The first cleavage of the zygote occurs around 23-31 hours after insemination (Maddox-Hyttel *et al.* 1988). The developing embryo leaves the oviduct and moves into the uterus approximately at Day 5 of pregnancy when it is about 16 cells (Betteridge *et al.* 1988).

Until hatching at Day 7-8, the embryo is surrounded by a protein coat called the zona pellucida (composed of three glycoproteins; ZP1-ZP3) that is important for

prevention of polyspermy, to keep the blastomeres together and to conserve the microenviroment of the peritvitelline space (Litscher *et al.* 2009; Van Soom *et al.* 2010). After hatching, the blastocyst is transformed to an ovoid form until elongation of the trophoblast is initiated between Days 12 and 14. Also around this period, gastrulation and specification of the germ layers occur in the embryonic disc (ED). By Day 24 the conceptus can fill the entire length of both uterine horns (Blomberg *et al.* 2008). Implantation starts on Day 20, when the trophectoderm (TE) adheres to the endometrial luminal epithelium of the mother (Blomberg *et al.* 2008; Bazer *et al.* 2009).

Changes in Gene Expression During Cleavage Stages

During early development, the embryonic genome is inactive and the embryo relies on maternal messenger ribonucleic acid (mRNA) for protein synthesis (Thelie *et al.* 2009). The recruitment mechanisms by which dormant RNA is either targeted for translation or decay are still largely uncharacterized. The current model involves lengthening of the poly(A) tail, which triggers binding of the poly(A) binding protein and binding of translation initiation factors (Memili and First 2000; Groisman *et al.* 2002). RNA concentration is highest in the germinal vesicle stage oocyte and from then until the 8-cell stage, RNA is gradually depleted (Gilbert *et al.* 2009; Vallée *et al.* 2009). Evidence in the mouse suggests that this decline is important for activation of the embryonic genome (Li *et al.* 2010). Depletion of maternal argonaute 2 (*Ago2*), which encodes a catalytic RNA hydrolase (RNase), disrupts gene expression and the 2 cell embryo fails to become a blastocyst (Li *et al.* 2010). In the bovine, embryonic genome activation (EGA) occurs at the 8 to 16-cell stage (Memili and First 2000) through an unknown mechanism.

Changes in Metabolism of the Early Embryo

The type of substrate metabolized by the embryo changes with development. At early stages, the fuel for ATP formation by the mitochondria is provided by pyruvate, while the uptake of glucose is low. As the embryo develops to the compact morula and blastocyst stage, ATP synthesis increases and glucose starts to contribute to the citric acid cycle through conversion to lactate and then pyruvate (Thompson 2000). Glucose can also generate ribose required for nucleic acid synthesis, and nicotinamide adenine dinucleotide phosphate (Thompson 2000). Furthermore, glucose, pyruvate and lactate production and/or consumption can be different between the two cell types of the Day 8 blastocyst. While inner cell mass (ICM) cells consume more glucose than pyruvate, the converse is true for TE cells (Gopichandran and Leese 2003). Lactate production is higher for TE than for ICM cells (Gopichandran and Leese 2003).

The number and activity of mitochondria also change as the embryo develops. A primordial oocyte contains as few as 10 mitochondrial DNA (mtDNA) copies whereas a fully grown oocyte can contain more than 100.000 copies of mtDNA, with one or two copies of mtDNA per organelle (Ferreira *et al.* 2009; Chiaratti *et al.* 2010). At this stage the mitochondria is immature and does not present any activity, therefore the energy is supplied through the granulosa cells (Tarazona *et al.* 2006). Initially present near the periphery of the cell, oocyte mitochondria become more dispersed at the germinal vesicle breakdown stage, presumably to better serve the cell (Ferreira *et al.* 2009; Marteil *et al.* 2009). At this point the levels of activity have highly increased and the mitochondria are capable of generating the necessary ATP (Tarazona *et al.* 2006). The bovine embryo does not gain the capacity for replenishing the mtDNA until the morula and blastocyst stages when there is increased expression of nuclear respiratory factor 1

(*NRF1*) and transcription factor A, mitochondria (*TFAM*) that are regulators of mtDNA replication and transcription (Chiaratti *et al.* 2010). Embryos with non-competent mitochondria stop development before the EGA (Tarazona *et al.* 2006).

High concentrations of glucose are toxic to bovine embryos, especially female embryos undergoing the transition from the morula to blastocyst stage (Gutiérrez-Adán *et al.* 2000). This fact along, with the possibility that male embryos develop faster in culture than female embryos (Gutiérrez-Adán *et al.* 2000; Kimura *et al.* 2005), could explain the skewing of the sex ratio towards males often seen in calves from IVP embryos (Block and Hansen 2007; Camargo *et al.* 2010).

The gender difference in growth rate and sensitivity to glucose could be due to differential gene expression either because of differences in activity of sex or autosomal chromosomes. Both X-chromosomes are active in the cleavage-stage female embryo (Mak *et al.* 2004). Unbalanced expression of X-linked genes can increase the activity of enzymes involved in energy metabolism and detoxification of oxygen radicals (Pérez-Crespo *et al.* 2005). For example, the gene for glucose 6-phosphate dehydrogenase (*G6PDH*), an enzyme that controls the entry of glucose into the pentose-phospate pathway, is located on the X-chromosome. With both X-chromosomes active in the female embryo there is more pentose-phospate pathway activity which leads to poor tolerated imbalance in carbohydrate metabolism (Gutiérrez-Adán *et al.* 2000). Hypoxanthine phosphoribosyl transferase 1, an enzyme involved in controlling the amount of oxygen radicals, is also on the X-chromosome (Goldammer *et al.* 2003). Free radical actions involve not only cellular damage but also cellular growth (Rieger 1992).

The retarded development in female embryos could be caused by a decrease in oxygen radical levels due to increased activity of the enzyme.

By the time the embryo reaches the blastocyst stage, 88% of the genes (193 genes) in the X-chromosome are upregulated in the female embryo comparing to the male embryo. However, only 10% of these genes had a 2 fold-increase and in fact, 70% of the genes had a fold-increase lower than 1.6 comparing to the male embryo (Bermejo-Alvarez *et al.* 2010). These suggest that at the blastocyst stage the X-chromosome is starting to be inactivated.

Glutathione is the most important cellular antioxidant in the cytosol. There is a large decline in its concentration after fertilization because it is consumed as part of the chromatin decondensation process (Lim *et al.* 1996; Luberda 2005). However, *de novo* synthesis of glutathione increases at the 16 cell stage (around EGA), and reaches its maximum peak at the hatched blastocyst stage (Lim *et al.* 1996).

Developmental Changes in Apoptosis

Apoptosis is a developmentally regulated process that plays an important role in the survival of the preimplantation embryo. Although the maturing oocyte can undergo apoptosis (Roth and Hansen 2004a; Roth and Hansen 2004b; Roth and Hansen 2005), the capacity for apoptosis is lost at the 2-cell stage and does not become reacquired until sometime between the 8 and 16-cell stages (Paula-Lopes and Hansen 2002a; Gjørret *et al.* 2005). As the embryo undergoes further development, there is little change in the degree of apoptosis (Loureiro *et al.* 2007). The lack of apoptosis at the two cell stage caused is in part by a block in activation of caspases (Brad *et al.* 2007; de Castro e Paula and Hansen 2008a), which in turn reflects increased resistance of the mitochondria to depolarization (Brad *et al.* 2007). A second block to the pathway exists

at the level of the nucleus since addition of carbonyl cyanide 3-chloro-phenylhydrazone, a mitochondria depolarization agent, did not cause increase in DNA fragmentation even though group II caspases were activated (Brad *et al.* 2007; Carambula *et al.* 2009).

The sensitivity of embryonic DNA to fragmentation coincides with its levels of methylation. DNA is highly methylated at the 2-cell stage and then becomes progressively more demethylated as development progresses until by the 8-16 cell stage the embryo has low methylation and transcription is activated (Dean *et al.* 2001; Park *et al.* 2007). The interaction between apoptosis and methylation was shown when sensitivity of 2-cell embryos to carbonyl cyanide 3-chloro-phenylhydrazone could be induced with 5-aza-20-deoxycytidine, a DNA methylation inhibitor (Carambula *et al.* 2009).

In the bovine embryo, the consequences of apoptosis are dependent upon stage of development. Induction of apoptosis is a major cause for the reduced oocyte competence for fertilization and development caused by heat shock (Roth and Hansen 2004b). Cell stressors like arsenic (Krininger *et al.* 2002), heat shock (Loureiro *et al.* 2007) and ceramide (de Castro e Paula and Hansen 2008b) induce apoptosis through the mitochondrial pathway that causes mitochondria depolarization and activation of caspases. It is likely that massive activation of apoptosis by these stresses compromises development. However, when the stress is less severe so that the increase in apoptosis is limited, the apoptotic process itself may be beneficial to embryo survival. At Day 4 of development, inhibition of apoptosis with a caspase inhibitor exacerbated deleterious effects of heat shock on development to the blastocyst stage (Paula-Lopes and Hansen 2002b).

Epigenetic Modifications

Cytosine methylation at the cytosine-guanine dinucleotide (CpG) islands in the genomic DNA is necessary for regulation of chromatin configuration and normal gene expression (Geiman and Muegge 2010). In general, hypermethylated DNA is transcriptionally inactive whereas hypomethylated DNA is highly transcribed (Corry *et al.* 2009). Conservation of methylation in only one parental allele is a form of epigenetic regulation known as imprinting (Wilkins 2006; Tveden-Nyborg *et al.* 2008). This monoallelic expression can be tissue specific. One example of tissue specific imprinting is the X-chromosome, in which the paternally derived copy is inactivated in extraembryonic tissue while its expression is random in fetal and adult somatic tissues (Wilkins 2006).

The degree of methylation change with development. In the bovine zygote, the male pronucleus is partially demethylated at 20 hours after fertilization so that paternal levels of reactivity to antibody to 5-methyl cytosine is 51% of that of maternal DNA (Park *et al.* 2007). Methylation of the paternal chromosomes increases to levels similar to the female by 28 hours after fertilization when embryos are at about the 2-cell stage (Park *et al.* 2007). Embryonic genome activation occurs at the 8 to16-cell stage (Gilbert *et al.* 2009). Up to this stage, embryos are highly methylated (Dean *et al.* 2001) and coincident with embryonic genome activation, methylation declines (Dean *et al.* 2001). *De novo* methylation after the 16-cell stage is not identical for all the nuclei. By the blastocyst stage, the ICM contains highly methylated nuclei and the TE lower amounts of methylated DNA (Dean *et al.* 2001). The degree of methylation influences cell potential for differentiation (Western *et al.* 2010).

Compaction and Blastocyst Formation

Around the 32-cell stage, at Day 5 of pregnancy, the bovine embryo forms a solid mass known as the morula that then undergoes a process called compaction (Betteridge *et al.* 1988). Prior to compaction, the blastomeres are spherical and lack specialized intercellular junctions. During compaction, cells become flattened against one another, thus maximizing intercellular contact and obscuring intercellular boundaries (Sheth *et al.* 2000; Johnson and McConnell 2004). This process is mediated primarily by activation of e-cadherin, a Ca^{2+} dependent adhesion molecule. For full adhesive function and cytoskeletal anchorage, e-cadherin forms a core adhesion complex with a protein known as catenin (Niessen and Gottardi 2008). This type of junction is responsible for generating contact dependent growth and polarity signals with apical and basolateral domains in all blastomeres (Johnson and McConnell 2004). Blastomere initiation of polarity seems to be mediated by the Par complex protein (Par-3 and -6), atypical protein kinase C (aPKC) and caudal cell division cycle homolog (S. cerevisiae; cdc42) which are localized on the embryo's apical domain (Eckert and Fleming 2008). Inhibition of aPKC and Par-3 causes failure of asymmetric cleavage latter in development (Plusa *et al.* 2005).

Another important junctional complex for epithelial differentiation is the tight junction. Formed by claudin, ocludin and zona ocludins proteins (ZO-1 and -2), this junction tightly connects opposing cell membranes, creating a barrier that is virtually impermeable to fluid diffusion through the intercellular space (Tsukita *et al.* 2001). In mouse embryos, after compaction ZO-1 binds to rab-guanosine triphosphate hydrolase (GTPase) and Par-3-6/aPKC to the e-cahdehirin-catenin complex. When cells start to differentiate, the peripheral membrane proteins cingulin and ZO-2 assemble to the

complex which in turn results in loss of Par-3-6/aPKC. Finally, during blastocoel formation, ZO-1α+ and the transmembrane proteins occludins and claudins join the complex. At this point the embryo generates an impermeable seal between TE cells and the blastocoel cavity (Sheth *et al.* 2000).

Cavitation, in which the fluid-filled blastocoel is formed, involves polarized transport of ions and water (Watson *et al.* 2004). Vectorial transport of Na^+ and Cl^- ions through the TE into the blastocoel generates an osmotic gradient that drives fluid across the epithelium (Kawagishi *et al.* 2004). In the mouse embryo, it is a carrier-mediated process that involves several types of ion transporters, including a Na^+ channel, Na^+/H^+ exchangers and ATPase Na^+/K^+ transporting, alpha 1 (*Atp1a1*). The *Atp1a1* are localized in the basolateral membrane region of the TE (Madan *et al.* 2007). Blastocoel expansion is significantly retarded in the absence of extraembryonic Na^+, in the presence of inhibitors of Na^+ channels or silencing RNA (siRNA) for *Atp1a1* (Kidder 2002; Madan *et al.* 2007). Also present in the TE membrane are water channels known as aquaporins (AQP). They allow rapid water flow across the membrane in the direction of the osmotic gradient (Liu and Wintour 2005). Murine preimplantation embryos express mRNA for multiple AQP throughout preimplantation development (Liu and Wintour 2005). *AQP-3* mRNA increases at the morula to blastocyst transition and AQP-8 protein is first detected in the cell margins at the morula stage (Barcroft *et al.* 2003).

Both AQP-3 and -8 are found in the basolateral membrane of the TE while AQP-9 is predominantly observed in the apical membrane domain of the TE (Liu and Wintour 2005). AQP-8 is highly selective for water molecules while AQP-3 and -9 are less selective, allowing the passage of small solutes (van Os *et al.* 2000).

Following compaction and cavitation, the bovine blastocyst at Days 6-7 starts to differentiate into ICM cells, which retain a pluripotent phenotype (Pant and Keefer 2009) and TE cells which will become the outer layer of the placenta (Hamilton 1946; Betteridge *et al.* 1988). TE cells are the first to differentiate and have the characteristics of an epithelium (Marikawa and Alarcón 2009). The ICM cells go through a second lineage segregation becoming the epiblast, which will give rise to the fetus itself, and primitive endoderm that becomes the parietal and visceral endoderm, which latter contributes do the yolk sac (Blomberg *et al.* 2008). Around the third week of development gastrulation, neurulation and formation of the somites will take place in the epiblast (Maddox-Hyttel *et al.* 2003).

In the bovine, it is not known how the blastomere decides to become a TE cell or ICM cell. There are two theories to explain TE and ICM differentiation in the mouse embryo. According to the inside-out theory, position determines cell fate. At the morula stage, cell to cell contact increases and some cells become enclosed by surrounding cells while other cells in the outside layer are in contact with the external environment for part of their surface. Asymmetric cell contact induces epithelial differentiation into TE whilst symmetric contact of the enclosed ICM inhibits differentiation (Eckert and Fleming 2008; Marikawa and Alarcón 2009). An alternative theory suggests that the cell decides its fate and the location in which it will reside, rather than the location deciding what fate the cell will have (Yamanaka *et al.* 2006). Several studies have corroborated the inside-outside model. For example, imunosurgically-isolated ICM have the potential to form TE *in vivo* (Rossant and Lis 1979) and tight junction and a blastocoel *in vitro* (Eckert *et al.* 2005).

A few genes have been identified as being responsible for formation of the ICM and TE in human and mouse. The first transcription factor to appear in the late morula ICM is Sex determining region Y-box2 (*Sox2*) (Guo *et al.* 2010). It is a key factor for maintenance of pluripotency and for reprogramming of differentiated cells into induced stem cells (Takahashi and Yamanaka 2006). Pou domain class 5 transcription factor-1 (*Pou5f1* or *Oct4*) is a transcription factor necessary for the maintenance of the pluripotency in the ICM and it prevents ICM transformation to TE (Zernicka-Goetz *et al.* 2009). Another stem cell marker present in the ICM is Nanog homeobox (*Nanog*) (Marikawa and Alarcón 2009). Caudal type homeobox 2 (*Cdx2*) is specifically expressed in TE and its presence is necessary to repress *Pou5f1* expression in TE. Loss of *Cdx2* results in failure to downregulate *Pou5f1* and *Nanog* in outer cells of the blastocyst and subsequent death of those cells (Strumpf *et al.* 2005). Nodal is found to be expressed in the ICM and primitive endoderm of the blastocyst and also latter in the epiblast and visceral epithelium (Mesnard *et al.* 2006).

In the bovine, the mechanism of early segregation and differentiation is different in some respects from the mouse. *POU5F1* is expressed in both ICM and TE of *in vitro* and *in vivo* produced blastocysts until Day 10 of development (Eijk *et al.* 1999; Kirchhof *et al.* 2000; Mesnard *et al.* 2006) whereas *CDX2* has a weak expression (Degrelle *et al.* 2005). *NANOG* mRNA and protein are also found in both ICM and TE of bovine blastocysts and elongated embryos, but with greater expression in the ICM and ED (Degrelle *et al.* 2005; Muñoz *et al.* 2008).

A microarray experiment that analyzed the transcriptome of ICM and TE of human blastocysts has identified new marker genes to complement the existing markers for

ICM/TE such as *POU5F1* and *CDX2*. Pathway analysis of the microarray data identified signaling pathways related to integrin mediated cell adhesion and overexpression of several NA+/K+-ATPases in TE (Adjaye *et al.* 2005), reflecting their role in controlling permeabilization and fluid transport across the epithelium. Keratin 18 (*KRT18*), a cytoeskeletal protein, was predominantly expressed in the TE cells and imunocytochemistry detected its expression only in the cell to cell contacts of the TE (Goossens *et al.* 2007). Pathways involved in cell cycle were also differentially expressed. Genes that activate the WNT signaling pathway were shown to be overexpressed in the ICM while genes encoding Casein kinase 1 alpha (*CSNK1A*) and disheveled activator of morphogenesis 1 (*DAAM1*), which are agonists of the WNT pathway, were both over expressed in the TE (Adjaye *et al.* 2005).

Hatching and Elongation

The blastocyst becomes fully expanded when it reaches about 160 to180-cells (Van Soom *et al.* 1997). Thereafter, the blastocyst hatches from the zona pellucida by a combination of cell growth and volume increase in the blastocoel (Van Soom *et al.* 1997; Houghton *et al.* 2003). The hatching process in the bovine embryo is apparently a mechanical process more than an enzymatic one (Fléchon and Renard 1978; Massip and Mulnard 1980; Massip *et al.* 1982).

Once the blastocyst has hatched, the ICM forms a protuberance called the ED, which is still covered with TE (Rauber's layer) until about Day 12. By Day 12, the layer of TE cells covering the ED is degraded via apoptosis and the disc cells are exposed to the maternal milieu (Williams and Biggers 1990; Guillomot *et al.* 2004).

Elongation begins between Days 12 and 14 (Betteridge *et al.* 1988; Vejlsted *et al.* 2006) and is concomitant with gastrulation (Hue *et al.* 2001). The development of the

trophoblast provides a large placental surface area that is better able to initiate the maternal-conceptus cross-talk and exchange essential nutrients for survival of the conceptus (Spencer and Bazer 2004). As part of the process of elongation, the conceptus undergoes shape changes going from spherical to ovoid, then tubular, and finally to the elongated stage. As a result, the conceptus increases in size more than 1000-fold by Day 24 of gestation so that it can extend the entire length of both uterine horns (Maddox-Hyttel *et al.* 2003). Elongation is accomplished by an increase in cell number accompanied by an increase in protein synthesis (Thomson 1998; Degrelle *et al.* 2005). Elongation of bovine embryos appears to be in part determined by uterine signals, given that extended *in vitro* culture beyond the blastocyst stages results in formation of TE outgrowths and attachment of the embryo to the bottom of the culture dish rather than elongation (Alexopoulos *et al.* 2005). However, *in vitro* produced blastocysts elongate when transferred to recipients (Block *et al.* 2007). One candidate as a uterine elongation factor is insulin-like growth factor binding protein 1 (IGFBP1). In the bovine endometrium, *IGFBP1* mRNA increases in amount by Day 16 of pregnancy and is significantly different in pregnant versus non pregnant animals (Simmons *et al.* 2009). *In vitro*, IGFBP1 stimulates migration and mediated attachment of ovine TE cells while had no effect on cell proliferation (Simmons *et al.* 2009).

Maintenance of the Corpus Luteum

One of the roles of the elongated conceptus is to produce the pregnancy recognition signal, interferon-tau (IFNT), which blocks luteal regression caused by uterine prostaglandin F2α (PGF2α) release and allowed for continued secretion of progesterone (Thatcher *et al.* 2001; Spencer *et al.* 2007). IFNT is secreted by the mononuclear cells of the primitive extra-embryonic trophoblast a few Days prior to when

the conceptus attaches to the uterine wall (Helmer *et al.* 1987). The peak production of IFNT occurs on Days 16 to 17 of pregnancy (Thatcher *et al.* 2001). The way IFNT prevents development of the luteolytic mechanism has been well documented in sheep. It inhibits transcription of the gene for the estrogen receptor α in the luminal and superficial ductal glandular epithelia (Spencer and Bazer 2004). This action prevents the induction of oxytocin-receptor transcription by estrogen and, therefore, oxytocin induced luteolytic pulses of $PGF_{2\alpha}$ (Asselin *et al.* 1997; Spencer *et al.* 1998; Pru *et al.* 2001; Spencer and Bazer 2004). In the cow the mechanism must be a little different, since there is no change in estrogen receptor mRNA in the endometrium from Day 16 pregnant cows and cyclic cows but there is a decrease in oxytocin receptor mRNA (Robinson *et al.* 1999). Therefore, in the cow, pregnancy may alter the oxytocin receptor regardless of the estrogen one. Between Days 8 and 17 of pregnancy is when the conceptus ordinarily inhibits pulsatile $PGF_{2\alpha}$ secretion but is also when at least 40% of total embryonic losses occur (Thatcher *et al.* 2001).

Attachment to the Endometrium

The ruminant conceptus does not actually implant in the uterus. Rather placentation occurs because of apposition and interdigitation with limited invasion of trophoblast cells into the endometrial epithelium. Placentation starts at about Day 20 of pregnancy (Chavatte-Palmer and Guillomot 2007). Cell contact is initiated in the region of the ED and extends towards the ends of the conceptus (Assis Neto *et al.* 2009a). In sheep and cow embryos, the trophoblast attaches to the caruncular epithelium mainly and to the intercaruncular mucosa to a lesser extent. In these areas, transitory villi grow on the trophoblast and invade the uterine glands. This process ensures an anchorage of

the conceptus in the uterine cavity and localized absorption sites of the glandular secretions (Chavatte-Palmer and Guillomot 2007).

The limited invasion of the maternal tissue is caused by trophoblast cells that migrate into the endometrial epithelium and fuse with uterine epithelial cells to form binucleated cells (BNC). The BNC represent 20% of the trophoblast and produce placental lactogen and a group of inative aspartyl proteinases called pregnancy associated glycoproteins that are delivered to the maternal compartment (Szenci *et al.* 1998; Klisch *et al.* 1999). Fusion of the BNC with uterine epithelial cells forms syncytial plaques (Schlafke and Enders 1975). Due to the presence of uterine syncytium, the ruminant placenta is classified as synepitheliochorial.

Placentomes

The definitive placenta is differentiated into two regions - cotyledons and intercotyledonary tissue. Placentomes are formed by interdigitation of cotyledons with corresponding structures on the endometrium called caruncles. Their primary role is nutrient and gas exchange between the fetus and the mother (Schlafer *et al.* 2000; Enders and Carter 2004). Cotyledons can be observed macroscopically after Day 37 of gestation and 80 to 120 cotyledons eventually form (Schlafer *et al.* 2000; Assis Neto *et al.* 2009). By the second trimester of gestation, the number of viable luteal cells decrease and the cotyledons are responsible for producing the progesterone necessary for maintenance of pregnancy (Shemesh 1990; Izhar *et al.* 1992).

Alterations in Embryonic Development *In vitro*

Pregnancy risk in recipients of IVP embryos are generally not superior to pregnancy risk following artificial insemination (Rodrigues *et al.* 2004; Sartori *et al.* 2006) and are less than following the transfer of *in vivo* derived embryos (Hasler 2000).

Furthermore, *in vitro* produced (IVP) embryos that survive the embryonic period are more likely to be lost later on. While pregnancy loss after the first two months of gestation for AI and superovulated embryos is generally around 5 to 14% (Santos *et al.* 2004; Demetrio *et al.* 2007; Jousan *et al.* 2007), pregnancy loss after Day 40 of gestation for IVP embryos ranged from 12 to 24% (Hasler 2000; Block *et al.* 2003; Demetrio *et al.* 2007).

A major problem with IVP embryos compared with embryos produced *in vivo* is poor survival to cryopreservation (Enright *et al.* 2000; Rizos *et al.* 2002). The high sensitivity of IVP embryos to chilling and freezing can be related to a higher accumulation of cytoplasmic lipid droplets (Ushijima *et al.* 1999). In addition, blastocysts cultured in synthetic oviduct fluid medium (SOF) had lower expression of connexin 43, a gap junction protein that is essential for the transport of cryoprotectants and fluids during freezing and thawing, when compared with embryos developed in coculture or *in vivo* (Rizos *et al.* 2002a).

The period of embryo development, rather than the period of maturation or fertilization, is the most critical one for perturbations resulting in reduced capacity of the blastocyst for cryopreservation. Survival rates after cryopreservation of blastocysts that were produced by maturation and fertilization *in vivo* but cultured *in vitro* were 0% compared with 70% for blastocysts that were matured, fertilized and cultured *in vivo* (Rizos *et al.* 2002a). A similar conclusion that the period of embryonic development is crucial for high competence for cryosurvival was acquired when cryopreservation rates of embryos produced by *in vitro* maturation, fertilization and embryo culture (0%) were compared with cryopreservation rates of embryos that were matured and fertilized *in*

vitro but cultured in the ewe oviduct (63%) (Enright *et al.* 2000; Rizos *et al.* 2010). Transcript abundance of 5 genes, elongation factor 1 gamma (*EEF1G*), guaninenucleotide binding protein (*GNB3*), forkhead transcription factor (*FOXP3*), represssor of estrogen receptor activity and high mobility group protein 2 (*ESR1*) were significantly lower for blastocysts cultured in SOF than for blastocysts produced *in vivo* or produced *in vitro* and allowed to develop in the ewe oviduct (Corcoran *et al.* 2007).

Abnormalities for calves resulting from IVP embryos have included increased calf birth weight (Lazzari *et al.* 2002), altered organ development (Farin and Farin 1995) and alterations in placentome number and placental structure (Miles *et al.* 2005). Those abnormalities can result in an increase in the cases of dystocia and cesarean section as well as perinatal mortality (van Wagtendonk-de Leeuw *et al.* 2000).

The low pregnancy risk and increased fetal loss that are characteristic of transfers with IVP embryos are probably connected to a variety of cellular and molecular deviations during early embryonic development. Morphological evaluation of Day 14 embryos revealed that IVP embryos have high incidence of no detectable ED (Bertolini *et al.* 2002; Fischer-Brown *et al.* 2002; Fischer-Brown *et al.* 2004). *In vitro* culture of bovine embryos in the presence of high concentrations of serum or bovine serum albumin resulted in increased number of cells in Day 7 blastocysts, size of blastocysts on Day 12, and the relative abundance of the transcripts for heat shock protein 70.1 (*HSP70.1)*, copper/zinc-superoxide dismutase, glucose transporters-3 and -4 (*SLC2A3* and *SLC2A4*), fiblroblast growth factor 2 (basic) (*FGF2*), and insulin like growth factor 1 receptor (*IGF1R)* when compared with *in vivo* derived embryos (Lazzari *et al.* 2002). Other studies show IVP embryos with alterations in the level of expression of X-linked

genes, increased chomosomally-abnormal cells (King 2008) and differential expression of IGF family genes (Bertolini *et al.* 2002; Sagirkaya *et al.* 2006; Moore *et al.* 2007). The expression of BCL2-associated X protein (*BAX)*, an apoptosis related gene, was higher in blastocysts produced *in vitro* using SOF than for those developed in coculture or *in vivo* (Rizos *et al.* 2002a). Oxidative stress genes are also differentially expressed, with mitochondrial manganese-superoxide dismutase, an important antioxidant defense in cells exposed to oxygen, strongly expressed in blastocyts developed *in vivo* when compared with IVP embryos while sarcosine oxidase, an oxidative enzyme, highly expressed on IVP embryos when compared with its *in vivo* counterparts (Rizos *et al.* 2002a).

Microarray techniques have been used to investigate differences in the transcriptome between IVP and *in vivo* embryos (Smith *et al.* 2009). *In vivo* produced embryos have significant overexpression of genes in the category 'response to stimulus', unfold protein and carboxy-lyase activity while genes in the G-protein coupled receptor signaling pathway were significantly lower for *in vivo* embryos. However, none of the genes differentially regulated in this study matched the imprinted genes thought to be responsible for large offspring syndrome. Transcripts for enzymes involved in the *de novo* methylation process were downregulate in the IVP embryos compared with the *in vivo* derived embryos; this could lead to aberrant methylation and subsequent fetal abnormalities (Smith *et al.* 2009).

Growth Factors and Cytokines as Uterine Regulators of Embryonic Development

Among the molecules secreted by the reproductive tract that can regulate embryonic development are various growth factors and cytokines. Originally described as protein molecules that promote cell proliferation and inhibit apoptosis, growth factors

are now known to play roles in endocrine, paracrine, and autocrine regulation of a wide variety of cell functions. Historically, cytokines are associated with hematopoietic and immune cells; however, they are now known to be secreted by a wide variety of cells and tissues including endocrinologically responsive tissues within the reproductive tract. While some cytokines can be growth factors, like CSF1 and CSF2, others have an inhibitory effect on cell growth, and they can target cells to undergo apoptosis and cell death. The embryo itself expresses receptors for an array of growth factors and cytokines. In bovine embryos, receptors for platelet derived growth factor α (*PDGFR-α*) and *IGF1R* and *IGF2R* are found at the oocyte stage and throughout embryo development (Yoshida *et al.* 1998; Wang *et al.* 2008a). Messenger RNAs for FGF2 receptor (*FGF2R)* are present in all stages of oocyte maturation and embryonic development up to the 2-cell stage, and again at the blastocyst stage (Yoshida *et al.* 1998). Transcripts for *FGFR1c, FGFR2b, FGFR3c and FGFR4* are found on *in vitro* produced blastocysts and the *in vivo* elongated Day 17 conceptus (Cooke *et al.* 2009). Epidermal growth factor receptor (*EGFR*) mRNA and protein have been shown to be present in spherical embryos on Day 13 and elongated embryos on Day 16 (Kliem *et al.* 1998). Expression of mRNA and protein of the CSF2 receptor alpha subunit (*CSF2R-α*) has only been shown in mouse embryos; it is present from the first cleavage through the blastocyst stage (Sjöblom *et al.* 2002).

The list of cytokines and growth factors present in the uterus is large and their physiological roles are still being resolved. The purpose of this section of the literature review is to provide examples of specific growth factors and cytokines implicated in

regulation of embryonic development in the cow and to illustrate the specific roles they may play.

Insulin-like Growth Factor 1

The main source of insulin like growth factor 1 (IGF1) is the liver, which secretes IGF1 in response to growth hormone (Scharf *et al.* 1996). However, it is not assured that changes in circulating IGF1 cause changes in uterine IGF1 concentrations since IGF1 concentrations in uterine fluid did not change in parallel with plasma concentrations of IGF1(Bilby *et al.* 2004). The uterus, oviduct and the embryo also produce IGF1 (Velazquez *et al.* 2008) although their contribution to the total IGF1 pool in uterine and oviductal fluid is unknown.

Addition of IGF1 to culture medium increases the proportion of IVP bovine embryos that develop to the blastocyst stage (Block *et al.* 2003; Lima *et al.* 2006). In addition, IGF1 increased blastocyst total cell number (Makarevich and Markkula 2002; Sirisathien *et al.* 2003) and altered the relative abundance of developmentally important mRNA transcripts at the blastocyst stage including increases in *IGFBP1-2*-3-5 (Prelle *et al.* 2001; Block *et al.* 2007), desmocollin 2, *ATP1A1* (Block *et al.* 2007) and *SLC2* genes (Oropeza *et al.* 2004) and decreases in expression of the gene for heat shock protein 70 (*HSP70*) (Block *et al.* 2007), and *IGF1R* (Enright *et al.* 2000; Prelle *et al.* 2001; Block *et al.* 2007).

In addition, IGF1 protected embryos from heat shock, allowing increased development and reduced apoptosis (Jousan and Hansen 2004; Jousan *et al.* 2007; Jousan *et al.* 2008), and protected bovine embryos from the anti-developmental actions of the prooxidant menadione (Moss *et al.* 2009). Furthermore, IGF1 treated embryos showed increased pregnancy rates after they were transferred to recipients exposed to

heat stress (Block *et al.* 2003; Block and Hansen 2007). Thus, IGF1 may function in pregnancy to increase embryonic development and protect embryos from specific stresses capable of disrupting development.

Interleukin 1

Interleukin1, beta (IL1B) is a polypeptide found in uterine flushes at least from Days 11, 14 and 17 of cyclic cows (Davidson *et al.* 1995). There is evidence that the source of this ILB1 is the luminal and glandular epithelium and stroma of the endometrium (Paula-Lopes *et al.* 1999). On the other hand, ILB1 could not be detected in pregnant cows on Days 25 and 30 of gestation (Davidson *et al.* 1995). When endometrium collected from pregnant cows was cultured with ILB1 it increased the secretion of PGE_2 and $PGF_{2\alpha}$ from epithelial cells (Betts and Hansen 1992; Davidson *et al.* 1995) and from stromal cells (Davidson *et al.* 1995). ILB1 treatment also decreased DNA synthesis in stromal cells but had no effect on epithelial cells (Davidson *et al.* 1995).

The presence of ILB1 in the uterus indicates that this cytokine might have a role in early embryonic development in cattle. Addition of ILB1 to cultured bovine embryos increased the percentageage of embryos becoming a blastocyst but only if ILB1 was added at the first Day of culture and if the embryos were cultured in high density (25-30 embryos/drop) (Paula-Lopes *et al.* 1998). The fact that effects are seen at high embryo density may mean that ILB1 acts to stimulate some embryo-derived growth factor that in turn increases embryonic development.

The importance of ILB1 for pregnancy was demonstrated in mouse (Simón *et al.* 1998). Injections of ILB1 receptor antagonist around the preimplantation period significantly decreased the number of implantation sites in this species. When flushed at

Day 8, 10 times more blastocysts were found in the uterine flush of the ILB1 receptor antagonist-treated mice. Even though all the blastocysts appeared to be morphologically normal, they were delayed in development comparing to the non injected group, were still surrounded by the zona pellucida and were not able to implant (Simón *et al.* 1998).

Fibroblast Growth Factor

Fibroblast growth factors (FGFs) are represented by more than 22 *FGF* genes and 4 *FGFR* genes with different temporal and spatial patterns of expression during development (Itoh and Ornitz 2004; Itoh and Ornitz 2008; Gotoh 2009). Specifics FGFs, *i.e. FGF2*, are required for self-renewal and maintenance of pluripotency activity in mouse and human embryos and embryonic stem (ES) cells (Gotoh 2009). Interestingly, *FGF4* is necessary for cellular differentiation in mouse and human ES cells (Kunath *et al.* 2007) but is needed to maintain multipotency and self renewal in trophoblast stem cells (Guzman-Ayala *et al.* 2004).

In the cow, FGF2 has been identified within the endometrium and in the uterine lumen at Days 17-18 of the estrous cycle in pregnant and non-pregnant females (Michael *et al.* 2006). Moreover, in the ewe flush, FGF2 concentrations in uterine flushings increase around Days 12-13 after estrus (Ocón-Grove *et al.* 2008).

In bovine embryos, the best characterized effect of FGF2 is on TE growth and IFNT secretion. Supplementation of a TE bovine cell line (CT-1) with FGF2 increases cell proliferation and IFNT secretion (Michael *et al.* 2006). In addition, treatment of IVP blastocysts with FGF2 increased the expression of *IFNT* mRNA but had no effect on blastocyst cell number. Other FGFs, *i.e.* FGF1 and FGF10, have also increased the

steady state amounts of mRNA for *IFNT* in CT-1 cells as well as IFNT biological activity (Cooke *et al.* 2009).

Tumor Necrosis Factor

Tumor necrosis factor (TNF) is a multifunctional cytokine that first identified as a regulator of immunological and inflammatory responses in several tissues, including the reproductive tract (Hunt *et al.* 1996). It is produced by macrophages and oviductal epithelial cells in the mouse (Hunt 1993), human (Morales *et al.* 2006) and bovine (Wijayagunawardane and Miyamoto 2004) and by blastocysts in mouse and human (Hunt *et al.* 1996). In the bovine endometrium, TNF can increase $PGF_{2\alpha}$ secretion and lead to luteolysis (Murakami 2001; Skarzynski *et al.* 2005; Siemieniuch *et al.* 2009).

Exposure of mouse blastocysts to TNF decreased cellular proliferation and increased the percentageage of blastomeres that were apoptotic (Pampfer *et al.* 1997). Furthermore, there was a decrease in the number of cells in the ICM and an increase in reabsorption rate when these blastocysts were transferred to recipients (Wuu *et al.* 1999). In bovine, TNF did not affect development of embryos to the blastocyst stage (Soto *et al.* 2003) but it did increased the percentageage of apoptotic blastomeres in embryos exposed to the cytokine at Days 4, 5 and 6 after insemination (Loureiro *et al.* 2007).

It is also possible that TNF is associated with mastitis, as elevated concentrations of TNF are found in the animals after infections in the mammary gland or infusion of lipopolysaccharide (Hansen *et al.* 2004). Cows that present mastitis have a reduction in fertility as there is an increase in the Days to first service, Days open and service per conception (Barker *et al.* 1998; Schrick *et al.* 2001). In mice, embryonic losses

associated with diabetes have been related to excessive production of TNF in the uterus (Pampfer 2001).

Colony Stimulating Factor 2

Biology and Signaling

CSF2 is a polypeptide growth factor of 124 amino acids and with a molecular weight of 14,138 (Metcalf 1985). Most adult organs synthesize detectable amounts of CSF2 (Burgess and Metcalf 1980); however increased secretion requires stimulation of cytokines, antigens, microbial products or inflammatory agents (Conti and Gessani 2008). In the human serum, concentrations of CSF2 range from 20 to 100 pg/ml (Conti and Gessani 2008). The main role of CSF2 is to promote survival and activation of neutrophils, eosinophils and macrophages, as well as dendritic cell maturation and differentiation of alveolar macrophages and invariant natural killer T cells (*i*NKT) (Barreda *et al.* 2004; Conti and Gessani 2008; Hercus *et al.* 2009). It is thought to promote the necessary communication between the hematopoietic cells and local tissues in the event of inflammation (Hercus *et al.* 2009) as well as to enhance proinflamatory cytokine production (Brissette *et al.* 1995) (Figure 1-1).

CSF2 deficient mice present deficient alveolar macrophage maturation, which leads to the development of abnormal lungs (Stanley *et al.* 1994), and compromised *i*NKT cellular differentiation (Bezbradica *et al.* 2006). Administration of exogenous CSF2 corrected these defects (Bezbradica *et al.* 2006). Furtheremore, CSF2 injection in mice caused an increase in the levels of circulatory neutrophils and cycling peritoneal macrophages (Hamilton 2002) and administration of CSF2 specific antibody caused a decrease in inflammation in the skin (Schön *et al.* 2000). Other inflammatory reactions

are promoted through activation of adhesion events and increased cell survival through inhibition of apoptosis (Hamilton 2002).

Many or all cells in the stem and progenitor compartments exhibit receptors for CSF2 and are responsive to stimulation by this molecule (Metcalf *et al.* 1980). CSF2 receptors are expressed at very low levels (100–1000 per cell) and comprise a cytokine-specific α subunit and a β common (βc) subunit that interacts with various receptor-associated proteins important for the signaling downstream of the receptor (Quelle *et al.* 1994; Guthridge *et al.* 1998; Carr *et al.* 2001; Mirza *et al.* 2010). Each α subunit binds the cytokine with low affinity but the presence of βc converts this to a high affinity reaction causing dimerization of both subunits and receptor activation (Carr *et al.* 2001; Mirza *et al.* 2010). Upon binding to CSF2, the CSF2 receptor complex is a high-order dodecamer composed of two hexamers with a stoichiometry of 2 CSF2, 2 α subunits and 2 βc subunits (Hansen *et al.* 2008) (Figure 1-1).

CSF2 and CSF2 receptors can be rapidly consumed by internalization of the complex followed by ligand endocytosis, lysosomal degradation and direct proteosomal degradation of the βc cytoplasmic domain. Furthermore, the α subunit mRNA is downregulated in response to the βc stimulation (Barreda *et al.* 2004).

One prominent interaction partner of CSF2 is Janus kinase 2 (JAK2), a tyrosine kinase that binds to the βc subunit for subsequent transphosphorylation and activation. Activated JAK2 phosphorylates tyrosine residues of the β subunit and generates binding sites for Src-homology 2 domains of other proteins such as members of the signal transducer and activator of transcription (STAT) family. JAK2 can also phosphorylate STAT proteins themselves. In parallel, additional signaling pathways can be activated,

such as the Ras–Raf–extracellular signal-regulated kinase (Ras-Raf-ERK) pathway, which is important for triggering the cell cycle and is activated by binding of the adapter proteins Shc and Grb2 to the βc (Degroot 1998; Barreda *et al.* 2004; Choi *et al.* 2007). While activation of JAK2/STAT is mainly responsible for CSF2 induced cell proliferation, phosphatidylinositol-3 kinase (PI3K), another signaling pathway triggered by the βc subunit has a role in regulation of apoptosis and cell survival (Degroot 1998; Dhar-Mascareno *et al.* 2005). One difference between these pathways in response to CSF2 is the concentration necessary for activation. For example, the apoptosis inhibition reaction occurs at significantly lower concentrations (100 fold) than those required for stimulation of cell proliferation (Barreda *et al.* 2004) (Figure 1-1).

Data indicate that the α subunit, but not the βc protein, is present in blastomere cell membranes of mouse and human preimplantation embryos (Robertson *et al.* 2001; Sjöblom *et al.* 2002).

CSF2 Secretion in the Uterine Tract

CSF2 is expressed at the protein and mRNA level in endometrial epithelial cells of humans and mice (Chegini *et al.* 1999; Robertson *et al.* 2001), in human fallopian tube (Zhao and Chegini 1994) and in human first trimester decidua (Segerer *et al.* 2009). In the cow, CSF2 has been localized in the oviductal epithelium (greater in ampulla) (de Moraes *et al.* 1999), endometrium (mostly in the luminal epithelium and the apical portions of the glands) (de Moraes *et al.* 1999; Emond *et al.* 2004) and in the myometrium after Day 30 of pregnancy (Emond *et al.* 2004).

On Days 14-17 after estrus, concentrations of CSF2 in uterine flushing in cattle tended to be higher in pregnant cows than cyclic cows (de Moraes *et al.* 1999). For

cyclic cows, concentrations of CSF2 tended to be lowest during estrus compared with Days 7, 13-16 and 18 of the cycle (de Moraes *et al.* 1999).

This pattern in the cow is unlike the mouse where CSF2 concentrations in the uterus peak during estrus (Robertson *et al.* 1996). Moreover, CSF2 release in the mouse is stimulated by specific factors in seminal plasma, including transforming growth factor (TGFB) (Tremellen *et al.* 1998). Expression declines at implantation under the inhibitory influence of progesterone (Robertson *et al.* 1996), but bioactive CSF2 can be detected in placental and decidual tissues for the duration of pregnancy (Crainie *et al.* 1990). In pregnant bitches, CSF2 mRNA was found at the onset of implantation/placentation (Beceriklisoy *et al.* 2009).

Actions of CSF2 on Embryo Development and Survival

The use of knock-out models in mice have indicated that CSF2 is important for normal embryonic development. Despite a regular number of implantation sites, offspring from CSF2 null mice are 25% of normal size, have a 4.5 fold increase in mortality rate during the first three weeks of life and a fetal growth retardation that persists through adulthood (Seymour *et al.* 1997; Robertson *et al.* 1999). Total cell number of blastocysts from CSF2-/- mice was reduced by 14-18% (Robertson *et al.* 2001). Cell number of embryos from CSF2 -/- mice was increased 24% when cultured with exogenous CSF2 (Robertson *et al.* 2001). Mitogenic actions of CSF2 were also demonstrated in a study in which mouse embryos were cultured in media supplemented with CSF2. The cytokine increased total cell numbers and ICM cell numbers of CF1 mice blastocysts (more sensitive strain), but only in the absence of human serum albumin (Karagenc *et al.* 2005). There was no effect of CSF2 on TE cell numbers.

Another line of evidence for beneficial effects of CSF2 on preimplantation embryonic development is the improvement in the proportion of cultured embryos that develop to the blastocyst stage *in vitro* in the cow (de Moraes and Hansen 1997a), human (Sjöblom *et al.* 1999), mouse (Robertson *et al.* 2001), and pig (Cui *et al.* 2004). Bovine embryos seem to be more responsive to CSF2 when it is added to culture at Day 5 after insemination than when added within 24 hours of insemination. When added at Day 5, CSF2 increased the proportion of oocytes becoming blastocysts (de Moraes and Hansen 1997a). When added after insemination, CSF2 had no effect on the proportion of embryos developing past the 8 cell stage (de Moraes and Hansen 1997a). The late effect of CSF2 coincides with the time that the embryo enters the uterus (Betteridge *et al.* 1988).

Murine embryos cultured in CSF2 have a higher rate of metabolic activity and requirements. CSF2 elicited a 50% increase in the uptake of the non-metabolizable glucose analogue, 3-O-methyl glucose, in mouse blastocysts (Robertson *et al.* 2001).

One possible reason for effects of CSF2 on the proportion of embryos becoming blastocysts may be the antiapoptotic effects of the cytokine. Culture of mouse embryos with CSF2 decreased the number of apoptotic blastomeres by 50% and increased the number of viable ICM cells by 30% as a result of both antiapoptotic and proliferative effects (Sjöblom *et al.* 2002). In another study, CSF2 protected one cell mouse embryos from freezing damage and decreased the apoptotic index to zero (Desai *et al.* 2007).

The means by which CSF2 protects against apoptosis in embryos is not clear. In neurons, polymorphonuclear neutrophils and HeLa cells, CSF2 can activate the PI3K-Akt pathway which decreases apoptosis by induction of BCL-2 and BCL2L1 (previous

known as BcL-XL) (Antignani and Youle 2007; Schäbitz *et al.* 2008). Using microarray analysis, CSF2 was recently shown to decrease expression of stress response genes and apoptosis genes in mouse blastocysts (Chin *et al.* 2009). *In vivo*, CSF2 null mutant mice had elevated expression of heat shock protein 1 (*Hsph1*) (Chin *et al.* 2009).

Sjöblom *et al.* (2005) found that survival of mouse embryos after transfer into recipient females was greater when embryos were cultured with CSF2 than when embryos were cultured without the cytokine (90% vs 76%). Along with this finding, there was evidence that CSF2 treatment during the preimplantation period affected placental development after transfer. Compared with values for fetuses produced *in vivo*, fetal placental weight ratio decreased by 8% for fetuses from CSF2 treated embryos versus 11% for fetuses from embryos cultured in medium alone.

CSF2 and Interferon-tau Secretion

One way in which CSF2 could improve post-transfer embryonic survival in cattle would be through increased secretion of IFNT by elongated conceptuses. Imakawa *et al.* (1993 and 1997) and Rooke *et al.* (2005) demonstrated that CSF2 stimulates production of IFNT by sheep trophoblast *in vitro*. Similar results were found when a cell line derived from bovine trophoblast cells (CT-1 cells) was treated with porcine CSF2 (Michael *et al.* 2006). On the other hand, de Moraes *et al.* (1997b) found no beneficial effect of bovine CSF2 on IFNT secretion by bovine blastocysts at Day 7-8 after insemination.

Goals of the Current Investigation

The aim of this dissertation is to understand the role of CSF2 in preimplantation embryonic development including its effects on blastocyst formation, the embryonic transcriptome, and long term embryonic survival. Based on the literature review, it is

likely that CSF2 can act as a regulator of preimplantation embryonic development, enhance embryo competence for survival, induce genes that promote proliferation, inhibit apoptosis and promote critical functions necessary for post-transfer survival.

To accomplish our goal we pursued the following research objectives:

Research objective 1: to determine the effects of CSF2 on blastocyst yield and capactity for survival after transfer into recipients, and evaluate properties of the blastocyst formed after CSF2 treatment.

Research objective 2: to determine the transcriptome of CSF2 treated embryos.

Research objective 3: test whether treatment of embryos with CSF2 increases post-transfer growth and IFNT secretion and alters the transcriptome of the ICM and TE.

Results from the proposed studies will add information regarding the biology of CSF2 during preimplantation embryonic development. The first objective establishes that CSF2 is an important regulator of embryonic development and post-transfer survival. The second objective seeks to identify genes and pathways through which CSF2 regulates embryo development and survival. The third objectives evaluates whether exposure to CSF2 during the preimplantation period leads to changes in gene expression later in pregnancy that could enhance subsequent embryonic and fetal development.

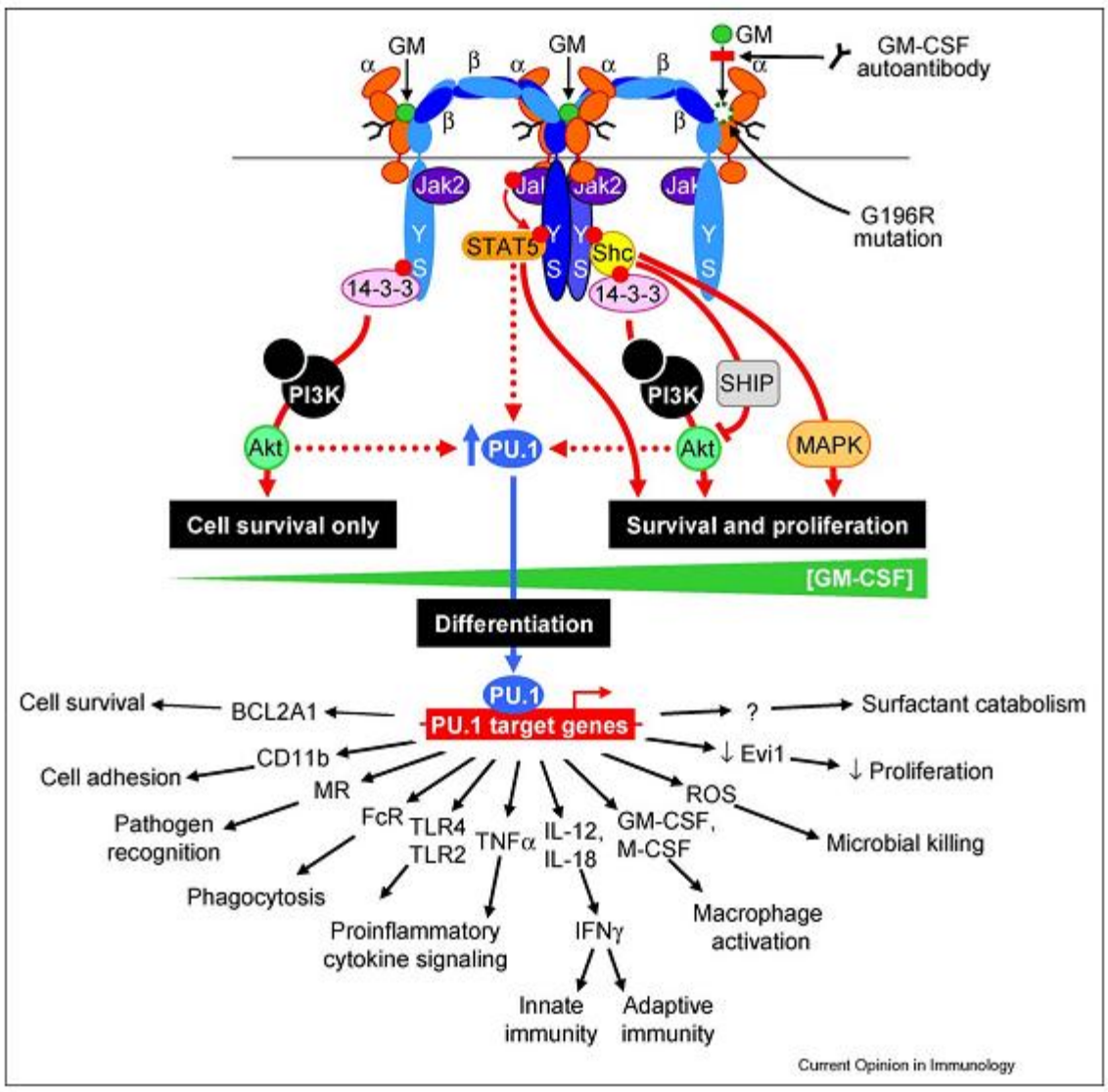

Figure 1-1. Mechanisms by which CSF2 regulates cellular survival, differentiation, functions and activation. CSF2 initiates signaling by first binding to the CSF2 receptor α, which then associates with homodimers of the affinity-enhancing CSF2 receptor βc. Jak2 is bound constitutively to the βc chain and signals through an intracytoplasmic βc chain motif. At low concentrations, CSF2 activates PI3K and Akt signaling resulting in cell survival without proliferation. At high concentrations, CSF2 activates STAT or Shc-dependent pathways stimulating cell survival, cellular activation and proliferation. Pulmonary CSF2 regulates the expression of numerous genes enabling multiple immune and non-immune functions. CSF2 is also known as GM-CSF. Figure reproduced with permission from Current Opinion in Immunology 2009, 21:514–521.

CHAPTER 2
COLONY STIMULATING FACTOR IMPROVES DEVELOPMENT AND POST-TRANSFER SURVIVAL OF BOVINE EMBRYOS PRODUCED *IN VITRO*

Introduction

The embryo executes its developmental program in the female reproductive tract in an environment largely dictated by the mother. An inadequate maternal environment can lead to reduced embryonic survival (Hansen 2007; Leroy *et al.* 2008; Robinson *et al.* 2008) and epigenetic changes that persist into adulthood (Sinclair *et al.* 2007). The maternal environment affects embryonic development by providing to the embryo an array of nutrients and regulatory molecules and by expression of cell adhesion molecules that facilitate eventual attachment and placentation (Spencer *et al.* 2008). Among the regulatory factors shown to affect preimplantation development in the cow, for example, are insulin-like growth factor-1 (Block 2007), interleukin 1, beta (Paula-Lopes *et al.* 1998), activin (Park *et al.* 2008) and granulocyte-macrophage colony stimulating factor (CSF2) (de Moraes and Hansen 1997a).

There are strong lines of evidence to implicate CSF2 as a physiologically-important regulator of early embryonic development. The cytokine is expressed in luminal epithelium and other tissues of the oviduct and endometrium (Zhao and Chegini 1994; Chegini *et al.* 1999; de Moraes *et al.* 1999; Emond *et al.* 2004). In mice, production of CSF2 fluctuates during the estrous cycle, peaking during estrus (Robertson *et al.* 1996). Expression declines at implantation under the inhibitory influence of progesterone (Robertson *et al.* 1996). Release of CSF2 into the uterine lumen is stimulated by specific factors in seminal plasma, including TGFB (Tremellen *et al.* 1998). In cattle, amounts of immunoreactive CSF2 in the endometrium of cyclic cows tends to be low during estrus and high from Days 13 to 17 after estrus before declining

as estrus approaches (de Moraes *et al.* 1999; Emond *et al.* 2004). Immunoreactive CSF2 in the endometrial luminal epithelium increases in response to the maternal recognition of pregnancy signal interferon-tau (*IFNT*) so that amounts of immunoreactive CSF2 are higher in pregnant cows at Day 16 and 18 after estrus compared with cyclic cows (Emond *et al.* 2004).

Addition of CSF2 to culture medium improved the proportion of *in vitro* embryos developing to the blastocyst stage in the cow (de Moraes and Hansen 1997a), human (Sjöblom *et al.* 1999) and pig (Cui *et al.* 2004) and increased post-transfer embryonic survival in mice (Sjöblom *et al.* 2005). Total cell numbers of blastocysts from CSF2-/- mice were significantly reduced by 14-18% compared with wild-type controls (Robertson *et al.* 2001). Despite a regular number of implantation sites, offspring from CSF2-/- mice were 25% of normal size, exhibited fetal growth retardation that persists through adulthood, and experienced increased mortality during the first three weeks of life (Seymour *et al.* 1997; Robertson *et al.* 1999).

The bovine preimplantation embryo is a good model for studying maternal regulation of embryonic development. Development to the blastocyst stage is not absolutely dependent upon regulatory molecules present in the reproductive tract because embryos that give rise to live calves after transfer to recipient can be produced in culture in growth-factor free media (Block and Hansen 2007). However, embryos produced by *in vitro* oocyte maturation, fertilization and culture have aberrant biochemical and molecular properties compared with embryos produced *in vivo* (Lazzari *et al.* 2002; Rizos *et al.* 2002). Deviation in embryonic function associated with production *in vitro* is due in part to an inadequate environment during the

preimplantation period. This is so because the gene expression and cryotolerance of bovine embryos produced by *in vitro* fertilization was enhanced when embryos were cultured *in vivo* in the sheep oviduct after insemination (Rizos *et al.* 2002; Rizos *et al.* 2002).

Here we tested the possible role of CSF2 as one of the regulatory molecules that mediate maternal effects during the preimplantation period on survival through the embryonic and fetal periods. The approach was to determine whether addition of CSF2 to culture medium enhances the development and post-transfer survival of bovine embryos produced *in vitro*. As a positive control, some embryos were treated with insulin-like growth factor-1 (IGF1), which to date, is the only growth factor shown to improve survival of bovine embryos transferred to recipients. This molecule protects embryos from the effects of elevated temperature (Jousan and Hansen 2004; Jousan *et al.* 2008) and pregnancy and calving rates of cows exposed to heat stress were improved when embryos used for transfer were treated with IGF1 (Block *et al.* 2003; Block and Hansen 2007). However, no improvement in post-transfer survival was seen when cows were not heat-stressed (Block and Hansen 2007). The current studies were conducted in both hot and cool seasons.

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (HEPES-TL), *in vitro* fertilization (IVF)-TL, and Sperm-TL were purchased from Caisson (Sugar City, ID) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP) and IVF-TALP as previously described (29). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Hyclone, Logan UT) supplemented with 2% (v/v)

bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Folltropin-V; Belleville, ON), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden).

Potassium simplex optimized medium (KSOM) that contained 1 mg/ml bovine serum albumin (BSA) was obtained from Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). On the Day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto *et al.* 2003). Prostaglandin $F_{2\alpha}$ (PGF) was Lutalyse® from Pfizer (New York, NY, USA) and gonadotrophin releasing hormone (GnRH) was Cystorelin® from Merial (Duluth, GA, USA). Dithiothreitol (DTT) was from Sigma-Aldrich (St. Louis MO, USA).

Recombinant bovine CSF2 was donated by CIBA-GEIGY (Basle, Switzerland). Recombinant human modified IGF1 (E3R) was obtained from Upstate Biotech (Lake Placid, NY, USA).

Effects of CSF2 on Embryo Development and Blastocyst Properties

Production of embryos

Embryo production was performed as previously described (Soto *et al.* 2003). Cumulus oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact

cumulus cells were washed two times in OCM, placed in groups of 10 in 50-μl microdrops of OMM overlaid with mineral oil and matured for 20–22 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in humidified air.

Matured COCs were washed once in HEPES-TALP and transferred in groups of 50 to 4-well plates containing 425 μl of IVF-TALP and 20 μl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with ~1 x 10^6 Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls. After 8-10 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex in HEPES-TALP, and placed in groups of 30 in 50-μl microdrops of KSOM-BE2. Putative zygotes were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 or 5% CO_2, 5% O_2, and 90% N_2. Embryos received treatment at Day 0 (i.e., immediately after insemination) or Day 5 after insemination according to the specific experimental design.

Interactions between oxygen concentration and presence of CSF2

This experiment was designed to determine the effects of CSF2 on embryonic development at culture conditions of high oxygen [5% (v/v) CO_2 in air] or low oxygen (5% CO_2, 5% O_2, and 90% N_2, v/v) and when added at Day 0 or 5 after insemination. After removal from fertilization drops, embryos were washed, placed in microdrops of KSOM-BE2 medium and cultured in high O_2 or low O_2. If treatment was Day 0 after insemination, embryos were placed in a 50 μl KSOM-BE2 drop ± 10 ng/ml CSF2. When treatment was added at Day 5, embryos were placed in a 45 μl KSOM-BE2 drop and 5 μl of KSOM-BE2 ± 10 ng/ml CSF2 were added to the drop at Day 5 after insemination.

The concentration was chosen based on results from other experiments that addition of CSF2 at this concentration would increase blastocyst yield (de Moraes and Hansen 1997a). Cleavage rate was assessed at Day 3 after insemination and the percentageage of oocytes that became blastocysts was assessed at Days 7 and 8 after insemination. The experiment was replicated 11 times using 2673 oocytes.

Cell number and differentiation of blastocysts

The experiment was designed to test whether CSF2 would increase cell number of bovine blastocysts and alter cell allocation between the trophectoderm (TE) and inner cell mass (ICM). After insemination, embryos were cultured in 45 µl microdrops of KSOM-BE2 at 38.5°C in a humidified atmosphere 5% CO_2, 5% O_2, and 90% N_2. At Day 5 after insemination, 5 µl of CSF2 (to create a final concentration of 10 ng/ml) or 5 µl of vehicle (KSOM-BE2) were added to the drops. Zona-intact blastocysts were removed from culture at Day 7, washed two times in 50-µl microdrops of 10 mM KPO_4, pH 7.4 containing 0.9% (w/v) NaCl and 1 mg/ml PVP (PBS-PVP) by transferring the embryos from microdrop to microdrop. To label TE cells, embryos were placed in 100 µl PBS-PVP containing 0.5% (v/v) Triton X-100 and 100 µg/ml propidium iodine for 30 s at 37°C. Embryos were immediately washed in PBS-PVP. Embryos were then incubated in 50 µl drops of PBS-PVP containing 4% (w/v) paraformaldehyde and 10 µg/ml Hoechst 33258 for 15 min at room temperature to fix the embryos and stain ICM. Embryos were washed in PBS-PVP and mounted on slides using Prolong Gold Antifade (Invitrogen, Eugene, Or, USA) and coverslips placed on the slides. Labeling of propidium iodine and Hoechst was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for the number of

ICM (blue nuclei) and TE (pink nuclei), and total cell number (blue + pink nuclei) with a DAPI filter using a 20x objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. The experiment was replicated 3 times using 18-36 embryos/group.

Apoptotic blastomeres

This experiment was designed to test the hypothesis that CSF2 decreases the percentageage of blastomeres that are positive for the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction. Embryos were cultured and treated with CSF2 as described above for analysis of TE and ICM. On Day 7 after insemination, blastocysts, expanded blastocysts and hatching blastocysts were removed from culture and washed two times in 50-μl microdrops of PBS-PVP. Embryos were fixed in a 50-μl microdrop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 600 μl of PBS-PVP at 4°C until analysis for TUNEL labeling as described previously (Jousan and Hansen 2004; Jousan *et al.* 2008). The experiment was replicated 3 times using 31-58 embryos/group.

Effects of CSF2 and IGF1 on Pregnancy and Calving Success after Transfer to Recipients

Production of Holstein embryos using X-sorted semen

Embryo production was performed as described above with slight modifications. Holstein COCs were purchased from one of three suppliers (Evergen, Storrs, CT, USA; Bomed, Madison, WI, USA; Trans Ova, Sioux City, IA, USA) and shipped overnight in a portable incubator at 38.5°C in maturation medium or collected from slaughter house ovaries. After 20-24 hours, COCs were removed from maturation medium, washed one

time in Hepes-TALP and transferred in groups of 30 to fertilization drops covered with mineral oil. Each drop contained 50 µl IVF-TALP and then 3 µl PHE and 20 µl sperm purified by Percoll gradient were added to this. The final concentration of sperm was $1x10^6$ per ml. For each replicate, two to four straws of X-sorted semen from one Holstein bull (Sexing Technologies Inc., Navasota TX) were used depending on the number of oocytes that had to be fertilized. In total 4 different bulls were used throughout the experiment.

After 20-22 h at 38.5°C and 5% (v/v) CO_2, presumptive zygotes were removed from fertilization drops, vortexed in 30 µl Hepes-TALP for 5 min in a microcentrifuge tube and washed two times to remove cumulus cells and associated spermatozoa. Putative zygotes (25-30/drop) were placed in 45 µl (treatment at Day 5) or 50 µl (treatment at Day 0) of KSOM-BE2 overlaid with mineral oil. Embryos were cultured at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (v/v). Treatments were added at either Day 0 (i.e., immediately after insemination) or Day 5 after insemination as described for each experiment.

The proportion of oocytes that cleaved was recorded at Day 3 after insemination. Morula, blastocyst and expanded blastocyst stage embryos classified as Grade 1 (Robertson and Nelson 1998) were harvested on Day 7 (Farm 1 and 2) and Day 8 (Farm 3) and loaded into 0.25 ml straws in HEPES-TALP supplemented with 10% fetal calf serum and 50 µM DTT. Straws containing selected embryos were then placed horizontally into a portable incubator (Cryologic, Mulgrave, Vic, Australia) at 38.5°C and transported to the respective farm.

Animals

The experiment was conducted at 3 locations: Farm 1 (Brooksco Dairy, Quitman, GA, USA; 30°47′5″ N, 83°33′39″ W), Farm 2 (University of Florida Dairy Research Unit, Hague, FL, USA; 29°46'21"N, 82°24'54"W) and Farm 3 (Alliance Dairy, Trenton, FL, USA; 29°34'35"N, 82°51'17"W). At Farm 1, 271 primiparous and multiparous lactating Holstein cows between 76 and 154 Days in milk (mean = 84) were used as recipients from June 29 - December, 12, 2007. Cows were housed in a free-stall barn equipped with fans and a sprinkler system. Overall, 15 replicates were completed with 8-30 recipients per replicate. At Farm 2, a total of 100 primiparous and multiparous lactating Holstein cows between 75 and 376 Days in milk (mean = 161) were used as recipients from December 7, 2007 - February 1, 2008. Recipients were housed in a free stall barn equipped with fans and sprinklers. Overall, 6 replicates were completed with 12–26 recipients per replicate. A single replicate was performed at farm 3 on December 23, 2007 using a total of 21 multiparous lactating Holstein cows between 168 and 468 Days in milk (mean = 267). Recipients were housed in a free stall barn equipped with fans and sprinklers.

Synchronization and timed embryo transfer

Each week, eligible cows were organized into a group (i.e. replicate) and ovulation synchronized for embryo transfer. The timed ovulation protocol was the Ovsynch-56 procedure (Brusveen *et al.* 2008). Day 0 was considered the Day of expected ovulation. Hormonal treatments consisted of 100 µg GnRH, i.m. on Day -10; 25 mg $PGF_{2\alpha}$, i.m. on Day -3; and 100 µg of GnRH i.m. at 56 hours after $PGF_{2\alpha}$. For first-service cows only, the timed ovulation protocol was preceded by a Presynch protocol (two injections of 25 mg $PGF_{2\alpha}$, i.m. 14 Days apart), with the last injection 14 Days before initiation of the

timed ovulation protocol. Cows were diagnosed for the presence of corpus luteum (CL) at Day 7 after anticipated ovulation using an Aloka 500 ultrasound equipped with a 5 MHz linear array transducer. Cows diagnosed with a CL received epidural anesthesia [5 ml of 2% (w/v) lidocaine] and a single embryo transferred to the uterine horn ipsilateral to the ovary via the transcervical route.

Effect of CSF2 and IGF1 added at Day **1 of culture on development and post-transfer survival of bovine embryos**

The experiment was conducted from June 29 through August 31, 2007 at Farm 1 in 7 replicates. At Day 1 after insemination, presumptive zygotes were randomly distributed to 50 µl KSOM-BE2 drops with one of three treatments: 1) KSOM-BE2 alone, 2) 10 ng/ml CSF2 or 3) 100 ng/ml IGF1. Cleavage rate was assessed at Day 3 after insemination. At Day 7 after insemination, Grade 1 morula, blastocyst and expanded blastocyst stage embryos, considered transferable embryos, were harvested and transferred to lactating dairy cows synchronized using the PreSynch/Ovsynch protocol. Pregnancy was diagnosed between Days 30-35 of gestation using ultrasonography and the incidence of calving recorded. Transfers were performed for 51-55 cows/treatment.

Effect of CSF2 added at Day **5 of culture and IGF1 added at** Day **1 of culture on development and post-transfer survival of bovine embryos**

The experiment was conducted from September 7, 2007 through February 1, 2008 at Farms 1-3 in 15 replicates. In 8 replicates, presumptive zygotes were randomly assigned to either 50 µl KSOM-BE2 (control) 2) 50 µl KSOM-BE2 + 100 ng/ml IGF1 added at Day 1 after insemination or 3) 50 µl KSOM-BE2 + 10 ng/ml CSF2 added at Day 5 after insemination. Embryos assigned to the CSF2 group were placed in 45 µl KSOM-BE2 drop at Day 1 after insemination. At Day 5 after insemination, an aliquot of 5 µl KSOM-BE2 + 100 ng/ml CSF2 was added to the drops to achieve a final

concentration of 10 ng/ml CSF2. The remaining 7 replicates used similar treatments except the control treatment was changed so that embryos were placed in 45 µl drops of KSOM-BE2 at Day 1 after insemination and 5 µl KSOM-BE2 were added to the drops at Day 5 after insemination. Because there was no statistical difference between the two types of control embryos, control data were pooled. Cleavage rate was assessed at Day 3 after insemination. At Day 7 after insemination, Grade 1 morula, blastocyst and expanded blastocyst stage embryos were harvested and transferred to lactating dairy cows synchronized using the PreSynch/Ovsynch-56 or Ovsynch-56 protocol. Pregnancy was diagnosed between Days 30-35 of gestation using ultrasonography and the incidence of calving recorded. Transfers were performed for 44-107 cows/treatment.

Statistical Analysis

Data on the percentageage of oocytes that cleaved and that became blastocysts and transferable embryos, the percentageage of cells that were TUNEL-positive and the ICM/TE ratio were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 9.0, Cary, NC). Data were transformed by arcsin transformation before analysis. The mathematical model included main effects and all interactions. Replicate was considered as a random effect and other main effects were considered fixed. Tests of significance were made using error terms determined by calculation of expected mean squares. All values reported are least-squares means ± SEM. Probability values are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

Data regarding the percentageage of cows that became pregnant after transfer were analyzed by logistic regression using the LOGISTIC procedure of SAS. Treatment

effects were separated into individual degree of freedom comparisons using orthogonal contrasts. Three sets of contrasts were compared. The first, testing the hypothesis that both CSF2 and IGF1 would increase pregnancy and calving rate, were control vs IGF1 and CSF2 and IGF1 vs CSF2. The second, testing the hypothesis that only CSF2 would increase pregnancy and calving rate, were control and IGF1 vs CSF2 and control vs IGF1. An additional contrast was also made to compare control to CSF2. Pregnancy loss was analyzed for the first and second experiment and for all the losses in both experiments by χ^2 analysis.

Results

Effects of CSF2 on Embryo Development

The experiment evaluated whether addition of CSF2 at Day 0 or 5 after insemination would increase the percentageage of oocytes that became blastocysts. Embryos were cultured in either high oxygen (air) or low oxygen (5%, v/v). Overall, there was no effect of CSF2 on cleavage rate at Day 3 after insemination, with averages varying from 73-80%. There was a tendency (P=0.09) for CSF2 to increase the percentage of oocytes that became blastocysts on Day 7 after insemination (Figure 2-1, Panel A). Development was higher in low oxygen (P<0.0001) but there was no interaction between Day of treatment or oxygen concentration. On Day 8 after insemination (Figure 2-1, Panel B), CSF2 increased (P=0.05) blastocyst development , development was higher (P<0.0001) in low oxygen and there was a treatment x Day x oxygen interaction (P=0.06). The observed 3 way interaction between treatment, Day and oxygen is due to the fact that the difference in response to addition of CSF2 at Day 0 vs Day 5 depended upon oxygen concentration. For embryos cultured in low oxygen, CSF2 increased blastocyst development to a greater extent when added at Day 5 rather

than at Day 1. For embryos in high oxygen, CSF2 increased blastocyst development to a greater extent when added at Day 1.

Effects of CSF2 on Blastocyst Total Cell Number, Cell Differentiation and Apoptosis

As shown in Table 2-1, blastocysts formed in the presence of CSF2 had a tendency for an increased (P=0.066) number of ICM cells and greater ICM/TE ratio (P< 0.02) when compared with control embryos. There was no significant effect of CSF2 on total cell number (Table 2-3) or on the percentage of blastomeres labeled as TUNEL-positive (Table 2-4).

Effects of CSF2 and IGF1 on Pregnancy and Calving Success after Transfer to Recipients

In the first experiment, treatments were added at Day 1 of culture and recipients were used in the summer during heat stress (Table 2-3). There was a significant increase in the percentageage of cleaved embryos that became transferable morulae or blastocyst on Day 7 after insemination when embryos were treated with CSF2 (control vs CSF2, P<0.01; control and IGF vs CSF2, P<0.02; control vs IGF and CSF2, P<0.02; Table 2-3). Treatment did not have a significant effect on pregnancy rate at Day 30-35 of gestation or on calving rate although, numerically, pregnancy and calving rates were higher in the IGF1 group than for the other two groups. Pregnancy loss between Day 30-35 and term was significantly lower for recipients that received a CSF2 treated embryo compared with control embryos (CSF2 vs control, P<0.05; CSF2 and IGF1 vs control, P<0.05; Table 2-3). Of the calves born, 85% were female.

The second experiment was conducted largely during the cool season. As for the first experiment, IGF1 was given to embryos at Day 1 after insemination. In contrast, CSF2 was added at Day 5 of culture. Treatment with CSF2 was modified because of

data indicating greater effects on development when added at Day 5 (see Figure 2-1) and the failure of CSF2 treatment at Day 1 to affect pregnancy rate (Table 2-3). There was a significant increase in the percentageage of cleaved embryos that became transferable morulae or blastocyst on Day 7 after insemination when embryos were treated with CSF2 at Day 5 of culture (CSF2 vs control, $P<0.01$; CSF2 vs IGF, $P<0.01$; CSF2 and IGF vs control, $P<0.02$; CSF2 vs control and IGF, $P<0.02$; Table 2-4). Moreover, pregnancy rate (CSF2 vs control and IGF1, $P<0.05$; CSF2 vs IGF, $P<0.06$) and calving rate (CSF2 vs control, $P<0.05$; CSF2 vs control and IGF, $P<0.05$) were higher for cows receiving embryos treated with CSF2 than embryos receiving control or IGF1 treated embryos. There was no effect of treatment on pregnancy loss for any of the treatments but again pregnancy loss was numerically lower for cows receiving embryos treated with IGF1 or CSF2 than for cows receiving control embryos. Of the calves born, 85% were female.

When data from the two transfer experiments were pooled, the difference in pregnancy loss between cows receiving control embryos (9/40; 22.5%) and cows receiving either an embryo treated with either IGF1(3/36; 8.3%) or CSF2 (8/98; 8.1%) was significant ($P<0.025$).

Discussion

Experiments reported here implicate CSF2 as an important regulator of preimplantation development. Treatment of preimplantation bovine embryos with CSF2 increased the proportion of embryos that became blastocysts, increased the cell number in the ICM and improved the survivability of embryos after transfer to recipients. The effect of CSF2 on post-transfer survival involved both an increase in the proportion of embryos that established pregnancy by Day 30-35 (when treatment was from Day 5-

7 after insemination) and a reduction in the proportion of embryos at Day 30-35 which were lost before completion of gestation (when treatment was from Day 1-7 or 5-7 after insemination). The fact that treatment with CSF2 during such a narrow window of development (from Day 1-7 or Day 5-7) altered embryonic function much later in pregnancy (after pregnancy diagnosis at Day 30-35) suggests that CSF2 is exerting epigenetic effects on the developing embryo that result in persistent changes in function during the embryonic and fetal periods of development.

The likelihood that actions of CSF2 during the preimplantation period on survival after Day 30-35 represent modifications of the epigenome implies that CSF2 may be one of the molecules through which changes in maternal physiology lead to alterations in fetal programming. An example of the importance of maternal function for conceptus development is induction of fetal overgrowth in sheep by transfer of embryos into an advanced uterine environment (Wilmut *et al.* 1981) or premature elevation of progesterone concentrations (Kleemann *et al.* 1994). Bovine embryos produced *in vitro*, which are not exposed to most of the regulatory molecules produced by the reproductive tract, are associated with an array of fetal abnormalities including increased rates of abortion, fetal overgrowth, and altered metabolism (Farin and Farin 1995; Lazzari *et al.* 2002; Miles *et al.* 2005). Maternal effects on conceptus development involve epigenetic alterations in DNA methylation patterns, as has been demonstrated for sheep exposed to nutritional stress (Sinclair *et al.* 2007) and for embryos produced *in vitro* in cattle (Suzuki *et al.* 2009).

Treatment with CSF2 increased the proportion of embryos becoming blastocysts regardless of whether it was added immediately after insemination or at Day 5 after

insemination (a time when embryos were at the morula stage of development). Similar results were seen in an earlier experiment with bovine embryos (de Moraes and Hansen 1997a). Thus, the action of CSF2 to increase the ability of embryos to advance to the blastocyst stage of development involves actions on the embryo during the transition from the morula to blastocyst stage of development. Perhaps, CSF2 is mitogenic and increases the number of embryos that have cell numbers sufficient for blastocoele formation. Another possibility, that CSF2 increases cell number by blocking apoptosis, is less likely. Although culture of human embryos with CSF2 decreased the number of apoptotic blastomeres by 50% (Sjöblom *et al.* 2002), no effect of CSF2 on apoptosis was observed in the present experiment.

Alternatively, CSF2 could increase one or more of the molecules involved in blastocyst formation. The observation that blastocysts from CSF2 treated embryos had proportionally more ICM cells relative to TE indicates the capacity of CSF2 to affect blastocyst differentiation. In the human (Sjöblom *et al.* 2002) and mouse (Karagenc *et al.* 2005), CSF2 treatment in culture resulted in more cells in the ICM of blastocysts.

The magnitude of the effect of CSF2 on blastocyst yield depended on the timing of exposure and the oxygen concentration used for culture. For embryos cultured in low oxygen, the increase in blastocyst yield caused by CSF2 was greater when the cytokine was added at Day 5 than at Day 0. The opposite was true when embryos were cultured in high oxygen. Differences in response between Day 0 and Day 5 may reflect degradation of CSF2 during culture or down-regulation of CSF2 receptors. The importance of oxygen as a determinant of the effect of timing of CSF2 precludes simple explanations, however.

The improvement in survival of embryos after transfer to recipients also varied with timing of CSF2 exposure. In the initial experiment, in which embryos were exposed to CSF2 from Day 1-7 after insemination, there was no effect of CSF2 on pregnancy risk at Day 30-35 of gestation. In the second experiment, however, when CSF2 treatment was from Day 5-7 after insemination, pregnancy risk at Day 30-35 was greater for cows receiving embryos treated with CSF2 than for cows receiving control embryos. Caution must be taken when interpreting these results. The first experiment was done during heat stress, a factor that can compromise embryonic survival (Hansen 2007) and involved relatively small numbers of transfers so that real treatment effects may not have been observed. What is clear is that, CSF2 reduced the loss of pregnancies occurring after Day 30-35 of gestation regardless of whether CSF2 was administered from Day 1-7 or 5-7 after insemination. In the mouse, as well, treatment of embryos with CSF2 in culture enhanced fetal and postnatal growth (Sjöblom *et al.* 2005). To our knowledge, CSF2 represents the only regulatory molecule shown to affect post-placentation events when acting during the preimplantation period.

As mentioned above, the effect of CSF2 to improve survivability of the conceptus after Day 30-35 of gestation is likely the result of alterations in the conceptus epigenome. Actions of CSF2 to affect pregnancy risk at Day 30-35 could involve changes in embryonic development related to functions important for establishment of pregnancy. One action of CSF2 that might enhance survival after transfer is the increase in number of ICM cells in the blastocyst. In the mouse, CSF2 increased the number of ICM cells (Karagenc *et al.* 2005) and post-transfer survival of embryos (Sjöblom *et al.* 2005). In the mouse, the number of cells in the ICM correlates with

viability after transfer (Lane and Gardner 1997). The ICM/TE ratio for bovine embryos produced *in vitro* differs considerably from embryos generated *in vivo* (Iwasaki *et al.* 1990; Crosier *et al.* 2001; Knijn *et al.* 2003). Moreover, a high proportion of embryos produced *in vitro* have no detectable embryonic disc by Day 14 of gestation (Fischer-Brown *et al.* 2004; Block *et al.* 2007). At Day 16 of gestation, embryonic discs were reported to be smaller for embryos produced *in vitro* than embryos produced *in vivo* (Bertolini *et al.* 2002). Perhaps, CSF2 minimizes the detrimental effects of culture on embryonic disc development or survival by increasing the ICM/TE ratio.

Another way in which CSF2 might improve post-transfer embryonic survival at Day 30-35 of gestation would be through increased secretion of the anti-luteolytic pregnancy recognition signal IFNT by elongated embryos. At least 40% of total embryonic losses have been estimated to occur between Days 8 and 17 of pregnancy (Thatcher *et al.* 1995) when the conceptus ordinarily inhibits pulsatile $PGF_{2\alpha}$ secretion. Treatment with CSF2 has been reported to increase IFNT production by elongated sheep embryos (Imakawa *et al.* 1993; Imakawa *et al.* 1997; Rooke *et al.* 2005) and a cell line derived from bovine trophoblast cells (Michael *et al.* 2006). On the other hand, de Moraes and Hansen (1997) found no beneficial effect of bovine CSF2 on IFNT secretion by bovine blastocysts at Day 7-8 after insemination. CSF2 could also affect expression of other genes during the preimplantation period. The production of embryos *in vitro* alters the expression of several developmentally important genes (Bertolini *et al.* 2002; Rizos *et al.* 2002; Sagirkaya *et al.* 2007; McHughes *et al.* 2009) and such alterations in gene expression can persist at least through Day 25 of gestation (Moore *et al.* 2007).

Treatment of embryos with IGF1 represents a positive control. This treatment has been reported to increase embryo survival when transfers to lactating cows are performed during periods of heat stress but to be without effect on embryonic survival when heat stress is not present (Block *et al.* 2003; Block and Hansen 2007). Similar results were obtained in the present study. In the first embryo transfer study, where cows were exposed to heat stress, pregnancy risk at Day 35 was highest for cows receiving embryos treated with IGF1. Treatment effects were not significant but the lack of significance may represent the small number of animals used for the study. In the second embryo transfer study, conducted mostly outside the time of year when heat stress is present, there was no improvement in pregnancy risk compared with the controls.

Interestingly, like CSF2, IGF1 reduced pregnancy loss after Day 30-35 in both studies. Thus, this growth factor may also cause changes in preimplantation development that result in changes in post-placentation development conducive for conceptus survival.

A practical outcome of this study is that CSF2 may prove useful as an additive to culture media for *in vitro* production of bovine embryos for commercial embryo transfer programs. There is a compelling need for such treatments. Pregnancy rates following transfer of embryos produced *in vitro* is lower than following transfer of *in vivo* derived embryos (Farin and Farin 1995). In addition, pregnancy loss after ~Day 40 of gestation is higher for embryos produced *in vitro* than for embryos produced *in vivo* IVP embryos that survive the fetal period are more likeable to be lost later on (Farin and Farin 1995).

Table 2-1. Effect of CSF2 on total cell number, inner cell mass (ICM), trophectoderm (TE) and ICM/TE ratio of blastocysts at Day 7 after insemination

	Control	CSF2	P-value
Total cell number	154 ± 9.02	170 ± 10.8	NS
ICM	41.9 ± 4.1	69.7 ± 4.6	0.066
TE	112 ± 5.6	106 ± 6.2	NS
Ratio ICM/TE	0.42 ± 0.023	0.66 ± 0.026	0.02

Values are expressed as the mean ± SEM

Replicates= 3; N= 18/36 embryos/group

Table 2-2. Effect of CSF2 on total cell number and TUNEL-positive blastomeres in blastocysts at Day 7 after insemination

	Control	CSF2	P-value
Total cell number	146 ± 5.3	154 ± 5.5	NS
Percentage of apoptosis	10.4 ± 1	8.7 ± 1	NS

Values are expressed as the mean ± SEM

Replicates= 3; N= 31/58 embryos/group

Table 2-3. Effect of CSF2 and IGF1 added at Day 1 of culture on embryonic development at Day 7, pregnancy risk at Day 30-35 (based on ultrasonography), calving rate and pregnancy loss among recipient cattle that received embryos that were cultured in 5% O_2.

	Transferable embryo yield[a] (percentage, %)	Pregnancy Risk	Calving Rate	Pregnancy Loss
Control (C)	17% ± 2	18/52 = 35%	14/52 = 27%	4/18 = 22%
IGF1 (I)	18% ± 2	24/55 = 43%	22/55 = 40%	2/24 = 8%
CSF2 (Day 1; G1)	25% ± 2	18/51 = 35%	18/51 = 35%	0/18 = 0%
		Orthogonal Contrasts		
SET 1: C vs I and G1	<0.02	NS	NS	<0.05
SET 1: I vs G1	<0.06	NS	NS	NS
SET 2: C and I vs G1	<0.02	NS	NS	NS
SET 2: C vs I	NS	NS	NS	NS
SET 3: C vs G1	<0.01	NS	NS	<0.05
SET 3: C and G1 vs I	NS	NS	NS	NS

[a]Transferable embryo yield: Grade 1 or 2 morulae and blastocysts at Day 7.

Table 2-4. Effect of CSF2 added at Day 5 of culture and IGF1 added at Day 1 of culture on embryonic development at Day 7, pregnancy risk at Day 30-35 (based on ultrasonography), calving rate and pregnancy loss among recipient cattle that received embryos that were cultured in 5% O_2.

	Transferable embryo yield[a] (percentage, %)	Pregnancy risk	Calving rate	Pregnancy loss
Control (C)	10% ± 1	27/79 = 34%	17/74 = 23%	5/22 = 22%
IGF1 (I)	14% ± 2	12/44 = 27%	11/44 = 25%	1/12 = 8%
CSF2 (Day 5; G5)	14% ± 1	47/107 = 43%	39/104 = 37%	5/44 = 11%
		Orthogonal Contrasts		
SET 1: C and I vs G5	<0.02	<0.05	<0.05	NS
SET 1: C vs I	NS	NS	NS	NS
SET 2: C vs I and G5	<0.02	NS	NS	NS
SET 2: I vs G5	<0.05	<0.06	NS	NS
SET 3: C vs G5	<0.01	NS	<0.05	NS
SET 3: C and G1 vs I	NS	NS	NS	NS

[a] Transferable embryo yield: Grade 1 or 2 morulae or blastocysts at Day 7.

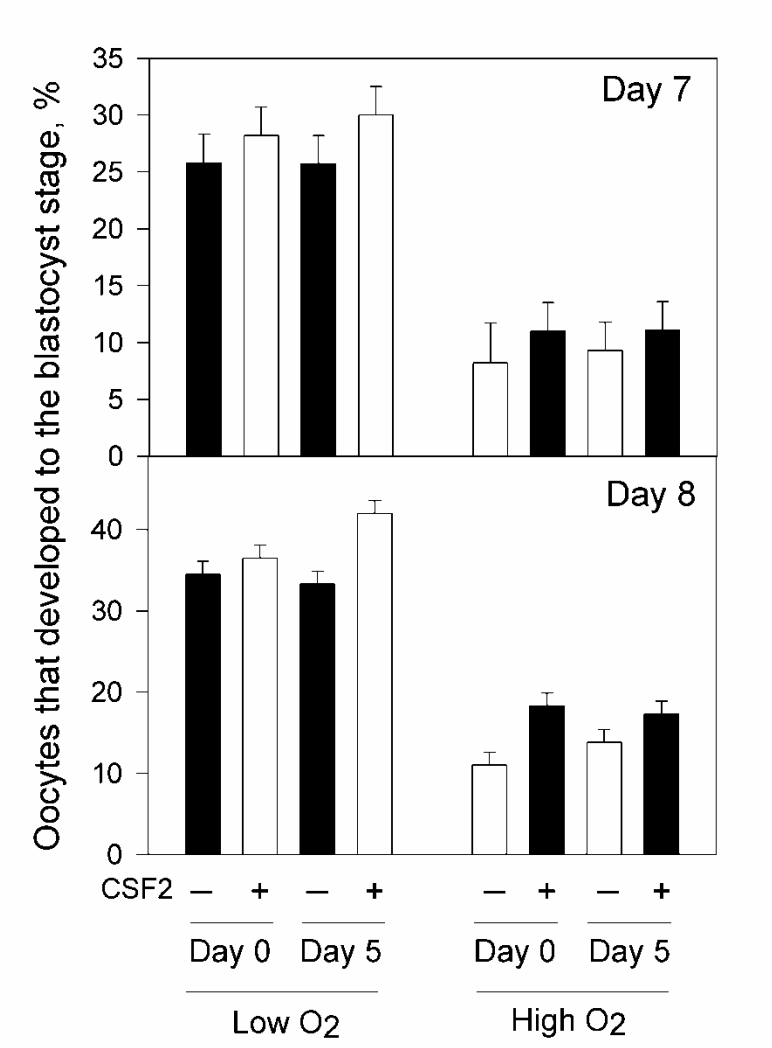

Figure 2-1. Percentageage of oocytes that developed to the blastocyst stage at Day 7 (Panel **A**) and 8 after insemination (Panel **B**). There was a tendency for an increase in the percentageage of blastocysts at Day 7 (treatment, P=0.09; treatment x Day x oxygen= NS) and a significant increase in blastocyst development at Day 8 (treatment, P=0.05; treatment x Day x oxygen= 0.06) when embryos were treated with CSF2. There was an effect of oxygen on the percentageage of oocytes that became blastocysts at Days 7 and 8 on both Days of treatment (P<0.0001).

CHAPTER 3
COLONY STIMULATING FACTOR 2 CAUSES CHANGES IN THE TRANSCRIPTOME OF THE BOVINE PREIMPLANTATION EMBRYO INCLUDING ALTERATIONS IN EXPRESSION OF DEVELOPMENTAL AND APOPTOSIS GENES

Introduction

Colony-stimulating factor 2 (CSF2) is an important local regulator of embryonic function during early pregnancy in several mammalian species. It is expressed in the oviduct (Zhao and Chegini 1994; de Moraes *et al.* 1999), endometrium (Chegini *et al.* 1999; de Moraes *et al.* 1999; Robertson *et al.* 2001; Emond *et al.* 2004), and decidua (Segerer *et al.* 2009). Addition of CSF2 to culture medium improved the proportion of cultured embryos developing to the blastocyst stage in the cow (de Moraes and Hansen 1997a; Chapter 2); human (Sjöblom *et al.* 1999), mouse (Robertson *et al.* 2001), and pig (Cui *et al.* 2004) and also causes a preferential increase in the number of cells in the inner cell mass(ICM) in the human (Sjöblom *et al.* 2002), mouse (Karagenc *et al.* 2005) and cow (Chapter 2). In the mouse blastocyst, CSF2 increased expression of genes involved in glucose transport (Robertson *et al.* 2001) and decreased incidence of apoptosis (Sjöblom *et al.* 2002; Desai *et al.* 2007).

A recent report using the cow as a model indicates that changes in blastocyst function caused by CSF2 are sufficient to increase the competence of the embryo for sustained development through the embryonic and fetal periods of pregnancy. In particular, addition of CSF2 to culture medium at Day 5 after insemination increased post-transfer survival of bovine *in vitro* produced (IVP) embryos by increasing pregnancy rate at Day 30-35 of gestation and decreasing loss of pregnancies after Day 30-35 (Chapter 2). The actions of CSF2 to enhance competence of the embryo for post-blastocyst development could involve changes in the embryonic transcriptome that

alter important functions for establishment of pregnancy and for differentiation and growth of the conceptus. The objective of the present study was to determine changes in the transcriptome of the bovine embryo caused by CSF2 and thereby identify gene pathways and ontologies that are involved in actions of CSF2 on formation of the blastocyst and post-transfer pregnancy success.

Materials and Methods

In vitro Production of Embryos

Embryo *in vitro* production (IVP) was performed as previously described (Chapter 2) with modifications described below. Cumulus oocyte complexes (COCs) from ovaries from a mixture of beef and dairy cattle were collected in Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Hyclone, Logan UT) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocytes were allowed to mature for 20-22 h in groups of 10 in 50 μl microdrops of TCM-199 (Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Folltropin-V; Belleville, ON, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine.

Matured oocytes were then washed in HEPES-TALP (Parrish *et al.* 1986; Caisson, Sugar City ID, USA) and transferred in groups of 50 to four-well plates containing 600 μL of IVF-TALP supplemented with 25 μL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl), and fertilized with 30 μL Percoll-purified spermatozoa (~ $1x10^6$ sperm cells). Sperm were prepared from a pool

of frozen-thawed semen from three different bulls; a different set of bulls was generally used for each replicate). Fertilization proceeded for 18-20 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in humidified air. Putative zygotes were removed from fertilization plates, denuded of cumulus cells by vortexing in HEPES-TALP, and placed in groups of 30 in 45-μl microdrops of KSOM-BE2 (Soto *et al.* 2003).

Embryos were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 or 5% CO_2, 5% O_2, and 90% N_2 (v/v). Cleavage rate was assessed at Day 3 after insemination. At Day 5 after insemination, 5 µl of KSOM-BE2 or 5 µl of KSOM-BE2 containing 100 ng/ml CSF2 (a gift from Novartis, Basle Switzerland) were added to each drop to achieve a final CSF2 concentration of 0 or 10 ng/ml. The concentration of CSF2 was one that increased blastocyst yield and post-transfer pregnancy rates when added at Day 5 after insemination (de Moraes and Hansen 1997a; Chapter 2).

On Day 6, 24 hours after treatment, morulae and early blastocysts were selected and washed three times in 50 μl microdrops of 10 mM PO_4 buffer, pH 7.4 containing 0.9% (w/v) NaCl and 1 mg/ml polyvinylpyrrolidone (PBS-PVP) by transferring the embryos from microdrop to microdrop. Embryos were frozen at -80°C in PBS-PVP in groups of 50. A total of 4 groups of embryos from each treatment were prepared in a total of 6 replicates of *in vitro* production.

RNA Purification and Processing

Total cellular RNA was extracted from embryos with the RNeasy Plus Micro kit (Qiagen-Inc, CA, USA) following manufacturer's instructions. Concentration of the input RNA was determined by Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity was determined by Agilent 2100 Bioanalyzer

with RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara CA, USA). Only samples that showed RNA integrity > 7 were used for the microarray hybridization and quantitative PCR analysis. Extracted RNA was stored at -80°C until microarray analysis.

Microarray Hybridization

The effect of CSF2 on gene expression was assessed using the *Bos taurus* Two Color Microarray Chip from Agilent v1 (Agilent Technologies). This array contains 21,475 unique 60-mer probes, representing approximately 19,500 distinct bovine genes arranged on a slide as 4 arrays in a 4 x 44K format. The probes were developed by clustering more than 450,000 mRNA and EST sequences of the bovine genome (btau 2.1). A total of four separate samples of RNA from CSF2 treated embryos and four separate samples of RNA from control embryos were subjected to microarray analysis. All microarray protocols were carried out by Mogene LLC (St. Louis MO, USA), an Agilent Certified Service Provider.

Prior to labeling, samples of RNA (1.5-3.0 pg/μl) were concentrated to 5 μl using a Savant SpeedVac (Therno Scientific) at low heat and amplified into ss-cDNA using the NuGEN WT-Ovation Pico RNA amplification System (NuGen Technologies, Inc, San Carlos, CA, USA) following manufacturer's instructions. Amplified ss-cDNA was purified using Zymo Spin IIC columns (Zymo Research, Orange, California, USA) and stored at -20 ^{o}C overnight. Product yield and purity were determined by the Nanodrop 1000 spectrophotometer assuming that 1 absorbance unit at 260 nm of ss-cDNA is equal to 33 μg/ml. All 260/230 values were greater than 2.

Aliquots containing 2 μg of ss-cDNA were labeled with the Agilent Genomic DNA Enzymatic Labeling kit to incorporate cyanine 3- or 5-labeled CTP. For half the

replicates, ss-cDNA from control embryos was labeled with Cy3 and ss-cDNA from CSF2-treated embryos was labeled with Cy5. For the other replicates, control embryos were labeled with Cy 5 and CSF2-treated embryos with Cy3.

Hybridizations were set up using 2 µg of each sample (Cy3 and Cy5 components) for a total of 4 µg per array. Volumes were brought to 44 µl with Diethylpyrocarbonate (DEPC)-water and then 11 µl of Agilent 10x GE Blocking Agent was added. The mixtures were incubated at 98^{o}C for 3 minutes and then cooled to room temperature for 5 minutes before adding 55 µl of Agilent 2x Hi-RPM Hybridization buffer. Tubes were flash-spinned on a microfuge and lightly vortexed before loading 100 µl of the hybridization mixture onto each array. Hybridization was carried out for 17 h at 65°C and 10 rpm in a SureHyb gasket slide (Agilent). Washing and scanning procedures were carried out using standard Agilent guidelines for gene expression microarray processing.

At the end of hybridization, microarray slides were sequentially washed using standard Agilent guidelines for gene expression microarray processing. Microarray slides were scanned immediately using an Agilent G2505B scanner.

Analysis of Microarray Data

Images were extracted and pre-processed using the Agilent Feature Extraction Software v 9.5 with default analysis parameters for the initial extraction, signal quantifications, and scaling of the generated data. The software produces background adjustment and normalizations for the dye of individual genes.

The intensity of each spot was summarized as the median pixel intensity. All the generated values were then transformed to $\log_2$. The Lowess method was used for intensity normalization within each array. JMP® Genomics 3.1 for SAS® 9.1.3 software

(SAS Inst., Inc., Cary, NC) was used for data global normalization and identification of differentially expressed genes. The PROC ANOVA procedure was used for simultaneous comparisons and the quantile method for intensity normalization. The model included replicate and treatment. Replicate (array) was considered random and treatment was considered fixed. Correction for false discovery rate was performed by the Benjamini and Hochberg method (Benjamini and Hochegerg 1995). Only genes with median pixel intensity of at least 2.8 were considered to be expressed. To increase the reliability of the results, only genes with a 1.5-fold difference and false discovery rate ≤ 0.01 were considered differentially expressed.

David Bioinformatics Database (http://david.abcc.ncifcrf.gov/; Huang *et al.* 2009) was used to categorize the genes into different ontologies. The Ingenuity pathway analysis software (http://www.ingenuity.com) was used to determine differentially regulated pathways and to modify existing canonical pathways to better fit the findings from the literature. The significance of the association between the list of genes and the canonical pathway was measured by the ratio of the number of molecules from the data set that mapped to the pathway divided by the total number of molecules in the pathway and a p-value calculated by Fisher's exact test that determined the probability that the association between the genes and the pathway is explained by chance alone. To more completely fit differentially-expressed genes into pathways, analyses of each gene based on available literature was used to modify certain canonical pathways to include additional genes.

Quantitative Real Time PCR

Quantitative Real Time PCR analysis (qPCR) of 16 differentially-expressed genes was performed to confirm microarray results. Also, qPCR was performed on one

housekeeping gene (GAPDH) for use as an internal control. Specific primers (Table 3-1) were designed using Integrated DNA Technologies software (http://idtdna.com).

The qPCR analysis was carried out by Mogene LLC with the Applied Biosystems Taqman Gene Expression Mastermix (Foster City, CA, USA). Each reaction consisted of a total volume of 25 µl, 25 ng of template cDNA and with the final concentration of each primer and probe being 200 nM. All probes had a 5' 6-Fam label and a 3' IA-Black Quencher. Following incubation at 95°C for 10 min, 40 cycles of denaturation (95°C for 15 s) and annealing/synthesis (60°C for 1 min) were completed. Each RNA sample was analyzed in triplicate. Responses were quantified based on the threshold cycle (CT).

All CT responses from genes of interest were normalized to the housekeeping GAPDH gene using the ΔC_T method. The $\Delta\Delta C_T$ for each sample was calculated by subtracting the ΔC_T of CSF2-treated group from the control. Fold change of genes inthe CSF2-treated group was determined by solving for $2^{-\Delta\Delta CT}$ relative to the controls. Treatment effects were analyzed by the median scores procedure of SAS (SAS for Windows, Version 9.0, Cary, NC, USA).

Regulation of Apoptosis by CSF2

This experiment was designed to verify whether CSF2 could regulate apoptosis by testing whether treatment of embryos with CSF2 reduced the induction of apoptosis caused by heat shock. The experimental design involved a 2 x 2 factorial arrangement of treatments (control vs heat shock and 0 or 10 ng/ml CSF2). Putative zygotes were randomly distributed in 45 µl microdrops of KSOM-BE2 at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% (v/v) N_2. At Day 5 after insemination, 5 µl of vehicle (KSOM-BE2) or CSF2 (to create a final concentration of 10 ng/ml) were added to the drops. On Day 6 (24 h after treatment), morulae and blastocysts were selected,

placed in previously-conditioned microdrops of the same treatment and then cultured at either 38.5°C or 42°C for 15 h in an atmosphere of 5% CO_2. Embryos were then returned to an environment of 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% (v/v) N_2 for 9 h back. Embryos were then removed from culture and washed three times in 50-μl microdrops of PBS-PVP. Embryos were fixed in a 50-μl microdrop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 600 μl of PBS-PVP at 4°C until analysis for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the in situ Cell Death Detection Kit TMR red (Roche Diagnostics, Indianapolis, USA) to determined apoptotic nuclei as described previously (Loureiro *et al.* 2007). The experiment was replicated 2 times using 65-81 embryos/group.

Results

Transcriptomal Profile

As shown in a Venn diagram (Figure 3-1A), there were a total of 17884 genes that were expressed by embryos from both treatments (control and CSF2), 276 genes expressed exclusively expressed in CSF2-treated embryos and 282 genes present only in control embryos (intensity cutoff for expression= 2.8). Hierarchical analysis (Figure3.1B) of microarray results indicated that the four CSF2 samples formed a distinct cluster from the four control samples.

Differentially expressed genes were determined as those genes where there were at least a 1.5-fold difference in expression and a significance of $P \leq 0.05$ (adjusted to a false discovery rate of 0.01). A total of 214 genes met these criteria and 160 of these could be annotated (67 genes upregulated by CSF2 and 93 genes downregulated).

The five most upregulated genes in terms of fold-change were calcium channel, voltage-dependent, alpha 1G subunit (*CACNA1G*; 9.2 fold increase), stearoyl-coenzyme A desaturase (*SCD*; 6.5 fold increase), Kruppel-like factor 8 (*KLF8*; 5.8 fold increase), secreted frizzled-related protein 4 (*SFRP4*; 4.0 fold increase), and alcohol sulfotransferase (*SULT2A1*; 3.2 fold increase). The five most downregulated genes, in terms of fold change were olfactomedin 4 (*OLFM4*; 4.2 fold decrease), wingless-type 16 (*WNT16*; 3.8 fold decrease), neural precursor cell expressed, developmentally down-regulated 4 (*NEDD4*; 3.8 fold decrease), MAP-kinase activating death domain (*MADD*; 3.7 fold decrease), and coiled-coil domain containing 103 (*CCDC103*; 2.9 fold decrease).

Biological Process Ontologies Affected by CSF2

Analysis using the David software categorized the differentially expressed genes into 13 biological process ontologies (Table 3-2). These ontologies could be grouped into four functional groups. One was for genes involved in development and differentiation. There were 42 differentially expressed genes in the developmental process ontology (26% of the differentially expressed annotated genes), 32 genes in the multicellular organ development ontology, 26 genes in the system development ontology, 26 genes in the anatomical structure development ontology, 23 genes in the cellular developmental process-cell differentiation ontology and 5 genes in the pattern specification process. The second was for differentially expressed genes involved in signal transduction and cell communication. There were 45 differentially expressed genes in the cell communication ontology (28% of the differentially expressed genes), 44 differentially expressed genes in the signal transduction ontology, and 25 genes in the cell surface receptor linked signal transduction ontology. The third was for genes

involved in apoptosis with 9 differentially expressed genes in the regulation of apoptosis ontology and 6 genes in the induction of programmed cell death ontology. The fourth group was for genes involved in cell adhesion with 13 differentially expressed genes in the biological adhesion-cell adhesion ontology. There was no pattern for CSF2 to regulate cell adhesion and migration genes in a way that would consistently promote or inhibit cell adhesion or migration.

Genes Involved in Cellular Development and Differentiation

Many of the differentially-regulated genes identified by DAVID as being involved in developmental processes were involved in neurogenesis, mesoderm or muscle formation, and pluripotency. A total of 13 genes identified as being involved in neurogenesis were differentially regulated by CSF2, with 4 upregulated genes (*MAB21L2*, 3.0-fold increase; *HUWE*, 2.5-fold increase; *NOTCH2,* 2.2-fold increase *RTN4*, 1.6-fold increase) and 8 downregulated genes (*DTX3*, 2.2 fold decrease; *HUNK*, 2.1-fold decrease; *CELSR*, 1.6-fold decrease; *SEMA4*, 1.6 fold decrease; *ARSA*, 1.6-fold decrease; *GREM1*, 1.6-fold decrease; *GLIS2*, 1-6 fold decrease; *CHURC1*, 1.5-fold decrease; and *RGS12*, 1.5 fold decrease). A total of 8 differentially regulated genes were identified as being associated with mesenchyme, mesoderm or muscle cells, including regulation of the epithelial-mesenchymal transition. Upregulated genes were *KLF8* (5.8-fold increase), *MYF6* (3.1 fold increase), *HOXA5* (2.2 fold increase), *NOTCH2* (2.2 fold increase), *CD73/NT5E* (2.2 fold increase) and *FHL1* (2.1 fold increase) while *DTX3* (2.2 fold decrease) and *GREM1* (1.7-fold decrease) were downregulated by CSF2. Two of these genes are involved in hematopoiesis (*KLF8* and *NOTCH2*). In addition, CSF2 upregulated expression of two other genes involved in hematopoiesis, *CCL23 (2.5* fold increase*)* and *HOXA5* (2.2 fold increase), and

decreased expression of another gene involved in hematopoiesis (*CXCL12*, 1.9 fold decrease).

Genes Involved in Signal Transduction and Cell Communication

Analysis of differentially-regulated genes in signal transduction and cell communication ontologies (Table 3-2) indicates a broad range of effects of CSF2 on expression of genes encoding ligands (*TNFSF8, CXCL2, CXCL12*), receptors (*OR51E1, CCRL1, GIPR, , NCOA7, GRID1, OR2T11, ITPR2, PTGER4, GPR143*), receptor tyrosine kinases (*ROR2, PTPRK, RIPK3*), other protein kinases (*PTPN22, MARK2, PRKA2B, PTPRK, DAPK1, CSNK2B, MADD*), transcription factors (*KLF8, MYF6*) and cAMP regulators (*NOTCH2, PDE7B, CREM, PTGER4*).

Genes Involved in WNT Signaling

One signaling system that was represented often in the list of differentially genes regulated in the most consistent manner was the WNT system. As shown in Table 3-3, expression of a total of 10 genes involved in WNT signaling were affected by CSF2. Of the 6 upregulated genes, four (*SFRP4/FrpHE, NOTCH2, PPP2R3A*, and *PCDH24*) are inhibitory to β-catenin-dependent signaling. Of the 4 downregulated genes, two activate the β-catenin-dependent signaling pathway (*WNT16* and *CSNK2B)* and all 4 activate at least one β-catenin-independent signaling pathway (*WNT16, CSNK2B, ROR2*, and *CELSR2).*

The data set of differentially expressed genes was also examined for 70 genes reported to be upregulated by WNT signaling (Ambrosetti *et al.* 2008; Segditsas *et al.* 2008; Chien *et al.* 2009). Only three genes in this list were found to be differentially

regulated by CSF2. *GREM1* (1.7 fold decrease), *SEMA4* (1.6 Fold decrease) and *MAB21L2*, (3.0 fold increase).

Genes Involved in Apoptosis Signaling Pathway

Effects of CSF2 on genes involved in apoptosis signaling are shown in Table 3-4. A total of 16 genes were differentially regulated. Of the 7 upregulated genes, 3 are anti-apoptotic (*TNFSF8, PRKAR2B, CD73/NT5E,* and *PGR*), one (*NOTCH2*) is usually, but not always, anti-apoptotic, and 2 (*CASP7* and *RTN4*) are pro-apoptotic. Of the 9 genes downregulated by CSF2, 6 are pro-apoptotic (*NOD2, PIK3IP1, RIPK3, MADD, DAPK1,* and *CREM*) and 3 are anti-apoptotic (*RNF7, CXCL12,* and *PLD2*).

Quantitative Real Time PCR

Quantitative real time PCR (qPCR) was used to confirm the findings of the microarray analysis (Figure 3-2). For 11 of the 16 genes examined, the fold change caused by CSF2 as determined by qPCR microarray was in the same direction as the fold change as determined by microarray hybridization. The effect of CSF2 as determined by qPCR was significant (P<0.001) for one of these genes (*SLC16A10*), approached significance (P=0.09) for 7 genes (*PPP2R3A, PMM2, ANKRD37, NOTCH2, MAB21L2, MRSPS12,*and *MADD*) and was not significant for three genes (*VGLL2, ECE1,* and *TNFSF8*). One gene, *WNT16*. whose expression was decreased by CSF2 as determined by microarray hybridization was increased by CSF2 (P<0.001) as determined by qPCR. While not significant, the same trend was apparent for *RIPK3* and *HK1*. Two other genes affected by CSF2 as determined by microarray, had a fold change near 1.0 as determined by qPCR (*MASP2* and *CSNK2B).*

Actions of CSF2 to Block Heat-Shock Induced Apoptosis

As another validation of the microarray results, an experiment was performed to test whether embryonic function was changed by CSF2 in a way predicted from the microarray results. In particular, since CSF2 altered gene expression in a way that would inhibit apoptosis, it was tested whether culture of embryos with CSF2 beginning at Day 5 after insemination would block induction of apoptosis caused by heat shock initiated at Day 6. As shown in Figure 3-3E, culture at 42°C for 15 h increased the percentage of blastomeres that were TUNEL-positive and reduced total cell number ($P<0.0001$). While CSF2 did not affect the percentage of cells that were TUNEL positive at 38.5°C, it blocked the increase in TUNEL labeling caused by heat shock (CSF2, $P<0.005$).

Discussion

Colony stimulating factor 2 is an important regulator of preimplantation embryonic development. In the cow, it can increase the proportion of embryos that develop to the blastocyst stage, increase the number of cells in the ICM, improve the competence of the embryo to establish pregnancy after transfer to a recipient female, and reduce the probability of fetal loss after Day 30-35 of pregnancy (de Moraes and Hansen 1997a; Chapter 2). As indicated by changes in the embryonic transcriptome described in this paper, these actions of CSF2 involve changes in expression of genes controlling developmental and apoptotic processes. Changes in expression of genes controlling development are likely to lead to a blastocyst whose developmental trajectory favors embryonic survival in the embryonic and fetal period. Actions of CSF2 to increase resistance to induction of apoptosis could conceivably contribute to growth of the embryo and survival after transfer into a recipient with suboptimal uterine environment.

A large number (42) of the genes whose expression was altered by CSF2 are genes implicated in developmental processes. A total of 26% of annotated genes that were differentially expressed were in the developmental process ontology. Thus, it is likely that CSF2 acts on the embryo to control cell fate and differentiation in the blastocyst. Two major actions of CSF2 on development could be inferred by examination of the developmental genes regulated by CSF2. The first was regulation of genes involved in neurogenesis in a manner that would act to inhibit neural cell formation and differentiation. The second was stimulation of genes involved in formation or differentiation of mesoderm or mesoderm derived cells.

That CSF2 acts to inhibit processes involved in neurogenesis is evident from an examination of the 4 genes involved in neurogenesis increased by CSF2 (*MAB21L2, HUWE, NOTCH2* and *RTN4*) and the 9 genes that were downregulated by CSF2 (*DTX3, HUNK*, *CELSR*, *SEMA4, ARSA*, *GREM1*, *GLIS2, CHURC1*, and *RGS12*). Two of the upregulated genes are required for neurogenesis. Use of antisense oligonucleotides to deplete MAB21L2 impaired notochord and neural tube differentiation (Wong and Chow 2002). HUWE1 causes ubiquitination of N-myc and induces ES cells to differentiate into neural tissue (Zhao *et al.* 2009). Conversely, RTN4 is a neural stem cell marker that prevents neurite outgrowth (Zheng *et al.* 2010) and NOTCH signaling can inhibit mitosis of neuronal precursors (le Roux *et al.* 2003). However, it is not clear whether NOTCH signaling is enhanced in CSF2 treated embryos, since one of the genes downregulated by CSF2, *DTX3*, is a component of the NOTCH signaling pathway (Pampeno *et al.* 2001). Moreover, NOTCH signaling is involved in a wide array of developmental events and the phenotype caused by activation of NOTCH pathways

depends upon inputs from multiple inputs (Lewis *et al.* 2009). The other 8 genes involved in neurogenesis that were downregulated by CSF2 are all involved in neural cell function. Two of these genes, the bone morphogenic protein (BMP) inhibitor *GREM1* (Wordinger *et al.* 2008) and the transcription factor *CHURC1*, participate in neurulation. Early in development, ectoderm is inhibited from differentiating into neural cells by the actions of BMP4. Secretion of BMP antagonists relieves this inhibition and contributes to neural induction (Hemmati-Brivanlou and Melton 1997). Neural formation also requires actions of fibroblast growth factors, which act through the transcription factor CHURC1 to enhance actions of BMP antagonists and activate expression of *SOX2* (Sheng *et al.* 2003). Inhibition of *GREM1* and *CHURC1* expression by CSF2 could, therefore, result in maintenance of the inhibition of neural cell formation. The other genes involved in neurogenesis inhibited by CSF2 are the transcription factor G*LIS2* (Zhang *et al.* 2002), *RGS12*, a component of the signaling system for nerve growth factor (Willard *et al.* 2007), *CELSR*, a cadherin involved in planar polarity and neural tube formation (Curtin *et al.* 2003; Zhou *et al.* 2007a), *SEMA4*, a member of the semaphorin family of axon guidance proteins (Pasterkamp and Giger 2009), *HUNK*, a protein kinase expressed in fetal brain (Gardner *et al.* 2000), and *ARSA*, a lysosomal enzyme involved in myelin formation (Gieselmann *et al.* 1991).

In contrast to the apparent inhibition of neural formation caused by CSF2, there was evidence that CSF2 regulated gene expression in a way that would increase mesoderm formation and differentiation. One gene increased by CSF2 was *KLF8,* a Krüppel-like transcription factor that can induce epithelial-mesenchymal transition (Wang *et al.* 2007). The process of epithelial-mesenchymal transition occurs at several

developmental events including gastrulation and leads to epithelial cells becoming migratory and differentiating into mesenchymal cells (Baum *et al.* 2008). CSF2 also caused upregulation of *HOXA5*, a mesenchymally-restricted morphogen involved in pattern formation (Boucherat *et al.* 2009), *MYF6*, a member of the basic helix-loop-helix family of transcription factors that activates genes involved in muscle cell formation (Rescan 2001), *CD73/NT5E,* an extracellular nucleotidase and mesenchymal stem cell marker which can induce proliferation, migration, invasion, and adhesion of breast cancer cells (Barry *et al.* 2001; Babiychuk and Draeger 2006; Zhou *et al.* 2007b; Wang *et al.* 2008b), and *FHL1*, a four-and-a-half-LIM only protein involved in muscle development (McGrath *et al.* 2003) (2.1 fold increase). One effect of CSF2 that was inconsistent with promotion of differentiation of mesoderm derivatives was the inhibition of *GREM1* expression because this BMP antagonist promotes the epithelial-mesenchymal transition in kidney and limb bud (Michos *et al.* 2004).

The first differentiation event in the embryo after formation of the blastocyst is gastrulation whereby the three germ layers (ectoderm, mesoderm, and endoderm) are formed. In the cow, this event occurs as early at Day 9 of development when mesoderm can be identified (Maddox-Hyttel *et al.* 2003). Neurulation occurs later in development and the neural groove forms betweens Day 14 and 21 (Maddox-Hyttel *et al.* 2003). Taken together, the net result of actions of CSF2 on expression of genes involved in developmental processes could be a blastocyst in which formation of mesoderm and mesoderm-derived cell types is enhanced while differentiation of the ectoderm into the neural plate is delayed. Perhaps, CSF2 favors embryonic survival by ensuring allocation

of a sufficient number of ICM cells towards mesoderm early in development and by inhibiting neurulation until the appropriate stage of development.

A total of 10 genes involved in WNT signaling were differentially expressed by CSF2. Given the involvement of WNT in epidermal fate determination, BMP4 regulation and endoderm formation (Wilson *et al.* 2001; Hansson *et al.* 2009), such changes could conceivably contribute to changes in the process of gastrulation. Analysis of the WNT-related genes regulated by CSF2 would indicate inhibition of β-catenin dependent WNT signaling. Four genes inhibitory to this pathway (*SFRP4/FrpHE, NOTCH2, PPP2R3A*, and *PCDH24*) were upregulated by CSF2 and two of the genes downregulated by CSF2 *(WNT16* and *CSNK2B)* promote β-catenin dependent signaling. Actions of proteins involved in WNT signaling are complex, however, and depend upon the array of other WNT signaling proteins present in the cell (Chien *et al.* 2009). Moreover, all four of the WNT-signaling genes that were downregulated by CSF2 are involved in one or more β-catenin-independent signaling pathways (*WNT16, CSNK2B, ROR2*, and *CELSR2)* and activation of these pathways leads to an inhibition of β-catenin dependent gene expression (Chien *et al.* 2009). Expression of only 3 of 70 WNT-induced genes were significantly affected by CSF2 and only two of those (*GREM1* and *SEMA4*) experienced the decrease in expression expected if CSF2 decreased β-catenin dependent gene expression. Further research is required to delineate regulation of WNT signaling by CSF2 in the preimplantation embryo.

It has been demonstrated previously that CSF2 can reduce apoptosis in preimplantation mouse embryos (Sjöblom 2002). Similar results were obtained in the present experiment with bovine embryos. Expression of three genes (*CD73/NT5E,*

PGR, and *PRKAR2B*) that inhibit apoptosis were increased by CSF2. The ecto-nucleotidase CD73/NT5E blocks TRAIL-induced apoptosis, possibly through interaction with death receptor 5 (Mikhailov *et al.* 2010). Ligand dependent action of PGR leads to inhibition of apoptosis through an unknown mechanism (Friberg *et al.* 2009). The cAMP-dependent kinase PRKAR2B can inhibits apoptosis through a p53-dependent mechanism (Srivastava *et al.* 1999). CSF2 also increased expression of two genes that can be pro- or anti-apoptotic, *NOTCH2* (Quillard *et al.* 2009; Yoon *et al.* 2009) and the TNF-receptor superfamily member TNFSF8 (CD30) (Al-Shamkhani 2004). Treatment with CSF2 also inhibited expression of 6 pro-apoptotic genes (*MADD, RIPK3, PIK3IP, NOD2, DAPK1* and *CREM1*). MADD is a death domain-containing adaptor protein, DAPK1 is a death associated protein kinase 1 (Martoriati *et al.* 2005; Okamoto *et al.* 2009) and NOD2 is a caspase recruitment domain family (Geddes *et al.* 2009). PIK3IP1 (He *et al.* 2008) and RIPK3 are kinase proteins that can trigger apoptotic signaling (Declercq *et al.* 2009) and CREM decreases amounts of the anti-apoptotic protein BCL2 (Jaworski *et al.* 2003; Mioduszewska *et al.* 2003)).

Consistent with the idea that CSF2 inhibits apoptosis was the finding that induction of apoptosis in Day 6 embryos by heat shock was reduced by treatment with CSF2. Heat shock induces apoptosis in preimplantation embryos through mitochondrial depolarization and activation of group III caspases such as caspase 9 (Loureiro *et al.* 2007).

It is possible that the increased number of ICM cells and increased ICM/TE ratio seen in CSF2 treated embryos (Chapter 2) is a result, at least in part, in inhibition of apoptosis responses because the incidence of apoptosis is greater in ICM than TE

(Knijn *et al.* 2003; Fouladi-Nashta *et al.* 2005; Pomar *et al.* 2005). Increased survival after transfer to recipients (Chapter 2) could also be due, in part, to increased resistance of embryos to adverse maternal environments that could induce blastomere apoptosis after transfer.

Quantitative PCR was used to confirm the microarray results. While 11 of 16 genes showed fold changes in the same direction as for microarray analysis, differences were significant in one case only and approached significance (P=0.09) in 7 other cases. Lack of significance was likely due to part to the small sample size and between-sample variation. It could also be, for some genes, the microarray and the qPCR probes recognize different splice variants. The most notable discrepancy between PCR and microarray results was for *WNT16,* where expression was reduced by CSF2 as determined by microarray hybridization and increased by CSF2 as determined by qPCR. This gene was not very abundant and the large fold-change differences may reflect sampling error.

While the lack of uniform agreement between microarray and qPCR results means that definitive conclusions regarding effects of CSF2 on individual genes is not possible, it is likely that systems and pathways found by microarray hybridization to be regulated by CSF2 reflect biological actions of CSF2. Indeed, the experiment on induction of apoptosis by heat shock represents a biological confirmation of the results from microarray analysis with respect to regulation of genes involved in apoptosis. Even though a small percentageage of genes were confirmed in the qPCR we were able to confirm the regulation of apoptosis by CSF2 evaluating the percentageage of apoptotic blastomers by a well characterized assay.

It is concluded from the present study that CSF2 can regulated embryonic development by altering the expression of genes controlling developmental process and apoptosis. Results indicate that CSF2 act to increase expression of genes regulating epithelial to mesenchymal transition and decrease neural cell differentiation. Other CSF2 effects include a decrease in genes involved in apoptosis and an increase in genes that regulate cell survival. Perhaps this change in cellular fate and the decrease in cellular death are responsible for the higher pregnancy rates at Day 35 and the lower embryonic losses seem on recipients that receive a CSF2 treated embryo.

Table 3-1. Primers and Probes used on qPCR.

Gene Name	Accession	Primer/Probe	Sequence (5'-3')
Ankyrin repeat domain 37 (*ANKRD37*)	XM_586036	Forward[a]	GTG GGA AAA GGA AGT GTT GAT G
		Probe[b]	TGG TCA TGA AGA GGT GAG GAG AAG GT
		Reverse[C]	CTT GTA GCT GAA CGG TAG ACC
Casein kinase 2, beta polypeptide (*CSNK2B*)	XM_585826	Forward[a]	GTT TCC CTC ACA TGC TCT TCA
		Probe[b]	TGG ATC TTG AAA CCG TAA AGC CTG GG
		Reverse[C]	TCA CTG GGC TCT TGA AGT TG
Endothelin converting enzyme 1 (*ECE1*)	NM_181009	Forward[a]	CAT TCT ACA CCC GCT CTT CAC
		Probe[b]	CCG ATG CCG CCG AAG TTT AAG G
		Reverse[C]	GTT CCC ATC CTT GTC GTA CTC
Hexokinase 1 (*HK1*)	NM_001012668	Forward[a]	CCA AAG TGT AAT GTG TCC TTC C
		Probe[b]	TGC CGC TGC CGT CTT CAG ATA A
		Reverse[C]	GAA GAG AGA AGT GCT GGA AGG
Mab-21-like 2 (*MAB21L2*)	XM_585738	Forward[a]	TCT CAC CAA TCC CAA AAG CC
		Probe[b]	AGC AGT CCC CAG CAC CCT ATA GT
		Reverse[C]	TCT GGA GTT CTC GCA GTT TG
MAP-kinase activating death domain (*MADD*)	XM_867337	Forward[a]	AGT GCA ATA CAG TCC GAG G
		Probe[b]	AGT ACA AGA CAC CGA TGG CCC AC
		Reverse[C]	CAA CAC GGA GTA GCA GAT C

Table 3-1. Continued

Gene Name	Accession	Primer/Probe	Sequence (5'-3')
Mannan-binding lectin serine peptidase 2 (*MASP2*)	XM_582170	Forward[a]	TGG TTT GTG GGA GGA ATA GTG
		Probe[b]	AGT ACA AGA CAC CGA TGG CCC AC
		Reverse[C]	CAA CAC GGA GTA GCA GAT C
Phosphomannomutase 2 (*PMM2*)	NM_001035095	Forward[a]	TCT AAC CCA GTC TCC CCT C
		Probe[b]	CCT CCT GCA AGT TCC TGT GGC T
		Reverse[C]	GGG ACC AAA GCT GAA CAA TG
Notch homolog 2 (*NOTCH2*)	XM_867242	Forward[a]	ACA CAT GTC TGA GCC ACC
		Probe[b]	FAM/TCT ATG CAT GAA AGA GTC TGC CTC CA
		Reverse[C]	TTT CCC GGA TGA CCT TCA
Protein phosphatase 2, regulatory subunit B, alpha 1 (*PPP2R3A*)	XM_599849	Forward[a]	CAA GAT GAC CAG CAC AGT
		Probe[b]	TGA TCC CAG AAC TCT AAA AGA TGT CCA GC
		Reverse[C]	GTT AGT GGC TGC GAT TGA
Receptor-interacting serine-threonine kinase 3 (*RIPK3*)	XM_584025	Forward[a]	CTG ACA GAT TTG ATG CAG AAG TG
		Probe[b]	AAG GGC TTT CTT GGT GTT TAT TCG GC
		Reverse[C]	
Solute carrier family 16, member 10 (*SLC16A10*)	XM_615239	Forward[a]	GCT CGG ATT CAT GTC TAT ACC C
		Probe[b]	TGA TGT GGC CTT CTA CCT CGC TG
		Reverse[C]	AAC AGC ACC TCC AAT AAG GG

Table 3-1. Continued

Gene Name	Accession	Primer/Probe	Sequence (5'-3')
Tumor necrosis factor (ligand) superfamily, member 8 (*TNFSF8*)	NM_001025207	Forward[a]	GCA AAA CTG ACC ATC CTG AAT C
		Probe[b]	CTG CCG GAA CTG AGA CTG ACA ATA AGA C
		Reverse[C]	CTG ACG GGA AAC AAA AGC TG
Vestigial like 1 (Drosophila) (*VGLL1*)	XM_872858	Forward[a]	AGC TCT GGG CAA TGT CAA G
		Probe[b]	AGT GGC GTT TCT CTG CTC CGT
		Reverse[C]	GCA AAA GAT ACT TCC GGC TG
Wingless-type MMTV integration site family, 16 isoform 1 (*WNT16*)	NM_016087	Forward[a]	GAG GTG TGA AAG CAT GAC TG
		Probe[b]	TGC CGC TGC CGT CTT CAG ATA A
		Reverse[C]	GAA GAG AGA AGT GCT GGA AGG
Mitochondrial ribosomal protein S12 (*MRPS12*)	NM_01077101	Forward[a]	TCT AGA AGT AGC TGG TCT GGG
		Probe[b]	CTA TGG GTT AAG GGT CCA CTG GGC
		Reverse[C]	GAT GGC AGT ACA GAG TCT TGT C

[a]Forward=sense (5') primer

[b]Each probe was synthesized with a 5' 6-FAM reporter dye and 3' IA-Black quencher

[c]Reverse=antisense (3') primer

Table 3-2. Gene ontologies in the biological process category that were regulated by CSF2[a].

Ontology	Effect of CSF2	Genes
GO:0032502: Developmental process	Up	*MAB21, HOXA5, CASP7, PGR, PDE3B, IBSP, NOTCH2, PCDH1, PTPN2, CD2, RTN4, MYF6, FHL1, SFRP4, SLC26, FGD3, TNFSF8*
	Down	*CYLC1, CELSR, ECE2, SEMA4, GREM1, FEZF1, POLL, ARSA, ROR2, PHGDH, GLIS2, GYPC, PTGER, HUNK, CREM, WNT16, BOLL, CHURC1, RIPK3, CENPI, MADD, NOD2, RNF7, GPR14, DAPK1*
GO:0007275: Multicellular organism development	Up	*MAB21, HOXA5, CASP7, PGR, PDE3B, IBSP, NOTCH2, PCDH1, PTPN2, CD2, RTN4, MYF6, FHL1, SFRP4*
	Down	*CYLC1, CELSR2, ECE2, SEMA4, GREM1, FEZF1, POLL, ARSA, ROR2, PHGDH, GLIS2, GYPC, PTGER, HUNK, CREM, WNT16, BOLL, CHURC1*
GO:0048731: System development	Up	*MAB21L, HOXA5, CASP7, PGR, PDE3B, IBSP, NOTCH2, PCDH1, PTPN2, CD2, RTN4, MYF6, FHL1, SLC26, FGD3*
	Down	*ECE2, SEMA4, GREM1, FEZF1, POLL, ARSA, ROR2, PHGDH, GLIS2, GYPC, PTGER4,*
GO:0048856: Anatomical structure development	Up	*MAB21L, HOXA5, CASP7, PGR, PDE3B, IBSP, NOTCH2, PCDH1, PTPN2, CD2, RTN4, MYF6, FHL1, SLC26, FGD3*
	Down	*ECE2, SEMA4, GREM1, FEZF1, POLL, ARSA, ROR2, PHGDH, GLIS2, GYPC, PTGER4*
GO:0007389: Pattern specification process	Up	*HOXA5, NOTCH2, MYF6*
	Down	*GREM1, ROR2*

Table 3-2. Continued

Ontology	Effect of CSF2	Genes
GO:0048518: Positive regulation of biological process	Up	*CD2, MASP2, MYF6, NOTCH2, PTPN2, RIPK3, RNF7, TNFSF8*
	Down	*BOLL, CHURC1, DAPK1, GLIS2, NOD2, STUB1, VAV1*
GO:0007165: Signal transduction	Up	*CASP7, CD2, CCRL, FGD3, GIPR, MARK1, NOTCH2, NXPH4, PDE3B, PRKAR, PTPN2, PDE7B, PGR, PYGO1, OR51E,TNFSF8*
	Down	*CREM, CELSR2, CXCL2, CXCL1, CSNK2, DTX3, DAPK1, ECE2, FYB, GPR14, HUNK, ITPR2, LRRN2, MADD, MPP2, RIPK3, NOD2, OR2T1, PLD2, STUB1, TBC1D2, VAV1, WNT16, SFRP4, PTGER, RALGP, ROR2, RGS12*
GO:0007154: Cell communication	Up	*CASP7, CD2, CCRL1, ECE1, FGD3, GIPR, MARK1, NOTCH2, NXPH4, PDE3B, PRKAR, PTPN2, PDE7B, PGR, PYGO1 SFRP4, TNFSF8, OR51E*
	Down	*CREM, CELSR2, CXCL2, CXCL1, CSNK2, DTX3, DAPK1, ECE2, FYB, GREM1, GPR14, HUNK, ITPR2, LRRN2, MADD, MPP2, NOD2, OR2T1, PLD2, STUB1, VAV1, WNT16, PTGER, RIPK3, RALGP, ROR2, RGS12*
GO:0007166: Cell surface receptor linked signal transduction	Up	*CD2, CCRL1, GIPR, NOTCH2, NXPH4, PTPN2, PYGO1, SFRP4, OR51E*
	Down	*CELSR2, CXCL2, CXCL1, CSNK2, DTX3, ECE2, GPR14, MADD, OR2T1, PLD2, STUB1, VAV1, WNT16, PTGER, ROR2, RGS12*

Table 3-2. Continued

Ontology	Effect of CSF2	Genes
GO:0012502: Induction of programmed cell death	Up	*CD2, TNFSF8, NOTCH2*
	Down	*RNF7, DAPK1, RIPK3*
GO:0043065: Regulation of apoptosis	Up	*CD2, TNFSF8, NOTCH2, RTN4*
	Down	*RNF7, DAPK1, RIPK3, MADD, NOD2*
GO:0022610: Biological adhesion-cell adhesion	Up	*CLDN2, IBSP, PCDH15, CD2, THBS3*
	Down	*CELSR2, LRRN2, VAV1, F5, OLFM4, ROR2, CXCL12, PPFIBP1*
GO:0048869: Cellular developmental process-cell differentiation	Up	*TNFSF8, SFRP4, CASP7, MYF6, NOTCH2, PCDH1, FHL1, CD2, RTN4, PTPN2*
	Down	*CYLC1, CREM, ECE2, NOD2, DAPK1, RIPK3, BOLL, SEMA4, FEZF1, MADD, ROR2, GLIS2, RNF7*

[a] Gene ontologies are from the David Bioinformatics Database GO and were those where the p value ≤ 0.05

Table 3-3. Differentially-regulated genes involved in WNT signaling.

Symbol	Least-squares mean CSF2	Least-squares mean Control	Fold Change	P-value	Role in WNT signaling	Reference
Upregulated						
SFRP4/FrpHE	13.07	3.26	4.00	0.04	Binds to and inhibits WNT	Chien *et al.*, 2009
NOTCH2	84.22	38.10	2.21	0.03	Blocks induction of WNT-induced genes	Walsh and Andrews, 2003
PDE7B	4.88	2.89	1.69	0.02	Promotes β-catenin independent and dependent signaling	Ahumada *et al.*, 2002; Li *et al.*, 2002
PYGO1	135.6	82.08	1.65	0.01	Nuclear cofactor for β-catenin	Jessen *et al.*, 2008
PPP2R3A (PP2A)	28.53	18.09	1.58	0.04	Phosphatase that inhibits β-catenin dependent signaling	Creyghton *et al.*, 2005
PCDH24	320.2	211.28	1.52	0.01	Protocadherin that inhibits activation of β-catenin	Ose *et al.*, 2009
Downregulated						
WNT16	3.76	14.28	-3.80	0.01	WNT ligand for β-catenin dependent and independent pathways	Mazieres *et al.*, 2005; Binet *et al.*, 2009
CSNK2B (CK2)	119.2	256.51	-2.20	0.02	Enhances activation of β-catenin dependent transcription; enhances activation of planar cell polarity; inhibits RAC-1 mediated effects of WNT	Wang and Jones, 2006; Bryja *et al.*, 2008
ROR2	34.89	63.48	-1.80	0.01	WNT receptor or co-receptor for planar cell polarity pathway	Chien *et al.*, 2009
CELSR2 (Flamingo)	206.1	316.26	-1.60	0.01	Cadherins that interact with WNT and activate planar cell polarity signaling	Saburi *et al.*, 2005

Table 3-4. Differentially-regulated genes involved in apoptosis.

Symbol	Least-squares mean CSF2	Least-squares mean Control	Fold Change	P-value	Role in apoptosis
Upregulated					
NOTCH2	84.22	38.10	2.21	0.03	anti/pro-apoptotic
CD73/NT5E	27.11	12.20	2.22	0.03	anti-apoptotic
PGR	63.08	34.46	1.83	0.05	anti-apoptotic
TNFSF8 (CD30)	64.96	37.64	1.73	0.03	anti/pro-apoptotic
CASP7	82.11	49.07	1.67	0.03	pro-apoptotic
PRKAR2B	182.85	119.97	1.52	0.04	anti-apoptotic
RTN4	492.97	311.20	1.58	0.02	pro-apoptotic
Downregulated					
MADD	8.18	29.55	-3.60	0.03	pro-apoptotic
RIPK3	43.02	85.65	-2.00	0.03	pro-apoptotic
PIK3IP1	34.58	62.71	-1.80	0.01	pro-apoptotic
CXCL12	75.16	138.34	-1.80	0.02	anti-apoptotic
NOD2	5.49	9.77	-1.80	0.05	pro-apoptotic
DAPK1	20.42	31.88	-1.60	0.02	pro-apoptotic
CREM	53.9	83.1	-1.60	0.02	pro-apoptotic
RNF7	95.80	155.50	-1.60	0.03	anti-apoptotic
PLD2	308	493.9	-1.60	0.02	anti-apoptotic

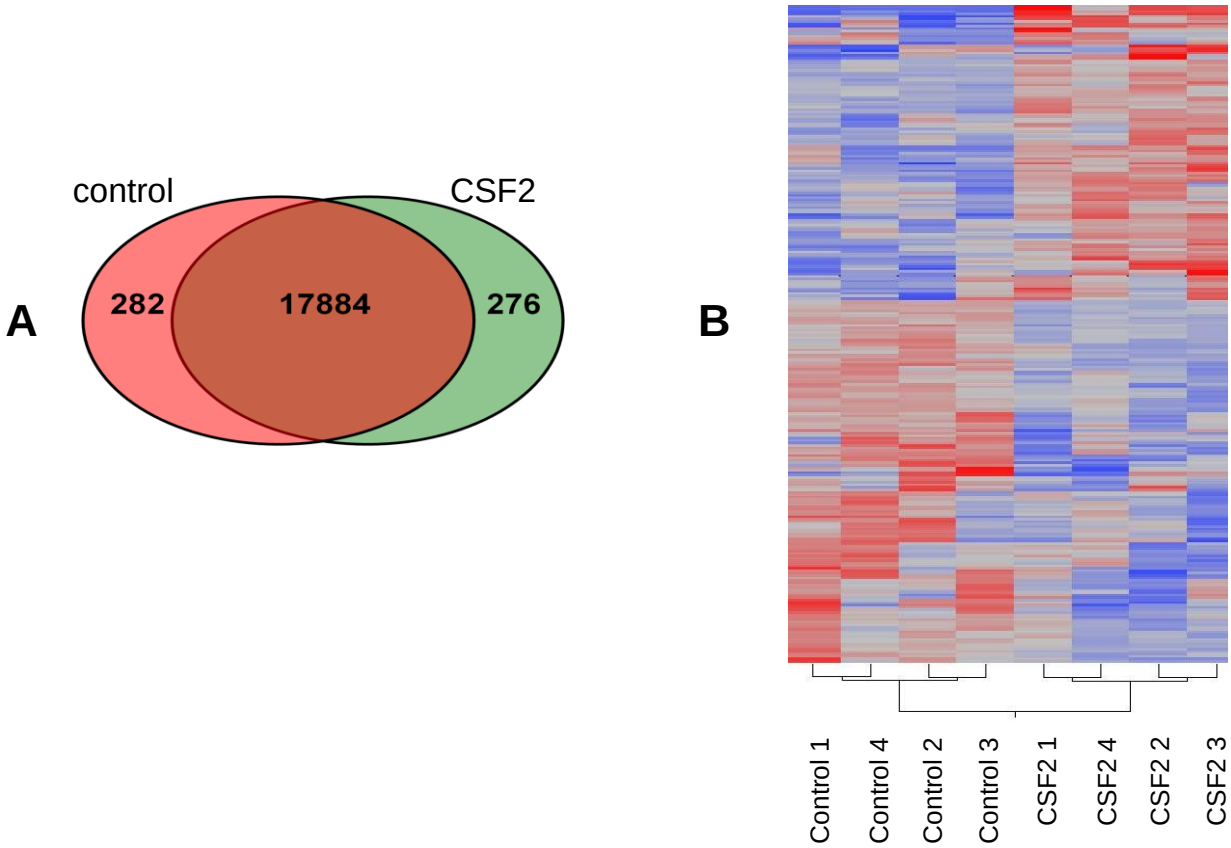

Figure 3-1. Genes expressed in control and CSF2-treated embryos at Day 6 of development. Shown in panel A is a Venn diagram of genes expressed in embryos of both treatments (brown), only in control embryos (red) or only in CSF2-treated embryos (green). Intensity cutoff for expression = 2.8. Shown in panel B is the hierarchical cluster of differentially-expressed genes.

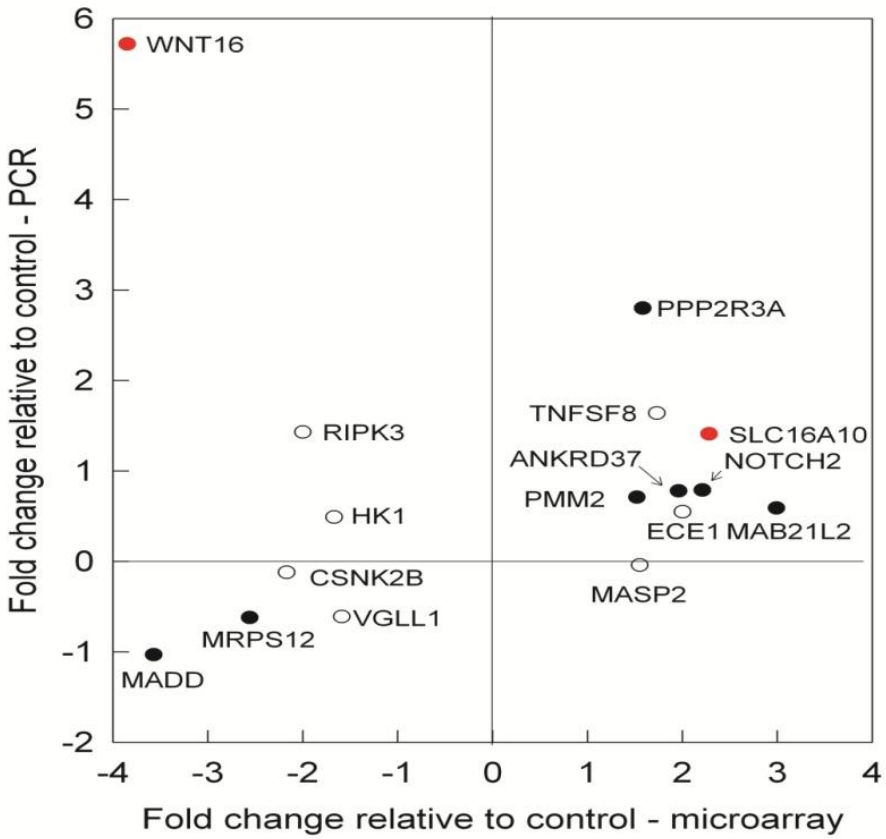

Figure 3-2. Validation of microarray results using quantitative PCR. Shown are the fold-change increases (positive) or decreases (negative) in expression caused by CSF2 as determined by microrarray hybridization (x-axis) and qPCR 9y-axis). Probability values for CSF2 effects in the PCR analysis are designated by the color of the circle (red, P<0.001; black, P=0.09; open, P>0.10).

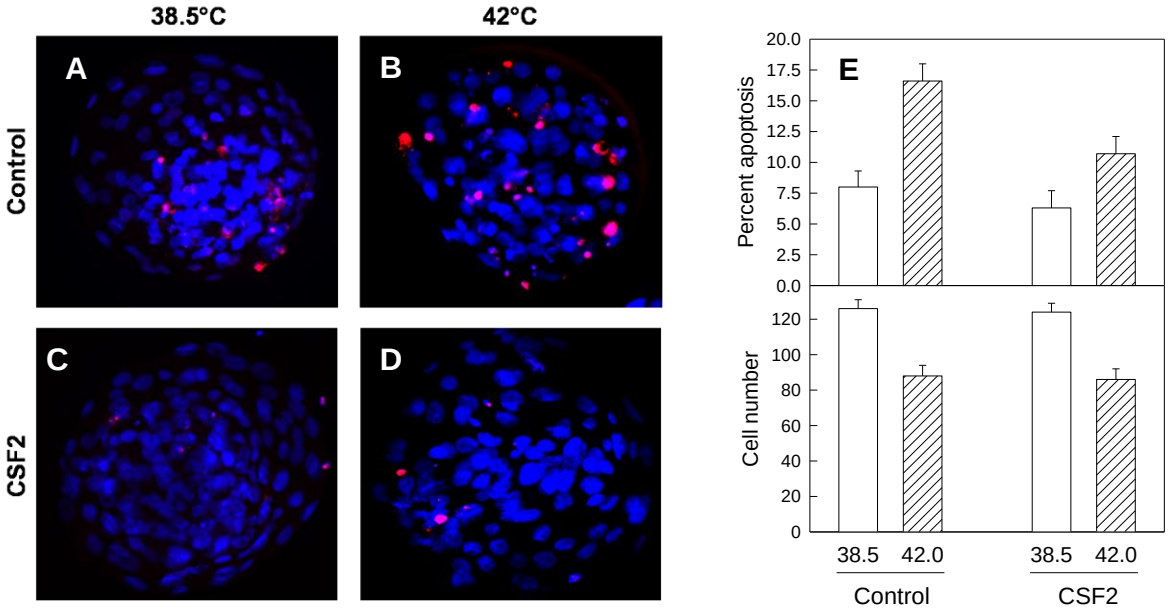

Figure 3-3. Regulation of heat-shock induced apoptosis in Day 6 bovine embryos by CSF. Panels A-D are representative photomicrographs illustrating the frequency of apoptotic nuclei in embryos at 38.5°C (A, D) and 42°C (B, E). as affected by heat shock at 42°C and treatment with colony stimulating factor 2 (CSF2). Detection of apoptosis was by TUNEL analysis using TMR red-conjugated dUTP to identify apoptotic nuclei (red) and Hoescht 33342 to identify all nuclei (blue). Note that exposure of embryos to 42°C for 15 h (B) increased the frequency of TUNEL-positive cells compared with embryos cultured at 38.5°C (A). In the presence of CSF2, however, there was no difference in TUNEL labeling between embryos cultured at 38.5°C (C) or 42°C (D). Panel E shows least-squares means for the percentage of cells that were apoptotic (top panel) and total cell number (bottom panel). Data represent results from 65-81 embryos per treatment. Heat shock at 42°C for 15 h increased the proportion of cells that were TUNEL-positive ($P<0.0001$) and decreased the total cell number ($P<0.0001$). Treatment with CSF2 decreased the percentage of cells that were TUNEL-positive ($P<0.005$) but did not affect total cell number.

CHAPTER 4
CONSEQUENCES OF EMBRYONIC EXPOSURE TO CSF2 FROM DAY 5 TO 7 AFTER INSEMINATION ON TROPHOBLAST ELONGATION, INTERFERON-TAU SECRETION AND GENE EXPRESSION IN THE EMBRYONIC DISC AND TROPHECTODERM

Introduction

Colony-stimulating factor 2 (CSF2) is an important regulator of embryonic development in the cow. It improves the proportion of cultured embryos that develop to the blastocyst stage (de Moraes and Hansen 1997a; Chapter 2), increases the number of cells in the inner cell mass (Chapter 2), and decreases the percentage of blastomeres undergoing apoptosis in response to heat shock (Chapter 3). Moreover, addition of CSF2 to culture medium Days 5 to 7 after insemination improved the competence of in vitro produced embryos to establish pregnancy after transfer to a recipient female while also reducing the probability of fetal loss after Day 30-35 of pregnancy (Chapter 2).

The primary objective of this study was to evaluate possible mechanisms by which CSF2 acts during Day 5 to 7 of development to improve embryonic and fetal survival. One hypothesis was that CSF2 causes increased secretion of interferon-tau (IFNT) by the trophoblast of elongated conceptuses. CSF2 has been shown to stimulate production of IFNT by sheep trophoblast (Imakawa *et al.* 1993; Imakawa *et al.* 1997; Rooke *et al.* 2005) and by a bovine trophoblast cell line (CT-1 cells) (Michael *et al.* 2006). CSF2 might also affect elongation of the trophoblast because of evidence that treatment of embryos from Day 5-6 with CSF2 caused differential regulation of a large number of genes involved in developmental processes (Chapter 3). Analysis of the genes involved in developmental processes regulated by CSF2 indicates a propensity for CSF2 to upregulate genes involved in mesoderm formation or differentiation while

decreasing expression of genes involved in neurogenesis (Chapter 3). This finding raises the possibility that CSF2 causes changes in gastrulation that could be reflected in changes in gene expression in the embryonic disk or trophoblast. A large proportion (~25%) of in vitro produced embryos that survive to Day 14-15 of gestation lose the embryonic disk (Fischer-Brown *et al.* 2004; Fischer-Brown *et al.* 2004; Block *et al.* 2007) and CSF2 might act to improve survival of the embryonic disc, either because of its antiapoptotic actions (apoptosis being more frequent in inner cell mass) (Knijn *et al.* 2003; Fouladi-Nashta *et al.* 2005; Pomar *et al.* 2005) or because of regulation of genes involved in developmental processes.

As part of this study, methods were developed to divide the elongated conceptus into 1) the embryonic disk and a small amount of adjacent trophoblast and 2) tissue containing trophoblast only. As a result, over 500 genes were identified that were preferentially expressed in the embryonic disk. These genes, or the proteins they encode, represent candidates markers for embryonic disk that should prove useful for studying the differentiation of the bovine conceptus through the periattachment period.

Materials and Methods

In vitro Production of Embryos

Embryo production was performed as described in Chapter 3 with modifications described below. Cumulus oocyte complexes (COCs) from ovaries from a mixture of beef and dairy cattle were collected in Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Hyclone, Logan UT) supplemented with 2% (v/v) bovine steer serum containing 2 U/ml heparin (Pel-Freez, Rogers, AR), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocytes were allowed to mature for 20-22 h in groups of 10 in 50 μl microdrops of TCM-199 (Invitrogen,

Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Folltropin-V; Belleville, ON, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine. Matured oocytes were then washed in HEPES-TALP (Parrish *et al.* 1986) (Caisson, Sugar City ID, USA) and transferred in groups of 50 to four-well plates containing 600 µL of IVF-TALP supplemented with 25 µL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl), and fertilized with 30 µL Percoll-purified spermatozoa (~ $1x10^6$ sperm cells). Sperm were prepared from a pool of frozen-thawed semen from three different bulls; a different set of bulls was generally used for each replicate). Fertilization proceeded for 18-20 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in humidified air. Putative zygotes were removed from fertilization plates, denuded of cumulus cells by vortexing in HEPES-TALP, and placed in groups of 30 in 45-μl microdrops of KSOM-BE2 (Soto *et al.* 2003).

Embryos were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 or 5% CO_2, 5% O_2, and 90% N_2 (v/v). Cleavage rate was assessed at Day 3 after insemination. At Day 5 after insemination, 5 µl of KSOM-BE2 or 5 µl of KSOM-BE2 containing 100 ng/ml recombinant bovine CSF2 (a gift from Novartis, Basle Switzerland) were added to each drop to achieve a final CSF2 concentration of 0 or 10 ng/ml.

Transfer Into Recipients

Morula, blastocyst and expanded blastocyst stage embryos (6, 9 and 5 in the control group and 5, 5 and 5 in the CSF2 group, respectively) classified as Grade 1 (Robertson and Nelson, 1998) were harvested on Day 7 after insemination and loaded into 0.25 ml straws in 250 µl HEPES-TALP supplemented with 10% (v/v) fetal calf

serum and 50 µM dithiothreitol (Sigma-Aldrich, St. Louis MO, USA). Straws containing selected embryos were then placed horizontally into a portable incubator (Cryologic, Mulgrave, Vic, Australia) at 38.5°C and transported to the farm.

Thirty five multiparous lactating Holstein cows were used as recipients. Cows were housed in a free-stall barn equipped with fans and a sprinkler system at a commercial dairy in Bell, Florida (29.75578N, 82.86188W). Overall, 4 replicates were completed with 5-11 recipients per replicate from June to October of 2009. For each replicate, eligible cows were synchronized for embryo transfer using the Ovsynch-56 procedure (Chapter 2). Day 0 was considered the Day of expected ovulation. Hormone treatments consisted of 100 µg gonadotrophin releasing hormone (GnRH; Merial; Duluth, GA, USA) i.m. on Day -10; 25 mg $PGF_{2\alpha}$ (Pfizer; New York, NY, USA) , i.m. on Day -3; and 100 µg of GnRH i.m. 56 hours after $PGF_{2\alpha}$. Cows were diagnosed for the presence of a corpus luteum (CL) at Day 7 after anticipated ovulation (Day of ovulation was considered Day 0) using an Aloka 500 ultrasound equipped with a 5 MHz linear array transducer. Cows diagnosed with a corpus luteum were given epidural anesthesia [5 ml of 2% (w/v) lidocaine] and a single embryo transferred to the uterine horn ipsilateral to the ovary via the transcervical route.

Embryo Recovery and Evaluation

At Day 15 after expected ovulation, the ovary ipsilateral to the uterine horn that received the embryo was examined using ultrasonography to confirm the presence of the corpus luteum. Cows that did not have a visible CL were not flushed. Embryos were recovered transcervically with a 20 French Foley catheter inserted with a stainless steel stylet and held in position by inflating the cuff at the end of the catheter. The uterine horn ipsilateral to the CL was flushed with DPBS (Sigma-Aldrich St. Louis MO, USA)

with 1% (v/v) polyvinyl alcohol (PVA). Flushing involved multiple injections and recovery of 60 ml DPBS using a 60 cc syringe attached to the Foley catheter. The procedure was continued until either an embryo was identified in the flush or 180 to 240 ml of DPBS-PVA had been flushed. The flushings recovered from the first 60 ml flush were centrifuged at 500 rpm for 10 min and stored at -20^{o}C for analysis of antiviral activity.

Following recovery, each embryo was assessed for length, stage, and the presence or absence of an embryonic disc (ED) by light microscopy using a stereomicroscope. Stage of development was classified based on shape as ovoid, tubular, or filamentous. After all measurements were recorded, embryos were washed once in DPBS-PVA and the embryo was dissected to produce a piece of tissue containing the ED and some nearby trophoblast (termed ED) and two pieces of trophoblast (Tr) that were on either end of the ED (Figure 4-1). Tissues were immediately snap frozen separately in liquid nitrogen.

Antiviral Assay

The quantity of biologically active IFNT in uterine flushings was determined indirectly using an antiviral assay based on the inhibition of vesicular stomatitis virus (VSV) induced lysis of Madin-Darby bovine kidney (MDBK) cells. The assay was performed as described by Micheal *et al.* (2006). Briefly, 1:3 serial dilutions of the uterine flushings was performed using DMEM containing 10% (v/v) FBS in 100 µl volume in 96 well plates. MDBK cells were resuspended in DMEM-FBS and 50 µl of cell preparation (~500.000/ml) was added to each well. The plate was incubated at 37°C and 5% CO_2 in air for 24 h. Medium was then aspirated from wells and VSV diluted in DMEM (without FBS) was added to each well. The plate was incubated for 1 h at 37°C.

The VSV solution was aspirated, replaced by 100 μl DMEM-FBS and the plate was incubated for 24 h at 37°C. After aspiration of medium, cells were fixed with 70% (v/v) ethanol and stained with 0.5% (w/v) gentian violet stain in 70% (w/w) ethanol (Fisher Scientific, Pittsburgh, PA, USA). Plates were washed two times in water and allowed to dry. Antiviral activity of each sample was assessed visually based on inhibition of lysis of MDBK cells. The antiviral activity (units/ml) was defined as the dilution of flushing that reduced by 50% the destruction of the monolayer by virus. Antiviral units were converted to IFN (IU/ml) using a standard curve with known amounts of human IFN-α standard included in the assay (EMD Biosciences, San Diego, CA). All samples (from cows with and without an embryo) were analyzed in duplicate.

Analysis of the Transcriptome of Trophectoderm and Embryonic Disc

Microarray analysis was performed using a subset of 8 ED and 8 Tr samples that were randomly selected from among the filamentous embryos. Tissues were disrupted by vortexing for 30 seconds and passed through a 20 gauge needle. Samples were homogenized using a QIAshredder (Qiagen-Inc, Valencia,CA, USA). Total cellular RNA was extracted with the RNeasy Plus Micro kit (Qiagen-Inc) following manufacturer's instructions. Concentration of the input RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and RNA integrity was determined by use of the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa clara, CA, USA). Only samples that showed high RNA integrity (RIN > 7) were used for the microarray hybridization and quantitative PCR analysis. Extracted RNA was stored at -80^{o}C until microarray analysis.

Microarray Hybridization

Procedures were performed by the University of Florida Gene Expression Division of the Interdisciplinary Center for Biotechnology Research. Labeled aRNA was generated using Amino Allyl MessageAmp II Amplification kit (Ambion Inc, Austin, TX, USA). First strand cDNA was synthesized from 425 ng of total RNA. Half of the first strand cDNA was used to generate labeled aRNA according to the manufacturer's protocol and the remainder was reserved for quantitative PCR (qPCR) verification. The microarray analysis was performed using the *Bos taurus* Two Color Microarray Chip from Agilent v 2 (Agilent Technologies, Santa Clara CA, USA). The 16 samples were distributed between the 2 microarray chips and the two dyes to avoid location biases. A total of 825 ng of labeled aRNA per sample was used for the hybridization. Hybridization and washing were performed according to the manufacturer's protocol using the Gene Expression Hybridization Kit and Gene Expression Wash Buffers (Agilent). Arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C, Agilent).

Analysis of Microarray Data

The microarray images were first analyzed with Agilent Feature Extraction Software v10.1 (Agilent Technologies, Inc). Spot signal intensities were adjusted by subtracting local background and normalizing using within-array lowess approach for dye-bias correction. The quantile approach was then used for between array normalization. Statistical tests were performed using BioConductor statistical software (http://www.bioconductor.org/), which is an open source and open development software project for analysis of microarray and other high-throughput data based primarily on the R programming language (Gentleman *et al.* 2004).

Differentially expressed genes were identified using Limma, a software package that implements linear models for microarray data (Smyth *et al.* 2005).In Limma, *p*-values are obtained from moderated *t* statistics or *F* statistics using empirical Bayesian methods. The primary analysis comparing differential expression was a 2 × 2 factorial design with tissue (ED, Tr) and treatment (control, CSF2) as main effects and the tissue x treatment interaction as another effect. Pairwise comparisons of interest were also performed to test for significant differential expression. The Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) was used to control false discovery rate (FDR) at the 0.01 level. Genes meeting this statistical threshold and showing a fold change equal or greater than 2 were considered as differentially expressed.

Ingenuity pathway analysis software (http://www.ingenuity.com) was used to identify canonical pathways associated with differentially expressed genes. Fisher's exact test was used to determine the probability that the association between the genes and the pathway was explained by chance alone.

Quantitative Real Time PCR

Quantitative Real Time PCR analysis (qPCR) of 11 differentially-expressed genes and one housekeeping gene (*GAPDH*) was performed to confirm microarray results. Specific primers (Table 4-1) were designed using Integrated DNA technologies software (http://idtdna.com). The SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) reaction chemistry and a CFX 96 Real-Time PCR Detection System (Bio-Rad) were used to quantify mRNA concentrations. After an initial activation step (95ºC for 30 sec), 45 cycles of a two-step amplification protocol (95ºC for 5 sec; 60ºC for 5 sec) were completed.

The amplification of a single product was verified by performing dissociation curve analysis (65-95ºC) in the thermocycler. In additional, agarose gel electrophoresis of the PCR product was performed. Identity of the amplicon was confirmed by sequencing the PCR product of the band product. The DNA was extracted from the gel fragment using the PureLink Quick gel Extraction and PCR Purification Combo Kit (Invitrogen Carlsbad, CA, USA). First, the gel with the DNA fragment was dissolved using Solubilization Buffer for 15 min at 50°C. The gel mixture was centrifuged for 1 min at 10.000 x g in a PureLink clean up spin column. After a wash, the DNA was extracted from the tube membrane by centrifuging the column with Elution Buffer for 1 min at 10.000 x g. The purified DNA was sequenced by the University of Florida Gennomics Division of the Interdisciplinary Center for Biotechnology Research.

Each sample was analyzed in duplicate reactions. All C_T values for genes of interest were normalized to the housekeeping gene *GAPDH* using the ΔC_T method. The $\Delta\Delta C_T$ for each sample was calculated by subtracting the average ΔC_T of the gene of interest for control embryos from the value for each individual embryo. Fold change was determined by solving for $2^{-\Delta\Delta CT}$.

Statistical Analysis

Categorical data were analyzed by logistic regression using the LOGISTIC procedure of the Statistical Analysis System (SAS® 9.1.3, SAS Inst., Cary, NC, USA) with backward selection (P=0.2). The statistical model included replicate, stage of the embryo at the time of transfer, and treatment. Effects on continuous variables were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS. Various mathematical models were utilized. All included treatment and, in addition, included replicate, stage of the embryo at the time of transfer, stage of the embryo at

Day 15 and, for embryo length, whether the embryo was intact. Data for embryo length and antiviral activity were subjected to log transformation before analysis to account for heterogeneity of variance. In addition, these data were analyzed using the Wilcoxon's and median nonparametric tests with the NPARWAY1 procedure of SAS.

Results

Embryo Survival After Transfer

Results are presented in Table 4-2. A total of 80% of cows receiving a control embryo and 93% of cows receiving a CS2F-treated embryo had a CL detected by ultrasound. For the other cows, the CL was either absent or small and regressing. Cows without a CL represent either cows that were not successfully synchronized, so that the presumed Day 15 was actually later in the estrous cycle, or cows in which the CL had undergone luteolysis by Day 15. When all cows were considered, including those without a large corpus luteum, there was a tendency (P=0.07) for the proportion of cows with a recovered embryo to be higher for those receiving a CSF2 treated embryo (35% for control vs. 66% for CSF2). The same trend was apparent when only cows with a CL were considered (44% for control vs 71% for CSF2) but the difference did not approach significance.

One cow in the control group did not have a detectable embryo but there was abundant antiviral activity in uterine flushings (59.049 IU IFN/ml). This cow either lost its pregnancy after the embryo initiated large scale IFNT secretion or the embryo was not recovered in the flushing. When this cow was considered pregnant, differences in survival between control and CSF2 groups remained but did not approach significance (Table 4-2).

Embryonic Growth and Development

Five of 7 control embryos (71%) and 8 of 10 (80%) CSF2-treated embryos were filamentous. The embryonic disc was visible in 6 of 7 (83%) of control embryos and 6 of 10 (60%) of CSF2-treated embryos. Differences between treatments in these two variables were not significant.

As illustrated in Figure 4-2, there was great variability in embryo length, even if the dataset was restricted to embryos that were filamentous. While there was no significant effect of treatment, CSF2-treated embryos tended to be longer than control embryos regardless of whether all embryos were considered, only those recovered intact were considered (4 embryos were recovered in pieces and were likely to be larger than measured), or only filamentous embryos were recovered. Overall, the average length was 39 mm for control embryos and 62 mm for CSF2-treated embryos.

Antiviral Activity in Uterine Flushings

Antiviral activity was also highly variable, ranging from non-detectable amounts (one cow in each group) to almost 9,000,000 IU IFN/ml (Figure 4-2). For all cows, there was a nonsignificant trend for antiviral activity to be higher for cows receiving CSF2-treated embryos than for cows receiving control embryos. This difference approached significance (P=0.07) if only cows with detectable antiviral activity were considered.

Changes in the Transcriptome of Embryonic Disc and Trophectoderm

To test the hypothesis that CSF2 alters the transcriptome of the filamentous embryo, gene expression was evaluated in two tissues - embryonic disc (which also contains adjacent trophoblast) and trophoblast using microarray technology. All 43.803 probes on the array produced signal above background. These probes represent 19,500 distinct genes or 88% of the bovine genome (btau 4.0). Hierarchical analysis

(Figure 4-3) indicated that the 8 ED samples formed a distinct cluster separated from the Tr. There was, however, no such distinction between samples from control and CSF2-treated embryos. Using a FDR ≤0.01 and a 2-fold difference as criteria, there were no genes affected by treatment or the treatment x tissue type interaction. There was, however, a total of 627 genes that were differentially expressed between ED and Tr and 576 of these could be annotated. Of the annotated genes, 538 genes were upregulated in ED and 38 genes were upregulated in Tr. One would expect a preponderance of differentially regulated genes that were upregulated in ED, and not in Tr, because the former tissue contains some Tr while the Tr sample was free of ED tissue.

Characteristics of Genes Differentially Expressed Between Embryonic Disc and Trophoblast

Ingenuity software was used to identify pathways that were significantly overrepresented in the set of genes that were differentially expressed between ED and Tr. Determination of significant pathways is based on the ratio of the number of genes in the differentially expressed dataset divided by the number of genes in the pathway and the P-value. A total of 31 canonical pathways were identified as above the threshhold ($P<0.05$; Table 4-3). The pathway with the highest score (smallest P-value) was Embryonic Stem Cell Pluripotency. Of the 148 genes in this pathway, 16 (*RAC2, NODAL, FGF2, FGFR1, SMAD6, FZD1, LEFTY2, TCF7, SOX2, NANOG, SMO, PDGFRA, BMP6, WNT11, POU5F1, FZD7*) were represented in the list of differentially expressed genes. Other pathways related to pluripotency were also significant, including Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency, with 7 of 45

genes in the pathway being differentially expressed, and Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency, with 9 of 114 genes being differentially expressed.

Several pathways that promote cellular differentiation were identified including pathways for Factors Promoting Cardiogenesis in Vertebrates, responsible for differentiation of the mesoderm into myocardium and endocardium, with 11 of 89 genes in the pathway being differentially expressed, Wnt/β-catenin Signaling (responsible for many differentiation events including cell polarity, neural tube patterning and cardiogenesis), with 15 of 168 genes being differentially expressed and Axonal Guidance Signaling, responsible for formation of neuronal connections, with 22 of 403 genes being differentially expressed.

Another type of pathway that was well represented was for cell signaling. Among these were pathways for Basal Cell Carcinoma Signaling, with 8 of 68 genes being differentially expressed, Ovarian Cancer Signaling, with 10 of 135 genes, Leukocyte Extravasation Signaling, with 12 of 194 genes, Prolactin Signaling, with 6 of75 genes, CTLA4 Signaling in Cytotoxic T Lymphocytes, with 7 of 94 genes, G-Protein Coupled Receptor Signaling, with 12 of 220 genes, HER-2 Signaling in Breast Cancer, with 6 of 79 genes, TGFB Signaling, with 6 of 83 genes, Acute Phase Response Signaling, with 10 of 178 genes, and Colorectal Cancer Metastasis Signaling, with 12 of 249 genes represented.

Identification of Likely Candidate Genes for Use as Embryonic Disc Markers

It is likely that many of the genes that are differentially expressed between ED and Tr will prove useful in future studies for identifying ED and Tr. Two different criteria were used to identify some of the 576 differentially expressed genes that might be particularly

useful as markers. The first criterion was to identify genes with the highest fold change between ED and Tr. The 15 genes with the highest fold difference for genes overexpressed in ED and the 15 genes with the highest fold difference for genes overexpressed in Tr are shown in Table 4-4. The fold difference for the 15 highest genes overexpressed in ED ranged from 120-fold to 45-fold, with the most overexpressed gene being fatty acid binding protein 1 (*FABP1).* Other genes with large fold increase for ED compared with Tr were transforming growth factor-β induced protein IG-H3 (*TGFBI1) (*95.2 fold increase), sepallata3 (*SEP3) (*89.9 fold increase) and nanog homeobox (*NANOG)* (75.7 fold increase). In contrast, the fold-difference for the 15 highest genes overexpressed in Tr ranged from -8.3 to -2.5, with the greatest fold change for ribosomal protein L36a (*RPL36A*) (-8.3 fold), ribosomal protein L10a (-5.2 fold) RAR-related orphan receptor B (*RORB*) (-4.7 fold).

The second approach to identify candidate gene markers was to identify the differentially expressed genes that were the most abundant. The rationale was that abundant genes, or their protein products, would be more easy to identify using a variety of molecular and immunochemical procedures. A list of the 15 most abundant genes for ED and Tr, based on signal intensity on the microarray, is presented in Table 4-5.

Validation of Microarray Results Using qPCR

A total of 11 genes that were differentially expressed between ED and Tr based on microarray analysis, were subjected to analysis by qPCR. For all 11 of the genes, the fold change for ED relative to Tr was in the same direction for qPCR as the fold change as determined by microarray hybridization. Differences between ED and Tr

were significant for 10 of the 11 genes, the exception being *GJC1.* For 7 genes (*BMPER, CLDN11, FGF2, GATA5, GDF3, IGFL1* and *NODAL*), the fold-change was of greater magnitude as determined by qPCR than as determined by microarray hybridization. There was no effect of treatment (control vs CSF2) or treatment x tissue interaction on mRNA abundance as determined by qPCR.

Discussion

Exposure of bovine embryos to CSF2 from Day 5 to 7 of development can have a profound effect on the subsequent developmental fate of the embryo. In particular, a greater percentage of embryos result in pregnancies at Day 30-35 of pregnancy and fewer of the pregnancies established at that point are lost thereafter (Chapter 2). The purpose of this experiment was to understand the mechanism by which CSF2 increases embryonic survival as measured at Day 30-35 and reduces fetal mortality thereafter. Results suggest that higher pregnancy rates at Day 30-35 represent increased embryonic survival before Day 15 and a greater capacity of the embryo to elongate and secrete IFNT at Day 15. Analysis of gene expression in filamentous embryos indicates little difference in transcription among this subset of embryos that survived to Day 15 and elongated successfully. Therefore, the reduction in embryonic and fetal loss after Day 30-35 caused by CSF2 is probably not a direct reflection of altered gene expression at Day 15.

Conclusions regarding embryonic survival, growth and IFNT secretion at Day 15 must be tentative because differences between control and CSF2 were largely non-significant. Nonetheless, it seems more likely that these traits were affected by CSF2. The proportion of transferred embryos that were recovered at Day 15 was twice as high for cows receiving CSF2 embryos (35% in control vs 66% in CSF2; $P<0.07$). Embryonic

length and antiviral activity in the uterus (a measure of IFNT bioactivity) (Maneglier *et al.* 2008) were highly variable, as is typical at this stage of pregnancy (Bilby *et al.* 2004; Bilby *et al.* 2006), but the largest embryos were in the CSF2 group and antiviral activity among cows that had detectable activity (excluding cows with tubular embryos where IFNT secretion would be low (Short *et al.* 1991) tended ($P<0.07$) to be greater for cows receiving CSF2 embryos.

Based on these findings, it can be postulated that CSF2 improves embryonic survival at two periods. The first is in the 8 Day period between transfer at Day 7 and flushing at Day 15. Embryos treated with CSF2 have large inner cell mass at Day 7 (Chapter 2) and are less prone to undergo apoptosis in response to heat shock (Chapter 3). The larger size of the inner cell mass and resistance to stress could promote early survival of the embryo. The second period is the period of maternal recognition of pregnancy, around Day 15-18, when the embryo acts to prevent luteolysis through secretion of IFNT (Thatcher *et al.* 2001) The larger size and greater IFNT secretory activity of the CSF2-treated embryos (as determined by uterine antiviral activity) would make the CSF2 embryo better able to prevent luteolysis and allow continued development of the embryo. Size of the embryo at this period does affect IFNT secretion (Bilby *et al.* 2004; Bilby *et al.* 2006). Indeed, the trend for a lower incidence of cows without functional CL (based on ultrasound) in cows receiving CSF2-treated embryos may reflect the greater antiluteolytic capacity of the CSF2-treated embryo.

The mechanism by which CSF2 treatment from Day 5-7 results in greater embryonic growth and IFNT secretion at Day 15 is not known. However, CSF2 can

increase expression of *IFNT* in sheep trophoblast (Imakawa *et al.* 1993; Imakawa *et al.* 1997; Rooke *et al.* 2005) and bovine trophoblast cells (CT-1 cells) *in vitro* (Michael *et al.* 2006). In addition, embryos treated with CSF2 at Day 5 had altered expression of genes involved in developmental processes at Day 6 (Chapter 3) characterized by an increase in expression of genes involved in mesoderm, mesenchyme and muscle formation and a decrease in genes involved in neurogenesis. Perhaps, effects of CSF2 on gastrulation lead to increased growth of trophoblast between Day 7 and 15 of development.

A relatively large number of embryos at Day 14 -15 do not have a detectable embryonic disc (Bertolini *et al.* 2002; Fischer-Brown *et al.* 2002; Fischer-Brown *et al.* 2004) and these embryos all eventually die (Fischer-Brown *et al.* 2004). Despite its effects on inner cell mass number, however, CSF2 did not affect the incidence of embryos without a disk and, numerically, the percentage of embryos without a disk was lower in the CSF2 group. It can be concluded from these observations that CSF2 is not improving embryonic survival by increasing the likelihood that the embryonic disc survives to Day 15.

There was no effect of CSF2 on expression of genes in either the ED or Tr at Day 15. The embryos used for this analysis were all filamentous embryos. Thus, CSF2 has no discernable effect on embryonic gene expression at Day 15 for the subset of embryos that has developed normally up to that point. It is unlikely, therefore, that the reduction in embryonic or fetal loss caused by CSF2 is the result of global changes in transcription. Instead, perhaps, altered trajectory of development caused by CSF2, as characterized by changes in expression of developmentally important genes at Day 6 (Chapter 3) could lead to formation of a fetus that is less likely to experience a

developmental defect leading to pregnancy loss after Day 35. It is also possible that CSF2 causes epigenetic changes in the developing embryo that result in altered gene expression at time points later than studied here. These changes would affect late embryonic and fetal loss.

One benefit of the transcriptomal analysis was that a large number of genes that are overexpressed in ED were identified. These genes represent good candidates for markers of the embryonic disc. The developmental processes involved in developmental events after embryonic hatching, including gastrulation and organ development, are not well understood in cattle and is the subject of investigation by several groups (Maddox-Hyttel *et al.* 2003; Alexopoulos *et al.* 2005; Tveden-Nyborg *et al.* 2005; Vejlsted *et al.* 2006). Identification of markers of the embryonic disc could simplify the study of post-hatching development. The genes that were differentially regulated between ED and Tr included large numbers of pluripotency genes, genes controlling differentiation and development and cell signaling genes. This is to be expected given the nature of the developmental processes underway in the ED at Day 15. Three genes involved in pluripotency serve as examples of the functional importance of the genes overexpressed in ED. *NANOG* was overexpressed 75 fold in the ED. In the mouse and human blastocyst, *NANOG* expression is limited to inner cell mass and epiblast and is necessary for pluripotency maintenance (Marikawa and Alarcón 2009; Guo *et al.* 2010). *NANOG* is downregulated in trophoblast by CDX2 (Strumpf *et al.* 2005). In the bovine, *NANOG* mRNA and protein are found in both inner cell mass and trophectoderm of the blastocyst, although *NANOG* expression is greater in the ICM and epiblast (Degrelle *et al.* 2005; Muñoz *et al.* 2008). Another gene that

serves as a marker of ICM and epiblast in mouse, human, and bovine,*SOX2* (Degrelle *et al.* 2005; Guo *et al.* 2010), was upregulated 57 fold in the ED compared with Tr in the present study. *SOX2* is the first transcription factor to appear in the late morula and is a key factor for maintenance of pluripotency and for reprogramming of differentiated cells into induced stem cells (Takahashi and Yamanaka 2006). Finally, *POU5F1,* also known as *Oct4*, was increased 29 fold in ED compared with Tr. Expression of *POU5F1* is limited to the ICM in human and mouse blastocysts (Zernicka-Goetz *et al.* 2009) but is in both ICM and Tr of *in vitro* and *in vivo* produced bovine blastocysts through Day 10 of development (Eijk *et al.* 1999; Kirchhof *et al.* 2000; Degrelle *et al.* 2005). POU5F1 is a transcription factor necessary for the maintenance of the pluripotency in the ICM and it prevents ICM transformation to TE (Zernicka-Goetz *et al.*, 2009).

Some markers of trophoblast were also identified although technical limitations of the procedure used to separate ED and Tr meant that many fewer of these were found than for ED.

In conclusion, results support the idea that the increased survival of embryos exposed to CSF2 from Day 5-7 of development is the result of increased embryonic survival before Day 15 and a greater capacity of the embryo to elongate and secrete IFNT at Day 15. The reduction in embryonic and fetal loss after Day 30-35 caused by CSF2 is probably not a direct reflection of altered gene expression at Day 15.

Table 4-1. Primers used for qPCR.

Accession #	Name	Sequence	Product size	Melting temp
NM_001077997	*BMPER*	F 5'-AGA GGA CTC CTA GTC CAA CAC TCT-3'	128 bp	77°C
		R 5'-GGA AAT GAG AGC AAG CAT GTA GAC C-3'		
NM_001030318	*CAST*	F 5'-AGA GGT CTA TGT GTT CCG TGC AGT-3'	136 bp	86°C
		R 5'-ACA GGC TTT CCG TCT TCT GGA TCT-3'		
NM_001035055	*CLDN11*	F 5'-CAT TCT GTG AGC TGT CTT GAA GTG-3'	81 bp	75°C
		R 5'-ATC AGT GTT TGC ACC CGT AAA GCC-3'		
NM_174056	*FGF2*	F 5'-TCT CTC GGG AAA CTG CTG ACT TGT-3'	110 bp	74°C
		R 5'-CCC ACT GTT TCA CTC ATA CAG AAT TT-3'		
NM_001034221	*GATA5*	F 5'-TCA CAT TGT AAT CAT CGT GGA CCC G-3'	80 bp	77°C
		R 5'-CAG AAC AAG GAA GGC TCT TTA CTG CC-3'		
NM_001025344	*GDF3*	F 5'-CTC CAT GCT CTA CCA GGA CAA TGA-3'	83 bp	78°C
		R 5'-ACC CAC ACC CAC ACT CAT CAA CTA-3'		
NM_001046076	*GJC1*	F 5'-AGA GAA CGG GAA ACA CAG CGT TC-3'	80 bp	77°C
		R 5'-CTG GAA GAC ACA AAT GTA AAG TTC TGC AAC-3'		
BE664075	*IGFL1*	F 5'-AGG CAC TCT GTG AAT TCT GCA ACC-3'	91 bp	79°C
		R 5'-TCT TGC CAC CTT TGG AAG TGG AGA-3'		
XM_609225	*NODAL*	F 5'-GAA GAC CAA GCC CTT GAG TAT GCT A-3'	91 bp	78°C
		R 5'-GCA CCC ACA TTC CTC CAC AAT CAT-3'		
NM_176646	*SERPINA5*	F 5'-GGG ATT GTA CTG TCC TGT GGG TTA-3'	80 bp	80°C
		R 5'-TAT TCG CGT CAG GCC TCC ATT CTT-3'		
XM_001788143	*TMEFF1*	F 5'-AAT AGA GGA CGA CGA CAG AAG CA-3'	80 bp	77°C
		R 5'-AAG TCA CCA GTT CAA ACC ATT CTG G-3'		
NM_001034034.1	*GAPDH*	F 5'-ACC CAG AAG ACT GTG GAT GG-3'	177 bp	88°C
		R5'-CAA CAG ACA CGT TGG GAG TG-3'		

Table 4-2. Estimates of effect of CSF2 on embryonic survival at Day 15 after expected ovulation.

	Control	CSF2	P-value
Number of cows receiving embryos	20	15	
Number of cows (percentage) with CL on Day 15	16/20 (80%)	14/15 (93%)	N.S.
Number (percentage) of cows with a recovered embryo, of all cows	7/20(35%)	10/15 (66%)	0.07
Number (percentage) of cows with a recovered embryo, of cows with a CL	7/16 (44%)	10/14 (71%)	N.S.
Number (percentage) pregnant[a], of all cows	8/20 (40%)	10/15 (67%)	N.S.
Number (percentage) pregnant[a], of cows with a CL	8/16 (50%)	10/14 (71%)	N.S.

[a] Cows were considered pregnant if there was a detectable embryo or antiviral activity in the flushing

Table 4-3. Canonical pathways containing a significant number of genes differentially expressed between embryonic disc and trophoblast.

Pathway[b]	P-value	Genes[a]
Human Embryonic Stem Cell Pluripotency	0.000001	*RAC2, NODAL, FGF2, FGFR1, SMAD6, FZD1, LEFTY2, TCF7, SOX2, NANOG, SMO, PDGFRA, BMP6, WNT11, POU5F1, FZD7*
Factors Promoting Cardiogenesis in Vertebrates	0.000032	*NODAL, CER1, SMO, FZD1, DKK1, BMP6, TCF7, WNT11, PRKD1, FZD7, PRKCB*
Coagulation System	0.000105	*F10, PROS1, SERPINA5, PROC, A2M, SERPINF2, SERPIND1*
Wnt/β-catenin Signaling	0.000141	*RAC2, GJA1, SFRP2, FZD1, SOX2, CDH2, DKK3, SMO, DVL3, PPP2R2C, SOX8, SFRP1, DKK1, WNT11, FZD7*
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	0.000380	*SOX2, TDH, NANOG, SPP1, PHC3, FBXO15, POU5F1*
Basal Cell Carcinoma Signaling	0.000741	*SMO, DVL3, FZD1, BMP6, GLI1, TCF7, WNT11, FZD7*
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.001738	*RAC2, SPP1, SFRP2, SMAD6, FZD1, TCF7, DKK3, NGFR, SMO, SFRP1, DKK1, BMP6, WNT11, TNFRSF11B, FZD7*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.001778	*COL1A2, IGFBP4, CD40, FGF2, NGFR, FGFR1, PDGFRA, EDNRA, MMP2, A2M, TNFRSF11B*
Axonal Guidance Signaling	0.001778	*DPYSL2, PRKACB, FYN, RAC2, PLXNC1, NRP2, EPHB2, DPYSL5, FZD1, ROBO1, SDC2, CXCL12, NGFR, SMO, RASSF5, BMP6, GLI1, WNT11, PRKD1, FZD7, PRKCB, UNC5C*
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.004365	*RAC2, SFRP2, FGF2, FZD1, TCF7, ROR2, DKK3, CXCL12, NGFR, SMO, ATF4, SFRP1, DKK1, WNT11, PRKD1, TNFRSF11B, FZD7, PRSS35, PRKCB*

Table 4-3. Continued

Pathway[b]	P-value	Genes[a]
Pantothenate and CoA Biosynthesis	0.004677	*DPYSL2, DPYS, CILP2, ENPP2*
Ovarian Cancer Signaling	0.004786	*PRKACB, RAC2, GJA1, SMO, EDNRA, MMP2, FZD1, TCF7, WNT11, FZD7*
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	0.005888	*SOX2, RAC2, NANOG, SMO, FZD1, BMP6, WNT11, FZD7, POU5F1*
LPS/IL-1 Mediated Inhibition of RXR Function	0.006607	*CYP2C9, APOC4, APOC2, FMO5, FABP2, ALDH1A1, UST, NGFR, FABP1, FABP7, ACSL1, MGST3, TNFRSF11B*
Leukocyte Extravasation Signaling	0.010965	*CLDN10, RAC2, CLDN11, CLDN5, MMP25, CXCL12, MMP2, RASSF5, ITGAL, PRKD1, MMP19, PRKCB*
RAR Activation	0.012023	*PRKACB, RAC2, ALDH1A1, CRABP2, ADCY3,*
Glycerolipid Metabolism	0.014454	*SMAD6, NCOR1, CRABP1, PRKD1, ADH4, PRKCB ADH6, PNLIPRP2, LIPA, ALDH1A1, LPL, DGAT2, APOC2, ADH4*
Prolactin Signaling	0.016982	*FYN, PRLR, PDK1, TCF7, PRKD1, PRKCB*
FXR/RXR Activation	0.017378	*RAC2, APOB, PCK2, APOC2, HNF4A, FOXA3, MTTP*
LXR/RXR Activation	0.018197	*APOC4, NGFR, LPL, NCOR1, APOC2, TNFRSF11B*
CTLA4 Signaling in Cytotoxic T Lymphocytes	0.018197	*FYN, CD3G, RAC2, LCK, AP1S2, PPP2R2C, CD3D*
PXR/RXR Activation	0.020417	*PRKACB, RAC2, ALDH1A1, CYP2C9, PCK2, HNF4A*
G-Protein Coupled Receptor Signaling	0.021380	*GPR161, PRKACB, FYN, RAC2, RASGRP1, RGS10, ADCY3, ATF4, EDNRA, DRD2, PDE4D, PRKCB*
HER-2 Signaling in Breast Cancer	0.026303	*RAC2, NRG1, MMP2, PARD6G, PRKD1, PRKCB*

Table 4-3. Continued

Pathway[b]	P-value	Genes[a]
Maturity Onset Diabetes of Young (MODY) Signaling	0.027542	*FABP2, FABP1, HNF4A*
TGF-β Signaling	0.029512	*ZNF423, NODAL, INHA, GSC, SMAD6, HNF4A*
Acute Phase Response Signaling	0.032359	*RAC2, TF, NGFR, APOA2, CRABP2, A2M, SERPINF2, CRABP1, TNFRSF11B, SERPIND1*
Calcium-induced T Lymphocyte Apoptosis	0.033113	*CD3G, LCK, CD3D, PRKD1, PRKCB*
Virus Entry via Endocytic Pathways	0.046774	*FYN, RAC2, ITGAL, PRKD1, FOLR1, PRKCB*
Molecular Mechanisms of Cancer	0.047863	*PRKACB, FYN, RAC2, DIRAS3, ADCY3, SMAD6, FZD1, RASGRP1, SMO, IHH, BMP6, GLI1, PRKD1, BCL2L11, PRKCB, FZD7*
Colorectal Cancer Metastasis Signaling	0.047863	*PRKACB, RAC2, MMP25, DIRAS3, ADCY3, SMO, MMP2, FZD1, TCF7, WNT11, FZD7, MMP19*

[a] Genes symbols in black are overexpressed in ED, genes symbols in red are overexpressed in Tr.

[b] Pathways are from Ingenuity (www.ingenuity.com)

Table 4-4. Genes with the greatest fold change for embryonic disc (ED) compared with trophoblast (Tr).

Accession #	Name	Fold Change	Intensity Means Tr	Intensity Means ED
Overexpressed in ED				
NM_175817	Fatty acid binding protein 1 (*FABP1*)	119.93	4.90	610.36
XR_028016	Transforming growth factor-Beta IG-H3 (*TGFBI*)	95.21	24.99	2379.0
NM_001076949	Septin 3 (*SEP3*)	89.88	7.44	669.04
NM_001025344	Nanog homeobox (*NANOG*)	75.69	11.51	1390.0
NM_174188	Secreted phosphoprotein 2 (*SPP2*)	68.26	7.32	499.21
NM_001077921	Amyloid beta precursor protein A 2 (*APBA2*)	64.85	7.25	470.18
NM_001105463	SRY (sex determining region Y)-box 2 (*SOX2*)	57.68	7.59	873.88
NM_001105411	GDNF family receptor alpha 1 (*GFRA1*)	56.96	4.59	261.73
NM_177484	Transferrin (*TF*)	54.89	31.07	6954.9
NM_001040502	Alpha-1 acid glycoprotein (*AGP*)	52.29	16.37	1006.4
NM_001083369	Transmembrane protein 130 (*TMEM130*)	50.70	4.74	240.07
NM_001078019	Solute carrier family 7, member 3 (*SLC7A3*)	49.32	13.38	660.05
XM_001789128	DAZ interacting protein 1 (*DZIP1*)	48.07	4.38	210.50
NM_001076945	Stearoyl-CoA desaturase 5 (*SCD5*)	46.19	7.65	460.06
XM_612940	Carboxypeptidase A6 (LOC540749)	45.16	4.53	238.31

Table 4-4. Continued

Accession #	Name	Fold Change	Intensity Means Tr	ED
Overexpressed in Tr				
XM_614458	CAP-GLY domain containing linker protein 1 (*CLIP1*)	-2.53	333.15	131.55
XM_866711	Nuclear receptor co-repressor 1 (*NCOR1*)	-2.57	184.07	71.62
XM_614825	Centrosomal protein (*CEP350*)	-2.59	104.34	40.32
NM_001034342	ATF4 activating transcription factor 4 (*ATF4*)	-2.60	342.05	131.44
XM_612376	Centromere protein F (*CENPF*)	-2.64	345.30	130.74
XM_585315	Biorientation of chromosomes in cell division 1 (*BOD1L*)	-2.71	296.27	109.40
NM_001102181	Cromodomain helicase DNA binding protein 2 (*CHD2*)	-2.81	287.45	102.12
NM_174436	Psrotein kinase, cGMP-dependent, I (*PRKG1*)	-2.88	73.98	25.68
XM_580572	Dickkopf homolog 1 (*DKK1*)	-2.92	2519.12	887.33
NM_174239	Aldehyde dehydrogenase 1, member A1 (*ALDH1A1*)	-3.02	457.08	151.46
XM_581155	Filamin A interacting protein 1 (*FILIP1L*)	-3.04	440.05	144.76
NM_001045901	Growth arrest and DNA-damage-inducible γ (*GADD45G*)	-3.56	2178.12	611.84
XM_606804	RAR-related orphan receptor B (*RORB*)	-4.74	36.98	7.79
XM_001256996	Ribosomal protein L10a (LOC790558)	-5.23	81.89	15.65
XM_864189	Ribosomal protein L36a (*RPL36A*)	-8.26	96.67	11.70

Table 4-5. The 15 most abundant genes overexpressed in embryonic disc or trophoblast.

Accession #	Name	Intensity Means		
Embryonic Disc		ED	Tr	Fold Change
NM_001034790	Stathmin 1 *(STMN1)*	39876.8	8984.0	4.4
NM_001166505	Tubulin, alpha 1a *(TUBA1A)*	39837.2	4416.9	9.0
NM_175801	Follistatin *(FST)*	29163.8	13194.4	2.4
XM_001251618	GL12416-like *(TUBA3D)*	26547.3	2820.9	9.4
NM_176646	Serpin peptidase inhibitor, clade A, member 5 *(SERPINA5)*	18250.3	1213.3	15.0
NM_174580	POU class 5 homeobox 1 *(POU5F1)*	17887.4	489.3	29.2
NM_001098378	Transmembrane protein 88 *(TMEM88)*	17535.0	7907.4	2.2
NM_205802	Lysosomal protein transmembrane 4 beta *(LAPTM4B)*	13950.3	2251.5	6.1
NM_001075171	Coiled-coil domain containing 109B *(CCDC109B)*	12955.7	3284.5	3.9
NM_001034231	Inhibitor of DNA binding 2 *(ID2)*	12754.3	4141.6	3.0
NM_001097568	Inhibitor of DNA binding 1 *(ID1)*	11206.3	2432.3	4.6
NM_174438	Protein S-alpha *(PROS1)*	9820.1	2079.4	4.7
NM_001114857	Metallothionein 1E *(MT1E)*	9815.9	393.8	18.9
NM_001101149	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 *(PLOD2)*	9500.8	4296.1	2.2
NM_001035046	Microsomal glutathione S-transferase 3 *(MGST3)*	8855.9	1347.2	6.6
Trophectoderm		ED	Tr	Fold Change
XM_580572	Dickkopf homolog 1 *(DKK1)*	887.33	2519.12	-2.92
NM_001045901	Growth arrest and DNA-damage-inducible, gamma *(GADD45G)*	611.84	2178.12	-3.56
XM_581232	Rho GTPase activating protein 21 *(ARHGAP21)*	392.39	841.24	-2.19
NW_877106.1	Retrovirus-related Pol polyprotein from transposon 297 *(LOC608201)*	339.91	783.89	-2.31
NM_174239	Aldehyde dehydrogenase 1 family, member A1 *(ALDH1A1)*	151.46	457.08	-3.02

Table 4-5. Continued

Accession #	Name	Intensity Means		
Trophectoderm		ED	Tr	Fold Change
XM_581155	Filamin A interacting protein 1-like *(FILIP1L)*	144.76	440.05	-3.04
NM_174824	Protein phosphatase 1, regulatory (inhibitor) subunit 16B *(PPP1R16B)*	182.95	386.36	-2.11
XM_612376	Centromere protein F, 350/400ka *(CENPF)*	130.74	345.30	-2.64
NM_001675.2	ATF4 activating transcription factor 4 *(ATF4)*	131.44	342.05	-2.60
XM_614458	CAP-GLY domain containing linker protein 1 *(CLIP1)*	131.55	333.15	-2.53
XM_585315	Biorientation of chromosomes in cell division 1-like *(BOD1L)*	109.40	296.27	-2.71
NM_001102181	Chromodomain helicase DNA binding protein 2 *(CHD2)*	102.12	287.45	-2.81
NM_001083753	SH3 domain containing ring finger 2 *(SH3RF2)*	115.48	236.26	-2.05
XM_589712	Tetratricopeptide repeat domain 17 *(TTC17)*	94.55	219.57	-2.39
NM_001103246	N-acetyltransferase 9 *(NAT9)*	82.36	202.23	-2.46

Table 4-6. Differences in expression of selected genes between embryonic disk and trophoblast as determined by microarray analysis and qPCR.

Name	Symbol	Fold Change [a]		P-value for qPCR
		Microarray	qPCR	
BMP binding endothelial regulation	*BMPER*	26.0	1389	<0.0001
Calpastatin	*CAST*	-2.1	-2.4	<0.006
Claudin 11	*CLDN11*	36.0	205.0	<0.0001
Fibroblast growth factor 2	*FGF2*	8.9	118.6	<0.0001
Gata binding protein 5	*GATA5*	15.4	61.4	<0.0001
Growth differentiation factor 3	*GDF3*	36.7	167.7	<0.0001
Gap junction protein, gamma 1	*GJC1*	11.4	3.2	N.S.
IGF-like family member 1	*IGFL1*	20.0	39.4	<0.0005
Nodal	*NODAL*	16.8	608.9	<0.0001
Serpin peptidase inhibitor, clade A	*SERPINA5*	15.0	12.8	<0.05
Transmembrane protein with EGF-like and two follistatin-like domain	*TMEFF1*	11.1	11.3	<0.0001

[a] ED/Tr

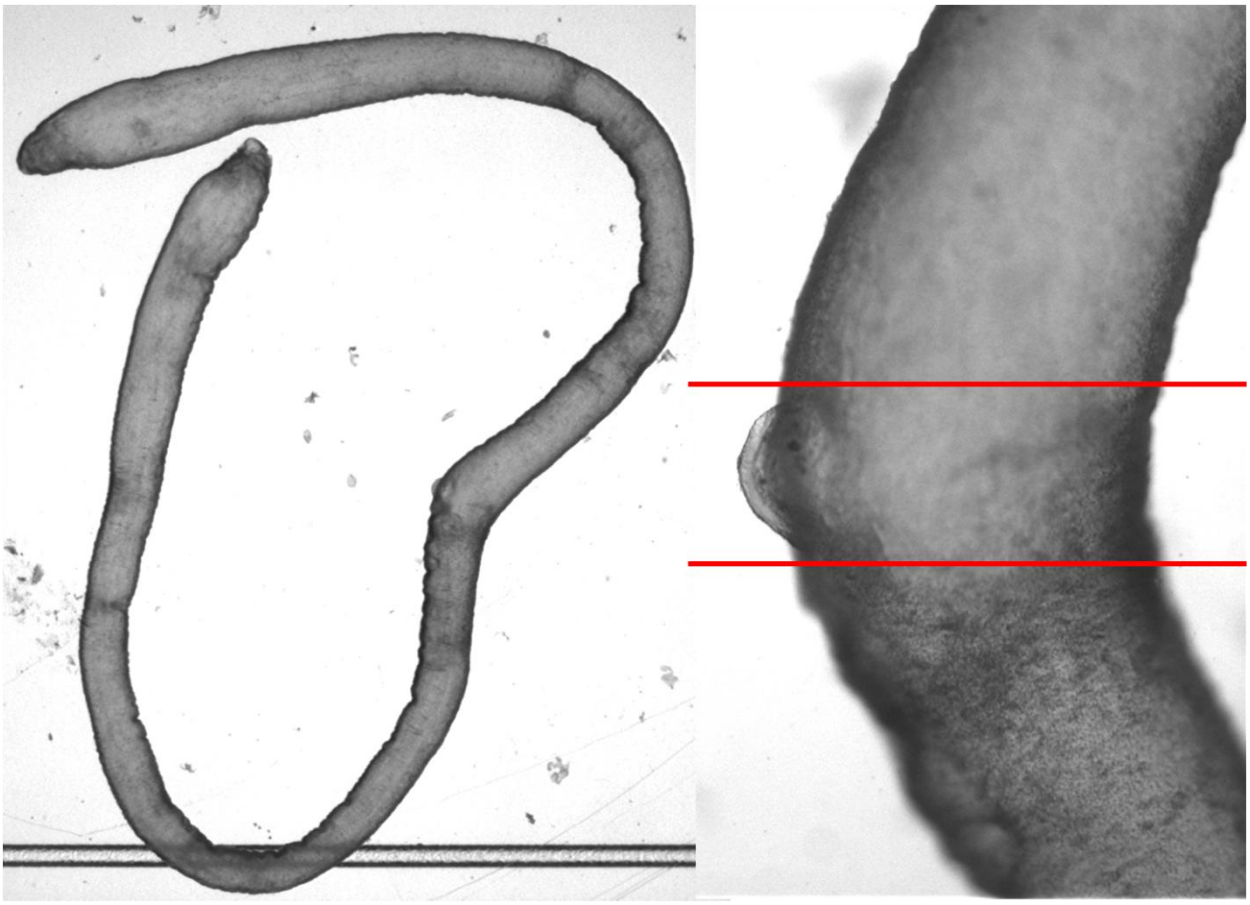

Figure 4-1. Separation of a Day 15 conceptus into embryonic disc and trophoblast. The left panel shows an entire Day 15 elongated embryo and the right panel shows an enlargement of the same embryo to visualize the embryonic disc. The embryo was bisected along the red lines.

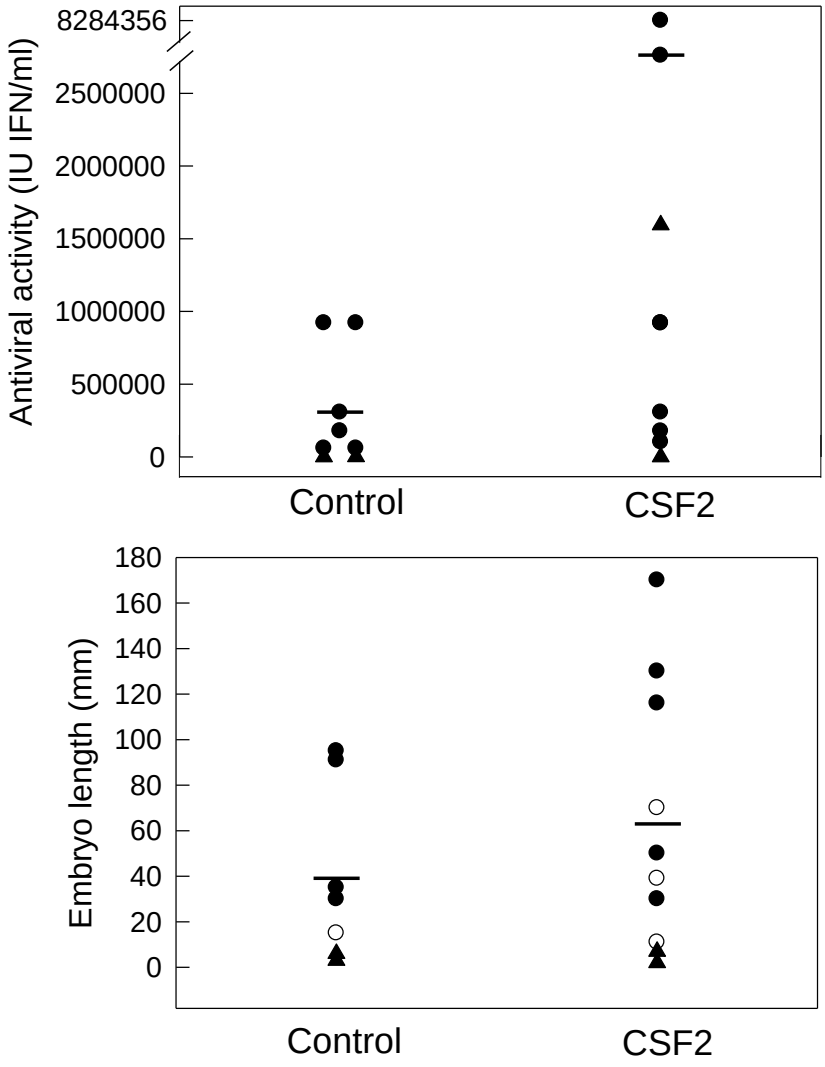

Figure 4-2. Individual values of antiviral activity in uterine flushings (top) and length of recovered embryos (bottom). Triangles represent tubular embryos and circles represent filamentous embryos. In the bottom panel, embryos that were not recovered intact are represented by open circles. The horizontal bars represent the mean value for each treatment. Embryo length was not significantly affected by treatment. Antiviral activity was also not affected by treatment. However, when considering only those cows in which detectable antiviral activity was present, there was a tendency for antiviral activity to be greater for cows receiving CSF2 treated embryos (P=0.07).

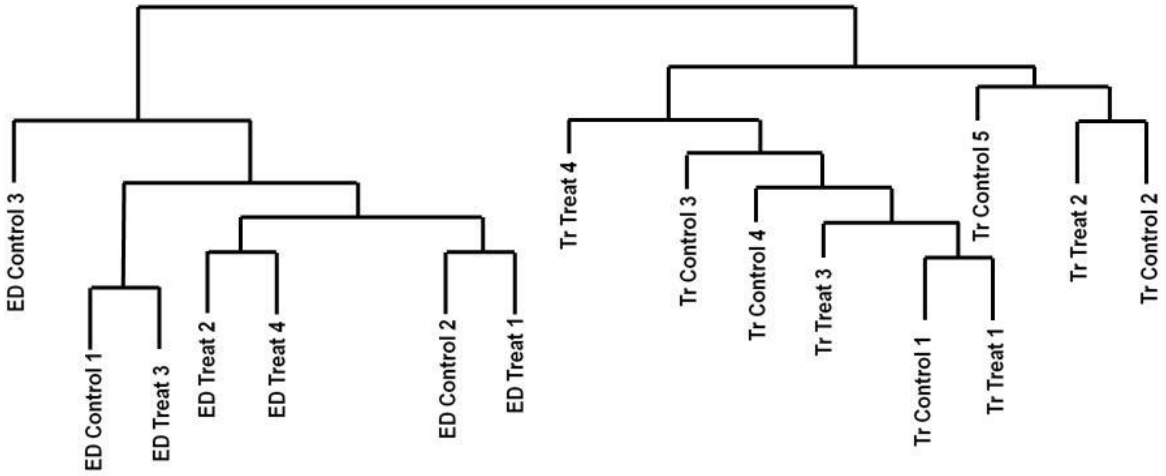

Figure 4-3. Hierarchical Cluster of the transcriptomes of samples of embryonic disc (ED) and trophoblast (Tr) for control and CSF2 treated embryos. ED samples form a separate cluster from Tr but there was no distinct clusters based on treatment.

CHAPTER 5
GENERAL DISCUSSION

Technological advances in manipulation of mammalian embryos outside the maternal environment have provided opportunities to study preimplantation embryo development and to optimize genetic selection and fertility. *In vitro* embryo production (IVP) has great potential to improve fertility and enhance breeding schemes in beef and dairy production systems. Nonetheless, transfer of IVP embryos has not met its potential because these embryos are altered in terms of ultrastructure (Rizos *et al.* 2002), metabolism (Khurana and Niemann 2000) and gene expression (Lonergan *et al.* 2006). These differences are associated with reduced embryo survival rates following transfer (Farin and Farin 1995; Drost *et al.* 1999; Hasler 2000), increased pregnancy loss (Hasler 2000; Block *et al.* 2003) and increased proportion of calves with congenital malformations and neonatal abnormalities (Farin *et al.* 2006).

One approach to improve post-transfer survival of IVP embryos is to modify culture medium with growth factors to more closely mimic the microenvironment found *in vivo*. Modification of embryo culture conditions can significantly impact the competence for development of the resulting blastocyst. Many attempts have been made to mimic aspects of the uterine environment in culture and decrease alterations in embryo morphology and physiology. Several growth factors and cytokines have been tested for their actions on bovine embryonic development *in vitro*, including epidermal growth factor (Sagirkaya *et al.* 2007), interleukin-1 (Paula-Lopes *et al.* 1998), IGF-1 (Block *et al.* 2003; Jousan and Hansen 2004; Lima *et al.* 2006) and CSF2 (de Moraes and Hansen 1997a). Only IGF-1 has been tested for improvement of post-transfer survival of bovine IVP embryos. In this case, IGF-1 treated embryos had increased survival rates after

transfer when transfers were performed in heat-stressed cows but not when performed in cows not subject to heat stress (Block *et al.* 2003; Block and Hansen 2007).

In this dissertation, we have identified CSF2 as another maternally-derived cytokine that can alter embryonic development in a way that increases the likelihood that a transferred embryo will continue to term. The actions of CSF2 on embryonic development that promote survival include changes in gene expression at Day 6 of development that block apoptosis and could conceivably result in alterations in gastrulation. In addition, CSF2 tended to increase embryonic survival between transfer and Day 15 and to increase the competence of the Day 15 embryo to exerts its antiluteolytic actions on the endometrium (Figure 5-1).

In Chapter 2, the ability of CSF2 to increase blastocyst development and post-transfer survival of bovine IVP embryos, as well as cell number, cell allocation and apoptosis were analyzed. The results indicate that addition of CSF2 to the culture medium at Day 5 after insemination increases blastocyst development, pregnancy rate at Day 35 and decreases pregnancy loss thereafter. On the other hand, addition of CSF2 at Day 1 increased blastocyst development at a lesser extent and did not have an effect on pregnancy rate. The differential effect of CSF2 may be dependent on stage of development of the embryo. Early addition of CSF2 could cause internalization and degradation of the ligand-receptor complex before the embryo has overcome the EGA process and is not ready to respond to the beneficial effects of CSF2.

Another effect of CSF2 that may be related to increased embryonic survival is a preferential increase in the number of cells in the ICM. The number of cells in the ICM correlates strongly with viability after transfer in mouse embryos (Lane and Gardner

1997). The ICM/TE ratio between bovine embryos produced *in vitro* differs considerably from embryos generated *in vivo* (Iwasaki *et al.* 1990; Crosier *et al.* 2001). Our results show that CSF2 can minimize the detrimental effects of *in vitro* culture by increasing the ICM/TE ratio.

Another particularity of this study is that embryos were produced using X-sorted semen. It is known that the female embryo undergoing the transition from the morula to blastocyst stage does not tolerate glucose as well as the male embryos (Gutiérrez-Adán *et al.* 2000). In addition, male embryos develop faster in culture than female embryos (Gutiérrez-Adán *et al.* 2000; Kimura *et al.* 2005). The gender difference in growth rate and sensitivity to glucose is probably due to differential expression of the X-chromosomes. In the female embryo, both X-chromosomes are active at the cleavage-stage (Mak *et al.* 2004) and the gene for glucose 6-phosphate dehydrogenase, an enzyme that controls the entry of glucose into the pentose-phospate pathway, is located on the X-chromosome. Another gene, hypoxanthine phosphoribosyl transferase 1, wich encodes an enzyme involved in controlling the amount of oxygen radicals, is also on the X-chromosome (Goldammer *et al.* 2003). The excess in free radical and the poor tolerance to glucose could retard the development of female embryos. Since the primary action of CSF2 is to promote cellular survival, there is a possibility that the effects of CSF2 in this study were amplified by the fact that at least 85% of the embryos were female. To further examine the changes in the embryo caused by CSF2 and the consequences of these changes into adulthood, a follow up study to investigate the reproduction and health status of the heifers born from the CSF2 treated embryos would be applicable.

Addition of CSF2 to mouse culture medium partially alleviates the long-term adverse consequences for postnatal growth caused by IVP. Adult mice that originated from embryos cultured in medium without any cytokine have increased body weight, increased central fat and increased fat relative to total body mass compared with mice that originated from embryos cultured in the presence of CSF2 or embryos developed *in vivo* (Sjöblom *et al.* 2005). When pregnant, female mice generated from *in vitro* culture have a larger placenta, which decreases the placenta/fetus weight ratio, compared with mothers that were developed from embryos produced *in vivo* or cultured *in vitro* in the presence of CSF2 (Sjöblom *et al.* 2005).

Thus far, two molecules, IGF1 and CSF2 have been reported to improve the post-transfer survival of bovine embryos, with IGF1 only being effective during the hot months of the year. This peculiarity of IGF1 was not specifically tested for CSF2; however the increased pregnancy rates found with addition of CSF2 at Day 5 after insemination were from transfers done in both the fall and winter seasons. While the transfer of embryos that were treated with CSF2 at Day 1 after insemination (when pregnancy rates were not increased) were done only during the summer season. The combination of IGF1 and CSF2 plus other growth factors and cytokines (IGF2, FGF2, TGFB and LIF) have been tested for embryo development and cell number. The addition of a single cytokine as well as the combination of all cytokines improved development and increased blastocyst cell number compared with medium alone (Neira *et al.* 2010). However, this study was not able to clarify whether the combination of multiple cytokines is better than the addition of a single cytokine since all the possible combinations were not compared at simultaneously. It is possible that the production of

bovine embryos in the presence of both IGF1 and CSF2 could compensate for the inability of IGF1to improve embryo survival during the winter and therefore result in greater post-transfer survival irrespective of season. IGF1 acts as a mitogenic and cellular survival factor, but it fails to surmount the negative effects of cryopreservation on embryos (Velazquez *et al.* 2009). While CSF2 can also act as a mitogenic factor, its central role is cell survival (Trapnell *et al.* 2009), including being able to protect one cell mouse embryos from freezing damage and decrease the apoptotic index to zero (Desai *et al.* 2007).

It is unlikely that the absence of CSF2 would cause absolute infertility in the cow. Given that the CSF2 receptor shares the βc activator subunit with IL3 and IL5, it is likely that in the absence of CSF2, the receptors would be activated by one or both of these two cytokines (Hansen *et al.* 2008). Moreover, CSF2 deficient mice (CSF2 -/-) can produce viable puppies (Robertson *et al.* 1999). On the other hand, in human and mice, excessive production of CSF2 has been reported to cause multiple pathologies, mostly related to autoimmune diseases (Hansen *et al.* 2008). An increased immune response, besides increasing the chances of diffuse inflammation, could also result in less tolerance for the fetus.

In Chapter 3, the transcriptome of the CSF2 embryo was analyzed. Embryos produced *in vitro* have altered gene expression patterns when compared with embryos produced *in vivo*. *In vitro* culture of bovine embryos results in increased abundance of the transcripts for heat shock protein 70.1, copper/zinc-superoxide dismutase, glucose transporters-3 and -4) (Lazzari *et al.* 2002), altered levels of expression of X-linked genes (King 2008) and differential expression of IGF family genes (Bertolini *et al.* 2002;

Sagirkaya *et al.* 2006; Moore *et al.* 2007). Furthermore, blastocysts produced *in vitro* have higher expression of BCL2-associated X protein, a pro-apoptotic protein, and arcosine oxidase, an oxidative enzyme and decreased expression of mitochondrial manganese-superoxide dismutase, an important antioxidant defense in cells exposed to oxygen (Rizos *et al.* 2002). The effects from embryo culture *in vitro* persist into fetal life being presented as fetal abnormalities and large offspring syndrome. These deformities could be consequence of alterations in the transcripts at the preimplantation stage or an epigenetic effect as IVP embryos have decreased expression of transcripts for enzymes involved in the de novo methylation process (Smith *et al.* 2009).

The results of Chapter 3 indicate that addition of CSF2 to embryo culture altered the expression of 3 major groups of genes (development and differentiation process, cell communication and apoptosis). The genes involved in the developmental process and differentiation were functioning to inhibit neurogenesis and stimulate mesoderm or muscle formation, and pluripotency. In cell communication process the signaling system that was most representative was the WNT system, analysis of the data indicates inhibition of β-catenin dependent WNT signaling. The apoptosis signaling pathway was also inhibited by CSF2. The biological effect of CSF2 on apoptosis genes was appropriately proved by submitting the embryos to heat shock and analyzing the percentageage of apoptotic blastomers by a well known assay. However, the differential expression of genes promoting mesoderm and delaying neural tissue development should be further studied.

The pre-gastrulation period is when the earliest structural decisions are being made; alterations of the genome during gastrulation may cause damage that will only

manifested in subsequent cell generations (Rutledge 1997). Certain changes in early development can also induce mid to late gestation anatomic malformations and fetal lethality (Rutledge 1997).

To further investigate the effects of CSF2 in the gastrulation process, a imunohistochemistry study of embryos recovered at different stages of development, such as Day 15 (when both mesoderm and neural tissue are beginning to form) and Day 21 (when gastrulation and neurulation are completed and somites are starting to form), could allow for properly visualization of the structures controlled by the referred genes. The use of antibodies that recognizes muscle specific α-actin isomers from cardiac, smooth and skeletal muscle or γ-actin from non-smooth muscle, cytokeratin for epithelial tissue and anti-neurofilament for neural tissue could reveal the preferential development of one tissue over the other. If the changes in these early stages are confirmed, a histomorphological study could be performed after the organs are formed (Day 45) to define if the damage persisted and if that would be one of the reasons for the pregnancy losses that happen after Day 35. The development and morphology of the placenta would also be of interest in this study.

The effects of CSF2 to inhibit WNT signaling pathway could be examined in a comparative study with control embryos, CSF2 treated embryos and embryos submitted to WNT inhibitors.

In Chapter 4, CSF2 treated embryos were tested for characteristics that could make them more likely to establish and maintain pregnancy. CSF2 treated embryos were greater in size, secrete more IFNT and have a higher recovery rate. However, there was no significant effect of treatment when expression of the embryonic disc and

trophectoderm dissected from elongated control and CSF2 embryos were. The lack of difference may be due to the fact that only filamentous embryos were selected in both groups. Perhaps if we had analyzed all the embryos recovered this bias could have been avoided. Another possibility is that the development of the placenta is facilitated by CSF2, as seen by a longer trophectoderm, and such an effect could improve implantation and possibly decrease embryonic loss.

The last study also resulted in the identification of over 500 genes preferentially expressed in the embryonic disc. Many of these have been described earlier as being ICM or trophectoderm markers in mouse and human embryos. Identification of new markers of ICM and TE could be used to facilitate development of the bovine embryonic stem cell, and advance the understanding of the embryonic developmental process.

Lastly, the study of different DNA methylation patterns may provide more insight into the actions of CSF2 to promote embryo development and survival. The knowledge attained by these studies could be pertinent for the development of more efficient systems in the production of embryos *in vitro*, which in turn will result in enhanced pregnancy success following embryo transfer in cattle. Understanding the mechanisms that regulate early bovine embryonic development may prove useful to study infertility in other species, including human.

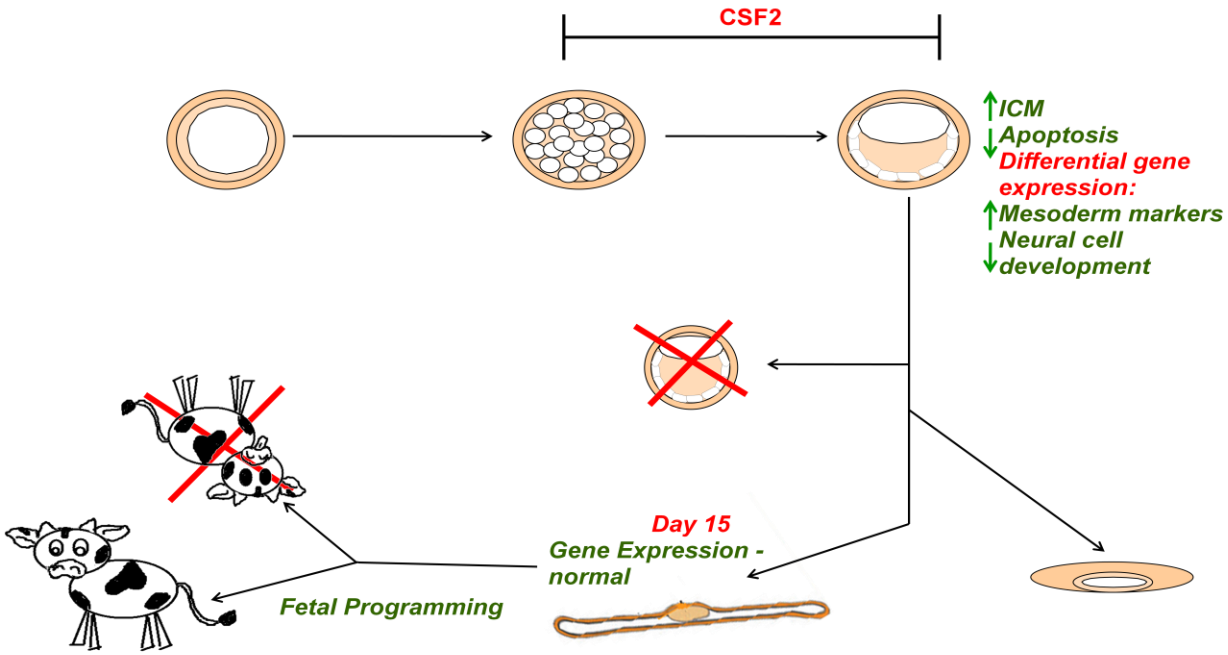

Figure 5-1. Summary of effects CSF2 on embryo development and post-transfer survival. Treatment of embryos with CSF2 from Days 5-7 after insemination increases development of oocytes to blastocyst, increases the number of cells in the inner cell mass, decreases apoptosis, increases expression of genes regulating mesoderm formation and epithelial mesenchymal transition and decrease expression of genes regulating neural cell development. CSF2 treatment increases recovery of embryos on Day 15, length, IFNT secretion and expression of IFNT and KRT18 mRNA. Recipients that receive an embryo treated with CSF2 have increased pregnancy rates at Days 30-35, decreased pregnancy losses after Days 30-35 and increased calving rate.

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BIOGRAPHICAL SKETCH

Bárbara Loureiro was born in 1980 in Linhares, Espírito Santo, Brazil, to Maria José and Carlos Alberto Barbosa Loureiro. The elder of two children, Bárbara lived her whole life in São Mateus, until she graduated from high school in 1997. Following graduation from high school, she entered the School of Veterinary Medicine at Universidade Federal Rural de Pernambuco, Brazil. There she was awarded the research scholarship “Brazilian Scientific Initiation Program” sponsored by the National Research Program (PIBIC/CNPq). During the three years of the scholarship she developed research in the Bovine Clinic and acquired good experience in scientific work. During her time in college, she was also interested in the educational process and she served as president of the Veterinary Students Association. She helped develop the academic program for the School of Veterinary Medicine and represented the students as a board member at the Educational Council of the School of Veterinary Medicine and at the Administrative Council of the Veterinary Department. She received her degree in Veterinary Medicine in July of 2003.

Concomitant with Veterinary School, she received education in the Special Education Program focused on teaching Agricultural Science, begning in 2002. There she had the opportunity to teach at the Federal Agricultural Technical School of Pernambuco in the Animal Science program and develop extension projects in Enviromental Health. She received her Teaching Specialist degree in May of 2004.

Immedialty after graduation from Veterinary School she entered a Master of Veterinary Science program at Universidade Federal Rural de Pernambuco. One year into her master’s program she received the opportunity to do an intership with Dr. P.J. Hansen at the University of Florida. Her research was focused on apoptosis in the

preimplantation embryo. After conclusion of her master's degree, she was awarded a CAPES/Fulbright scholarship to persue a Doctor of Philosophy Degree in the Animal Mollecular and Cellular Biology Graduate Program and continue her work with Dr. Hansen. In 2010 she received the Sigma Xi Graduate Research Award. After completing the requirements for the doctoral degree, Bárbara will continue her career as a research scientist back in Brazil.