To Lauren, Mom, Dad and Kory
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Peter J. Hansen, for mentoring me on what it takes to truly be a scientist. He always challenged me, which brought out my best, but did so while also being respectful and understanding. His advice, knowledge, expertise and willingness to take a chance on bringing me down to Florida are some things I will never forget and for which I am truly grateful. I would also like to thank my committee members, Dr. Stephanie Wohlgemuth, Dr. James Resnick and Dr. Kenneth Drury for all the support and advice that was given to me during my PhD program.

I also thank the members of the Hansen lab, both past and present, for all their help during my program. Among my lab mates deserving thanks are Anna Denicol, Miki Sakatani, Manabu Ozawa, James Moss, Luciano Bonilla, Maria Padua, Sofia Ortega, Sarah Cochran, Firdous Khan, Mateus Sudano, Juliana Delgado, Antonio Ruiz, Paula Tribulo, Veronica Negron-Perez, Luiz Siqueira, Jasmine Francis, Serdal Dikman, Christine Meyer and Marlon Rodriguez. Without you guys, I would not have been productive and for that, I am grateful.

I am very grateful to the lab of Dr. Marc-André Sirard and Dr. Claude Robert in Québec City, QC for their help and assistance teaching me how to do microarray analysis and helping me survive Québec with my broken French. Merci beaucoup! Thank you to Justin Fear and Dr. Alice Morse in the Lauren McInytre lab for their assistance with analyzing microarray data, specifically. I am also thankful to Dr. Alan Ealy and his lab for assistance on IFNT assays and to Stacey Jones and the IFAS communications office for designing figures for the dissertation.

Thanks also to Central Packing Co. in Center Hill, FL for providing the lab with ovaries and fetuses for my experiments and William Rembert for collecting these
samples. Special thanks go to Eric Diepersloot for his assistance and guidance at the University of Florida Dairy Research Unit (Hague, FL). I came into this lab with little experience handling cattle and Eric showed me the ropes.

I am also grateful for the Animal Molecular and Cellular Biology Graduate Program for the fellowship that provided me with funding to complete my PhD. Also, thanks to the Department of Animal Sciences for housing me and providing me with the resources for my research. I am also grateful to the graduate students of the department for their camaraderie and support including Dale Kelley, Rafael Bisinotto, Karun Kaniyamattam, Natalia Patiño, Eduardo Ribeiro and Tao Sha.

My thanks go out to all of my Gainesville friends outside of the lab that my wife and I were fortunate to meet, especially Rev. Dan and Jen Prugh as well as Dr. Peter and Natalie Carter. They made us feel like family and were always there for us.

I also want to thank my Mom and Dad. No one has been there for me throughout my whole life more than those two. They pushed me in all that I did and made me who I am today. I know they never thought the son of a CPA and cartographer would graduate with a PhD in science. I also thank the rest of my family, my brother Kory and my grandmothers for their love and support. Life was made a lot easier during my program with the love that was available 24/7 from our Jack-chi (dog), Lexi.

Finally, I thank my wife Lauren, for her love and support. It goes without saying that I could not have done this without her and I am forever indebted to her. She was and is always there for me and even moved 1,000+ miles from everyone and everything that she knew to support me.
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LIST OF ABBREVIATIONS

Note: Official gene symbols are used without definition as recommended by the HUGO Gene Nomenclature Committee (http://www.genenames.org)

<table>
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<th>Description</th>
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<tr>
<td>2i</td>
<td>Two inhibitors</td>
</tr>
<tr>
<td>ABAM</td>
<td>Anti-bacterial, anti-microbial</td>
</tr>
<tr>
<td>aRNA</td>
<td>Amplified ribonucleic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bST</td>
<td>Bovine somatotropin</td>
</tr>
<tr>
<td>BTA</td>
<td>Bos taurus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus oocyte complex</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for annotation, visualization and integrated discovery</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially expressed genes</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified eagle medium</td>
</tr>
<tr>
<td>DMR</td>
<td>Differentially methylated region</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>ED</td>
<td>Embryonic disc</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>EEM</td>
<td>Extra-embryonic membrane</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
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<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>ESM</td>
<td>Embryonic stem cell medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analysis</td>
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<tr>
<td>iPATH</td>
<td>Interactive pathway explorer</td>
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<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>IVP</td>
<td>In vitro produced</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
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<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MS-HRM</td>
<td>Methylation sensitive high resolution melting analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pi</td>
<td>post-insemination</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROX</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SOF-BE1</td>
<td>Synthetic oviductal fluid-bovine embryo 1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transduction and activation of transcription</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/Sucrose nonfermentable</td>
</tr>
<tr>
<td>TCJ</td>
<td>Tight cell junction</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase</td>
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The maternal environment is an important determinant of preimplantation development of the mammalian embryo. Many actions of the mother are mediated by alterations in secretion of regulatory molecules called embryokines produced by the endometrium. One of these molecules, colony stimulating factor 2 (CSF2), causes several actions on the embryo when added from days 5-7 of development including altering lineage commitment of the inner cell mass (ICM) and trophectoderm (TE) of the blastocyst, increasing competence of an embryo to become a blastocyst and to establish pregnancy after transfer. Multiple experiments were carried out to understand the mechanisms of CSF2 on the development of the preimplantation bovine embryo. First, experiments were conducted to evaluate regulation by CSF2 of pluripotency within the ICM and TE differentiation and growth. The proportion of isolated ICM colonies that survived until the end of the first cellular passage was greater for ICM derived from blastocysts produced in the presence of CSF2 than ICM from control blastocysts. There was little effect of CSF2 on characteristics of TE outgrowths from blastocysts. In addition, actions of CSF2 were found to occur through a signaling pathway that is likely
independent of CSF2RB because *CSF2RB* was undetectable from the zygote to blastocyst stages. Second, the actions of CSF2 from day 5-7 of development on characteristics of the embryo at day 15 of gestation to determine whether CSF2 causes long-term changes in embryonic development. CSF2 decreased length and IFNT secretion of female embryos at Day15 but increased length and IFNT secretion of male embryos. Similarly, CSF2 affected the transcriptome and methylome differently for female embryos than male embryos. Finally, immunofluorescent labeling with anti-5-methylcytosine was used to evaluate global DNA methylation during preimplantation development. Results suggest that DNA methylation undergoes dynamic changes during the preimplantation period in a manner that is dependent upon sex and that the TE of the blastocyst is more methylated than the ICM. There was no effect of CSF2 on global DNA methylation at the blastocyst stage. Overall, these results establish a mechanism by which the maternally secreted embryokine, CSF2, alters the developmental trajectory of the bovine preimplantation embryo.
CHAPTER 1  
LITERATURE REVIEW  

Introduction  

The mammalian embryo only spends a few days developing from the totipotent zygote stage to the blastocyst stage in which the first two lineage commitment events take place. In cattle, the blastocyst, consisting of ICM and TE, forms by Day 6-7 in vivo [1] and the hypoblast forms about 48 hours later [2]. Although development of the zygote to the blastocyst stage can occur in the absence of maternal signals (i.e., in culture media), the maternal environment is important for ensuring optimal development. Indeed, embryos produced in vitro have several characteristics that are altered compared to embryos that develop in vivo, including gene expression [3], lipid content [4, 5], ultrastructure [6, 7], and degree of methylation of CpG islands of specific genes [8]. Moreover, perturbations in the maternal environment during the preimplantation period can compromise embryonic survival and amend the developmental program of the embryo to cause long-term changes in physiology and health that extend into postnatal life.

In the cow, there are several lines of evidence indicating the importance of perturbations in the maternal environment for development of the preimplantation embryo. An experiment in which embryos were transferred into the oviduct indicated that lactation causes reduced ability of embryos to develop to the blastocyst stage [9]. Cows in which embryos were transferred into the reproductive tract at day 7 after estrus had a different pattern of endometrial gene expression on the day of transfer than cows in which the embryo did not survive [10]. The maternal hormonal environment can alter characteristics of the embryo as well, as shown by studies that premature elevation of
progesterone after ovulation hastened elongation of the trophoblast [11] and that treatment of superovulated cows with somatotropin increased the competence of the resultant embryos to establish pregnancy after embryos were recovered and transferred to recipients [12].

Experiments indicating that alterations in maternal environment can reconfigure the developmental program of the embryo come largely from experiments with rodents. Female mice or rats fed a low protein diet during the period corresponding to preimplantation development, produced changes in the resultant offspring, which persisted into adult life [13-15]. In the sheep, as well, change in nutritional environment during the peri-conception period (this time by reducing the bioavailability of methionine) caused changes in the adult offspring that included increased rate of obesity, altered immune response, elevated blood pressure and resistance to insulin [16].

One of the characteristics of developmental programming caused by changes in the maternal environment during the preimplantation period is that females are programmed differently than males. For example, feeding female mice diets low in protein during embryonic development affected females differently than males [13, 14]. Male adults born from mothers that were fed low protein diets had elevated angiotensin-converting enzyme [17], while female adults had increased blood pressure [14] and reduced heart to body weight ratio [13].

One of the assumptions of this dissertation is that a major mechanism by which changes in uterine environment affects embryonic development is change in the release of soluble regulators of embryonic development. These molecules, hereafter called embryokines, are produced by the oviduct and endometrium and are capable of altering
the course of embryo development. Indeed, several maternally-derived molecules have been identified that have beneficial effects on the preimplantation embryo. In the cow, these include CSF2 [18], IGF1 [19], TGFB [20], hyaluronan [21], LIF [20], FGF2 [22] and ILB1 [23].

The best-studied embryokine is CSF2, which can improve early development and embryonic competence for establishment of pregnancy in mice, cows and humans [18, 24-26]. CSF2 is secreted by the endometrial and oviductal epithelium [27-29]. Treatment of in vitro produced bovine embryos with CSF2 alters gene expression [30], decreases apoptosis [31] and increases ICM to TE cell ratio within the blastocyst [18]. At least two environmental factors can affect CSF2 synthesis in the uterus – seminal plasma [shown in mice; [32, 33]] and obesity (shown in cow) [34].

This literature review will be focused on understanding how the maternal reproductive milieu regulates development of the early embryo. The focus will be on the specific role of CSF2 as a maternal embryokine. While data from a variety of species will be dealt with, the major research model discussed will be the cow. For that reason, and to put research on maternal effects on embryonic development in context, the review will begin with an overview of the process of preimplantation development in the cow.

**Development of the Preimplantation Embryo in the Cow**

Preimplantation development involves a specific chain of events. Following fertilization of the oocyte, the embryo proceeds through symmetric cleavages, doubling its cell number and decreasing cell size with each cleavage. During this time, the embryo utilizes maternally inherited mRNA for protein production until transcription is initiated in a process called embryonic genome activation (EGA). Although the exact
timing is unclear, around the same time as EGA, the embryo loses all DNA methylation marks before regaining them during de novo methylation involving actions of embryo-specific methyltransferase enzymes. Finally, following blastocyst formation, the embryo hatches and begins to elongate, increasing its size more than 20 fold [35] and then begins to attach to the epithelium of the endometrium in a gradual process that is initiated around Day 20 [36].

**Time Course of Development**

Immediately after ovulation, the oocyte is picked up by the fimbria of the oviduct and moved into the oviductal lumen [37]. Fertilization takes place near the junction of the isthmus and ampulla [38]. Following fertilization, the two pronuclei fuse, and meiosis is completed by extrusion of the second polar body [39, 40]. The first round of cleavage following fertilization occurs within 28-35 hours [41]. This is followed by additional rounds of cell division so that the embryo reaches the 4-cell stage by 40-46 hours after fertilization, the 8-cell stage by 58-86 hours after fertilization and the 16-cell stage by 106 hours post-fertilization [41]. Cleavage throughout development is asynchronous [41].

By 134 hours post fertilization, the formation of tight cell junctions (TCJ) have formed [41]. TCJ formation involves the gathering of TCJ constituents to form a protein cluster on E-cadherin complex, which is located between blastomeres [42]. The TCJ becomes sealed during the 32-cell to early blastocyst stage following the addition of tight junction protein alpha [42]. TCJ formation represents the first step in differentiation of the embryo because the presence of tight junctions causes a different microenvironment for cells on the outside of the embryo from cells on the inside of the
Blastocyst formation occurs about 168 hours post insemination [1]. Accumulation of fluid in the blastocoelic cavity is the result of actions of Na⁺/K⁺ ATPase [44] as well as facilitated movement of water by aquaporin channel proteins [45]. The blastocyst represents the departure point for the embryo. Before the blastocyst stage, all cells of the embryo are totipotent. Beginning at the blastocyst stage, however, cells becoming increasingly restricted in the lineages they can form. The first differentiation is at the blastocyst stage where TE cells become committed towards a placental lineage [46]. Cells of the ICM give rise to the pluripotent epiblast and the hypoblast, which contributes to placental endoderm. The hypoblast can first be identified at day 8 after fertilization as a group of cells on the outer face of the ICM that label with GATA6 [47].

Competency of an embryo to develop to the blastocyst stage is in part due to the timing of cleavage. The longer the time between fertilization and the initial cleavage, the lower the likelihood that an embryo can develop to the blastocyst stage [48]. In vitro, embryos that reached the 16-cell stage by 72 hours after insemination produced more expanded and hatched blastocysts compared to embryos that were only at the 1-4 cell stage during the same time frame [48]. At least some of these differences in developmental potential are likely to be caused by differences in the transcriptome. Embryos that reached the 2-cell stage by 29.5 hours post-fertilization had increased expression of genes relating to cell-cycle control and DNA damage response factors compared to embryos requiring 46 hours to reach the same cell stage [49].
The timing of development is not the same for embryos produced in vitro versus those produced in vivo. In vitro produced embryos proceed through their first cleavage about 33 hours after fertilization, about 5-9 hours later than their in vivo counterparts [1]. This delay persists throughout development; for example blastocysts form about one day sooner in vivo than in vitro [1]. The timing of development is also dependent upon the sex of the embryo. Female embryos reach the 2-4-cell and blastocyst stages slower than male embryos [50]. In addition, female blastocysts are less likely to be expanded at day 8 compared to males [51].

At 192-240 hours post-insemination, the bovine embryo proceeds through a series of events that induces hatching of the embryo from the surrounding zona pellucida [48, 52]. Hatching involves two processes: lysis of the zona pellucida with the embryo-derived enzyme plasmin [1] and expansion of the blastocoel cavity to apply pressure to the zona pellucida and allows the blastocyst to protrude and eventually vacate from the structure. After hatching around day 8-10 after fertilization, the blastocyst undergoes a series of morphological changes to change shape from spherical to ovoidal [2]. By day 14 following fertilization, the embryo begins a dramatic elongation of the trophoblast. At first the embryo assumes a tubular shape, with sizes ranging from 0.5 mm to 19.0 mm in length [2]. Subsequently, the embryo assumes a filamentous shape and reaches lengths of 6.0 mm to 160.0 mm by day 16 [1]. The initiation of elongation is reliant upon the maternal environment, as in vitro produced embryos will not elongate [53].

Rapid elongation also requires rapid cell proliferation. Over 500 genes, predominantly associated with trophoblast cell proliferation, are upregulated in embryos
between day 7 and 14 [54]. By day 14-16, the process of gastrulation has begun with the formation of the three germ layers [2].

One function of the elongating embryo is production of IFNT to signal to the mother to block luteolysis and allow continued secretion of progesterone from the corpus luteum [55]. As the embryo increases in length, secretion of IFNT also increases [56, 57]. It is not clear whether the increase in IFNT with size is associated with an increase in INFT gene expression per cell or simply the larger size [54, 57].

Starting at gestational day 20, the elongating bovine embryo begins the process of apposition and adhesion to the endometrial epithelium [54]. The bovine embryo, like other species with an epitheliochorial placentation, does not invade through the epithelial basement membrane to cause implantation. Rather, some embryonic cells adhere to epithelial cells while others (binucleate cells) migrate into the endometrial epithelium and fuse with maternal cells [58]. The process of placentation in the cow is slow, requiring around 12 days to be completed. [59].

Embryonic Genome Activation

One of the key events in development is activation of the embryonic genome. Transcription in the oocyte ceases when it reaches a size of 110 μm [60]. Therefore, the oocyte and embryo are dependent upon preformed mRNA for direction of protein synthesis until the genome is reactivated. The embryo manages protein synthesis by modifying polyadenylation of maternally-donated mRNA and controlling the cell-cycle clock [61, 62]. The first round of transcription from the embryonic genome starts at the 2-4-cell stage, where a couple hundred genes are activated [63]. Some of the genes transcribed during this period of minor genome activation are SARS, IL18, CRABP1, ACO2, TXN2, SLC38A2 and SLC25A3 [63]. Minor activation is not required for
development to the blastocyst stage because treatment with alpha-amanitin to block transcription prior to major EGA had no effect on the percent of embryos that became blastocysts [64]. Embryonic RNA transcription begins at the 8-cell stage [65]. Full activation of the embryonic genome occurs between the 8-cell and 16-cell stage [66]. Development is blocked at this stage if the embryo is exposed to alpha-amanitin [64].

The mechanism by which EGA occurs in the cow is unknown. In other species such as mice, *Drosophila* and *C. elegans*, EGA is proposed to be initiated by removal of maternal transcripts through destabilization of the poly-adenylated tail [67]. Other mechanisms that are also likely to be involved including removal of transcriptional repressors and chromatin modifications such as the SWI/SNF complex from embryonic DNA, dilution of an unknown EGA repressor caused by cellular division, and the acquisition of the cellular machinery for transcription [67].

**DNA Methylation**

DNA methylation involves the addition of a methyl-group to the 5\textsuperscript{th} carbon position of cytosine when located 5' to guanine in a DNA sequence, also known as a CpG dinucleotide [68]. DNA methylation is an important mechanism for regulating gene expression. Methylated DNA elicits a transcriptionally-repressive response by recruiting binding proteins to the DNA strand and by physically impeding transcriptional machinery from proceeding 5’ to 3’ across the DNA [69, 70]. DNA methylation is thus an important mechanism for controlling a cell’s differentiation status, by controlling repression of specific DNA regions [71]. In addition, paternal imprinting involves DNA methylation on chromosomes of specific paternal origin [72]. Given the role of DNA methylation in controlling differentiation, it is necessary for the embryo to remove methylation marks as
part of the process to establish totipotency and restore methylation in a cell lineage-dependent manner.

The initial loss of global DNA methylation following fertilization occurs by both active and passive mechanisms [73, 74]. At least in the mouse, the maternally-derived genome is demethylated more slowly after fertilization than paternally-derived genome [75]. Loss of methylation in maternally-derived genome is passive due to the absence of a de novo DNA methyltransferase in the early embryo [76]. In contrast, DNA demethylation of the paternally-derived genome is accomplished by a host of DNA repair mechanisms such as base excision repair through DNA glycosylases [77], nucleotide excision repair [78], deamination of the methylated cytosine [79] in addition to 5-methylcytosine oxidation [80]. A specific DNA demethylation enzyme has not been identified. Before embryonic development, the methylation marks on imprinted genes are established and subsequently are maintained during global demethylation [72] and are only demethylated during primordial germ cell migration [81].

Following demethylation, the embryo reacquires methylation marks as development proceeds. Indeed, development will not occur unless those methylation marks are restored by DNA methyltransferases [82]. DNA methyltransferases Dnmt3a and Dnmt3b are activated in order to re-establish the methylation status of embryonic DNA [82]. Unique to the embryo is an imprinting maintenance DNA methyltransferases, DNMT1o. Although imprinting occurs during oogenesis in the absence of DNMT1o, these imprinted marks will disappear during early embryo development when DNMT1o is absent [83].
While DNA demethylation and *de novo* methylation are critical events, there is wide divergence in the developmental and lineage-specific characteristics of the process. In the mouse, demethylation is complete by the late morula stage, *de novo* methylation begins at the blastocyst stage of development, and the ICM is more methylated than the TE [84]. For sheep, in contrast, global methylation decreases continuously from the 2-cell stage until the blastocyst stage [85] while the pig embryo does not undergo a loss of methylation between the zygote and blastocyst stages [86]. For both pig and sheep, the ICM is more methylated than the TE [85, 86]. In the cow, it is unclear at what stage demethylation is complete or at what stage *de novo* methylation begins [87, 88].

**Differentiation of the First Two Cell Lineages in the Blastocyst**

Three cell populations emerge during the blastocyst stage: the ICM, TE and hypoblast [called primitive endoderm (PE) in mice]. The ICM is a population of cells that will become the embryo proper while the TE and hypoblast will develop into the extra-embryonic membranes forming the placenta and endodermal structures. Presence of specific transcription factors can be used to identify cells of the ICM (*NANOG* and *SOX2*) [89-91], TE (*CDX2*) or hypoblast (*GATA6*) [92] lineage. Embryos that have inactivation of *CDX2* will fail to develop a TE [93]. *CDX2* is required for differentiation of TE since inactivation of the gene prevents TE formation [93]. Unlike the mouse, however, *POU5F1* is not responsible for maintenance of ICM and is expressed in both ICM and TE fate [94]. Commitment to the TE lineage is a gradual process; cells at Day 7 of development retain the capacity to revert back to a pluripotent cell type [94]. By Day 11 of development, the expression of *CDX2* is more than 10 times higher than
POU5F1, which subsequently inhibits the activity of POU5F1 and causes irreversible differentiation of TE cells [94].

GATA6 is initially expressed throughout the blastocyst and then becomes increasingly restricted to the ICM which is characterized by a mixture of cells expressing either GATA6 or the epiblast marker NANOG [47]. GATA6 is not absent from the TE until Day 8 of development [47]. Heterogeneity of the ICM could be the result of variable responses to Fgf4 signaling as shown in the mouse. In that species, the second cells produced as a result of proliferation have greater expression of Fgfr2 than cells produced in the first wave [95, 96]. Cells from the second wave become hypoblast while the cells from the first wave stay dedicated to the ICM [95]. Interestingly, if Fgf4 signaling through mitogen-activated protein kinase (MAPK) is blocked in the epiblast cell population, all cells become Nanog positive and Gata6 negative [97]. In the bovine, as well, inhibition of MAPK leads to an ICM where all cells are NANOG positive [98]. Moreover, activation of FGF4 signaling using human recombinant FGF4, increased the number of cells in the ICM positive for GATA6 [47].

**Evidence for the Importance of the Maternal Environment for Regulation of Development**

That the reproductive tract environment established by the mother can affect the trajectory of development of the preimplantation embryo can be deduced from two lines of evidence. The first comes from examination of the characteristics of embryos produced in vitro. While an embryo can develop to the blastocyst stage in the complete absence of maternal signals, it is clear that such embryos experience a range of abnormalities that affect competence to establish pregnancy and characteristics of the resultant offspring. In some cases, such aberrations have been shown to be the result
of the culture conditions during embryo development rather than to disorders caused by in vitro maturation or fertilization. The second line of evidence comes from experiments indicating that alterations in maternal functions can alter embryonic development.

**In Vitro Produced Embryo**

Although preimplantation embryonic development ordinarily takes place in the microenvironment of the female reproductive tract, healthy offspring can result when the embryo resides in an artificial culture environment during the period up to and including formation of the blastocyst. The embryo produced in vitro (IVP) is derived from an oocyte that underwent maturation in the absence of maternal signals other than those provided by the surrounding cumulus cells. Examination of the characteristics of the IVP embryo make clear the importance of the maternal environment for regulation of development because embryos produced in vitro experience a variety of abnormalities compared to embryos produced in vivo. As a result, transfer of an IVP bovine embryo is associated with lower pregnancy rate [99] and a higher incidence of abnormal calves at birth [100].

In cattle, one of the consequences of in vitro production is an altered transcriptome [101]. Alterations in mRNA expression begin as early as the 4-cell stage [102]. By the blastocyst stage of development, there are hundreds of differentially expressed genes between embryos produced by in vitro and in vivo methods [101, 103-105]. Specific ontologies that are enriched for differentially expressed genes between IVP and in vivo blastocysts include cholesterol synthesis and cell differentiation [101] as well as apoptosis and stress response [105].

There is also aberrant DNA methylation in a fraction of IVP embryos [8, 88, 106]. Loss of methylation of the maternally-inherited allele of *KvDMR1* has been shown to be
associated with overgrowth of bovine fetuses [106]. Among the genes that experience decreased methylation following culture are genes involved in DNA methylation such as the *de novo* methyltransferases *DNMT3a* and *DNMT3b* and histone methyltransferases *G9a* and *SUV39H1* [8].

Ultrastructural morphology of the embryo can be disrupted by IVP. By the morula stage of development, embryos produced in vitro accumulate increased amount of intracellular lipid and have fewer mitochondria as compared to embryos produced in vivo [4]. Analysis of lipid profile revealed that IVP embryos had increased abundance of phosphatidylcholines 32:0 and 34:1 [107]. These lipids contain low amounts of double bonds so as to reduce membrane fluidity [107]. At the blastocyst stage, IVP has been reported to decrease the number of microvilli that did not fully cover the plasma membrane [7], increase the debris in the perivitelline space and lead to mitochondria that are translucent in appearance [108].

Experiments have been conducted to determine whether the disruption of development caused by IVP is the result of errors in oocyte maturation, fertilization or embryonic development. It is clear that maturation in vitro can disrupt the matured oocyte. First, the ultrastructure of the oocyte matured in vitro is different from oocytes matured in vivo [109]. In particular, release of cortical granules after fertilization, which are responsible for helping prevent polyspermy, are delayed [109]. Furthermore, in vitro matured oocytes have cumulus cells that are smaller in size and less expanded [109]. In addition, gene expression in oocytes and cumulus cells from cumulus oocyte complexes (COC) matured in vitro were found to differ from that of cumulus cells from COC matured in vivo [110]. Using microarray, a total of 64 genes were found to be
differentially expressed in cumulus cells from COCs in vivo and in vitro [110]. Genes that are downregulated in oocytes matured in vitro include \textit{PKP}, \textit{GLUT1} and \textit{DSC2} [111]. Among differentially expressed genes for in vitro COCs were upregulated genes related to stress response (\textit{HSPA5} and \textit{HSP90B1}) and downregulated genes related to anti-apoptosis and ATP binding (\textit{YWHAZ} and \textit{ACTG1}) [110]. Finally, oocytes that are matured in vivo followed by in vitro fertilization (IVF) and culture are more likely to become blastocysts on day 7 and 8 of development than oocytes that are matured in vitro [112]. In vitro maturation in other species is also associated with alterations in gene expression (mouse) [113], ultrastructure (pig) [114], reductions in oocyte competence to form a blastocyst (mouse) [115], and ability of resultant embryos to survive after transfer.

In vitro produced embryos also have reduced development because culture conditions for the embryo following fertilization are inadequate. As compared to embryos produced in vivo, Rizos et al. (2002c) observed a decrease in blastocyst yield for oocytes that were matured in vivo and then cultured for IVF and culture [112]. Survival of bovine embryos after freezing could be increased if embryos produced by in vitro maturation and IVF are placed inside the oviduct of female sheep [6, 105] following collection at the blastocyst stage.

One characteristic of in vitro produced embryos is increased susceptibility to damage after cryopreservation [6, 116, 117] and, as a result, cryopreserved IVP embryos have poor survival after embryo transfer [118]. One key aspect of how well a cryopreserved embryo develops is its lipid content prior to freezing [119]. Drugs such
as phenazine ethosulfate, which decrease the lipid content of the embryo, improve post-thaw survivability of the IVP embryo [119].

Recently, Gad et al. (2012) performed an extensive experiment to determine the specific stages of embryonic development relative to EGA in which culture environment disrupts gene expression in bovine blastocysts. The transcriptome of six groups of blastocysts were compared. Groups were as follows: 1) blastocysts produced in vitro, 2) blastocysts produced in vivo, 3) blastocysts produced by in vitro maturation and fertilization, cultured to the 4-cell stage and then transfer to recipients, 4) blastocysts produced as for treatment 3 except that embryos were transferred to recipients at the 16-cell stage, 5) blastocysts that were produced in vivo and then placed in culture at the 4-cell stage, and 6) blastocysts produced in vivo and then placed in culture at the 16-cell stage. The greatest deviation in the transcriptome occurred in the two groups that were in culture during EGA – embryos cultured until the 16-cell stage or produced in vivo and placed in culture at the 4-cell stage. In these two groups, there was upregulation of genes involved in the NRF2-mediated oxidative stress pathway. This result was interpreted to mean that the oviduct and the uterine environment provide some protection against reactive oxygen species and the absence of that protection leads to activation of oxidative stress genes. Additionally, lipid metabolism genes \textit{(MSMO1, ANXA1, ANXA3, HMGCR, HSD17B11, LDLR and ACAT2)}, were down-regulated when embryo culture spanned EGA [102].

It is important to recognize that the culture conditions associated with IVP not only compromise the ability of the embryo to establish pregnancy but also change the nature of the developmental program so that there is a greater risk for fetal loss,
neonatal death and developmental abnormalities in offspring produced as a result of IVP [99, 120-123]. Offspring from IVP have a higher rate of congenital malformations including incidence of hydroallantois and aberrant limb formation [124]. Fetal [125, 126] and birth weight [124, 127, 128] are increased in IVP offspring, which coincides with higher rates of dystocia in recipients of IVP embryos [124, 127]. Calves born from IVP embryos are also more likely to be born dead within the first 24 hours [129] or 20 days of life [120].

Disorders in development caused by IVP are not unique to cattle. In humans, IVP embryos have increased incidence of chromosomal abnormalities [130], implantation failure [131], loss of DNA methylation imprinting [87], abnormal cell division, cell allocation, cell death and embryonic arrest and death [132]. In the pig, IVP embryos also have decreased cell numbers [133], over 588 differentially expressed genes (DEG) [133], and aberrant ultrastructure including nucleoli located outside of the nuclear membrane and malformed smooth endoplasmic reticulum [134]. Mouse IVP embryos also have differential gene expression [135] and abnormal methylation at imprinted genes [136].

Consequences of being derived from an IVP embryo can persist into adulthood. This question has not been well examined in the cow but there is evidence for this idea from other species. Mice born from IVP embryos have increased blood pressure at 21 weeks of age [13] while children between the ages of 8-18 years old born from IVP embryos have increased blood pressure and blood glucose levels [137]. Sheep produced from IVP embryos are heavier by day 61 after birth and several major organs are larger in size than for offspring produced in vivo [138].
Alterations in Maternal Function

An additional line of experimentation to evaluate the importance of the reproductive tract environment for development of the embryo is to evaluate how changes in oviductal or uterine function during the preimplantation period affects embryonic development. Experiments described here indicate that alterations in the endocrine regulation of the reproductive tract by exogenous administration of hormones like progesterone and somatotropin can increase embryonic growth and the likelihood that the embryo will establish pregnancy. Other influences on the reproductive tract, in particular lactation, can alter the reproductive tract in a way that is detrimental to the developing embryo.

Supplemental Progesterone

The presence of progesterone during preimplantation embryo development modulates fertility and elongation of the bovine embryo. The importance of the reproductive tract for regulating embryonic development can be observed by evaluating the consequences of hastening the post-ovulation rise in progesterone by providing supplemental progesterone. Cows receiving supplemental progesterone on Days 1, 2, 3 and 4 of pregnancy have embryos which are developmentally advanced at Day 14 [139], larger in size and have premature IFNT secretion [57, 140].

The mechanism of action is unknown, but is likely to involve changes in characteristics of the uterine secretome. Forde et al. (2009) conducted a study where pregnant heifers received either an intravaginal device releasing either high or normal amounts of progesterone on day 3 after insemination [141]. Tissue was collected from the endometrium on days 5, 7, 13 or 16 of pregnancy and submitted to microarray analysis. On day 5 compared to day 7, heifers that received high progesterone had
more DEG (36 and 124 respectively) than on day 13 and 16, where heifers that received high progesterone had only 15 and 25 DEG respectively. The effects of higher progesterone were greater early in the cycle, when differences in progesterone between supplemented cows and normal progesterone cows would be greater than at later days of the cycle. Endometrial tissue collected from high progesterone heifers at day 5 had increased expression of genes associated with triglyceride synthesis and glucose transport. One gene that improves glucose secretion, MSTN, increased with high progesterone treatment. These studies suggest that embryo development is not increased by progesterone, but that the timeline of development can be shifted by supplemental progesterone so that embryos reach developmental milestones earlier.

**Somatotropin**

Another hormone that can improve the competence of the reproductive tract to support embryonic development is somatotropin. Bovine somatotropin (bST) increases the systemic and liver production of IGF1, leading to increased milk production [142]. Injections of bST improve pregnancy rates in lactating cows that act as embryo transfer recipients [143]. An experiment by Ribeiro et al. (2014) showed that injection of bST on day 0 and 14 following artificial insemination enhanced development of the embryo by increasing embryonic size at day 34 and 48, decreasing embryonic loss between day 31 and 66, increasing the number of pregnant cows at day 66 and increasing the number of live calves at birth [144]. The increase in embryonic size was also correlated with an increase in expression of ISG15 and RTP4 at day 31 and 66, both of which are markers of IFNT action [145, 146].
Lactation

The process of lactation can alter the reproductive tract environment in a way that compromises embryonic development. One reason lactating cows have decreased embryonic development during this time is due to corresponding low circulating progesterone levels [147]. Gene expression in the endometrium differs between lactating and non-lactating cows at day 17 after estrus in cyclic and pregnant cows [148]. Several of the upregulated genes in the endometrium of the lactating cow are involved in immune response and the WNT pathway [148]. That these changes in endometrial function compromise embryonic development is indicated by results of embryo transfer experiments. Fewer embryos develop to the blastocyst stage when two to four-cell embryos were transferred into the oviduct of lactating cows then when transferred into the oviducts of non-lactating cows [9]. Moreover, pregnancy rates were lower in lactating recipients when embryo transfer was done at Day 7 after estrus [149-151].

Developmental Programming

The idea that maternal environment can change the characteristics of development in a way that affects adult physiology and health is today often called the Barker hypothesis. This term refers to the scientist who first noted this phenomenon while studying epidemiological data from adults who were fetuses during the World War II Dutch famine crisis of 1944-1945 [152, 153]. Caloric intake in Netherlands during that winter was very low (around 400-800 kcal/d) because of the combination of a harsh winter and blockade of food delivery by Nazi Germany [152]. A study of middle-aged adults in the 1990s that were in utero during the famine revealed a variety of changes in physiology as compared to cohorts born before and after the famine. These changes
included increased circulating concentrations of glucose [154], high body mass index [155], reduced plasma concentrations of factor VII and higher plasma concentrations of fibrinogen [156].

Since these studies, other epidemiological studies in humans suggest the importance of nutrition as a programmer of development. For example, adults from mothers that consumed less protein during pregnancy had higher blood pressure than those who had a balanced diet [157]. Additionally, mothers that ingested less folic acid during embryonic development had offspring with an increase rate of congenital malformations due to defects in the formation of the neural tube [158].

Experimental studies in both laboratory and farm animals have confirmed the role of prenatal nutrition as a determinant of physiology of the mature animal. Rats fed a low protein diet during pregnancy had fewer offspring and those offspring had hypertension at 4 weeks of age [159]. In addition, offspring from rat mothers that were malnourished during prenatal development had insulinopenia due to a reduction in beta cells [160] and increased rates of hyperphagia [161]. Caloric restriction from day 28-78 of gestation resulted in offspring who as six-year adults had decreased insulin sensitivity, increased body weight and higher feed consumption than sheep from control mothers [162].

Besides nutrition, dehydration, psychological stress and heat stress that are imposed on the mother can also affect developmental programming of the mature animal. Sheep that were exposed to hypernatremia from days 110-150 in utero had offspring with higher plasma osmolality, sodium levels and arterial blood pressure as neonates [163]. Rhesus monkey mothers that were exposed to unpredictable audible noise from day 90-145 following conception had offspring with higher levels of
circulating ACTH and cortisol as juveniles [164]. Pigs that were exposed to heat stress in utero while fetuses regulated body temperature at a higher set point as adults than pigs from non-stressed mothers [165].

Developmental programing can occur very early during pregnancy including in the preimplantation period. Rat mothers that were fed a low protein diet (9% casein) from fertilization until day 4.25 of gestation had offspring that weighed more at week 4-7 following birth than offspring of mothers fed a control diet (18% casein) [15]. In sheep, feeding dams a diet deficient in cobalt and sulfur from 30 days before conception through 6 days following conception resulted in offspring with higher body weight and fat content as adults than control offspring [16]. Similar finding were found in mice [13].

One characteristic of developmental programming is that the effect can vary with sex. In one experiment in the rat, four types of offspring were examined at 110 days after birth 1) from mothers fed low protein (10% casein) during pregnancy and lactation, 2) from mothers fed low protein during pregnancy but a normal diet (20% casein) during lactation, 3) from mothers fed normal casein during pregnancy but low protein during lactation and 4) from mothers fed normal protein throughout pregnancy and lactation [166]. Males but not female offspring from mothers fed low protein during pregnancy and a normal diet during lactation had insulin resistance compared to controls. In another experiment, baboon mothers fed a 30% maternal nutrient restricted diet had male fetuses at mid-gestation with increased renal expression of \( AT1 \) compared to males from control mothers while there was no change in expression for female offspring [167]. In sheep restricted to 50% of their normal nutrient intake during early to
mid-gestation, there was a reduction in renal glomerulus numbers at gestational day 135 in male fetuses but did not affect female fetuses [168].

Sexual dimorphism in developmental programming also occurs during the preimplantation period. Female offspring from rats that were fed a low protein (9% casein) diet from day 0-4.25 after mating followed by a normal protein diet for the rest of gestation weighed less at birth than female offspring fed a normal protein (18% casein) diet, while male offspring were not affected by the level of protein within the diet [15]. On the other hand, while there was no effect in females, male pups from mothers fed a low protein diet also had increased systolic blood pressure at weeks 4 and 11 of age and a higher kidney and lower liver weight compared to the male pups fed the control diet [15]. Female mouse offspring that were from mothers restricted to a low protein diet during preimplantation development had higher expression of \( \text{Igf1r} \) in the retroperitoneal fat tissue at 1 year of age compared to females from mothers fed a normal protein diet; in males, however, there was no effect of protein restriction on \( \text{Igf1r} \) expression [14]. Male mouse offspring born to mothers that were fed a low protein diet (9% casein) during the pre-implantation period had elevated levels of lung angiotensin-converting enzyme in lung tissue compared to male offspring from control mothers while there was no effect of maternal diet on this characteristic in female offspring [17].

In another model, designed to disrupt DNA methylation, rat mothers were fed methyl-deficient diets that did not contain folic acid from 3 weeks prior to and 5 days after conception. At 6 months of age, male offspring from these mothers had higher concentrations of oral glucose than control male offspring while there was no difference
in the female offspring [169]. Additionally, sheep mothers that were fed a similar diet produced offspring with hypertension but only when the sex was male [16].

**CSF2 as an Embryokine**

Much of this literature review has focused on the evidence that the environment of the reproductive tract can affect competence of the preimplantation embryo for development and survival to term. What has not been discussed are the processes by which the reproductive tract affects embryonic development. The embryo receives all of its nutrition from the mother through reproductive tract secretions and blood plasma exudate. The nutritional components of uterine fluid are referred to as histotroph. There are changes in the composition of the histotroph during early pregnancy, including changes in amino acids, ions, enzymes, hormones, growth factors, proteases and protease inhibitors, vitamins, glucose, mitogens, lymphokines and cytokines [170]. In addition to nutritional support, the reproductive tract establishes the pH of the embryo’s environment as well as providing the epithelial surface to which the embryo will attach during placentation. The reproductive tract also produces a variety of regulatory molecules that can affect either endometrial function, other aspects of maternal physiology or development of the embryo. These include enzymes required for converting cortisone into cortisol in the sheep [171] and cow [172], prostaglandins, especially prostaglandin F2α and E2 [173], and a variety of protein growth factors and cytokines. In the cow, these include *IGF2, IGFBP2, PTGER2, VEGFR2* and *CST6* [174]. Here we propose the use of the term embryokine to describe regulatory molecules produced by the reproductive tract that regulate embryonic development. Several embryokines have been identified that can alter one or more aspects of
preimplantation development. In the cow, these include hyaluronan [21], IGF1 [19], FGF2 [22], LIF [20], TGFB [20] and ILB1 [23].

**Role of Colony Stimulating Factor 2 as an Embryokine**

Many molecules originally identified as being important for immune function have since been shown to be produced in the reproductive tract where they could either be involved in regulation of immune function in the uterus or play a role independent of regulation of immune function. The best-studied reproductive tract embryokine with respect to regulation of embryonic development is CSF2. CSF2 is a glycosylated, monomeric 23-kDa cytokine that was originally described as a product of T lymphocytes and macrophages, which causes differentiation of granulocytes and macrophages from hematopoietic stem cells [175-177]. CSF2 is also synthesized by fibroblasts [178]. As will be described in detail below, CSF2 is also produced by epithelial and stromal cells of the endometrium [27, 179, 180].

**Signal Transduction**

CSF2 binds to CSF2R and signals through the JAK2-STAT5ab pathway or the phosphatidylinositol 3-kinase kinase (PI3K) pathway. Initiation of CSF2 binding begins first with the attachment of the protein to the trans-membrane receptor colony stimulating factor alpha (CSF2RA). First described as a protein with 56% homology to IL-3 by Hayashida in 1990, CSF2RA is an 80 kDa, low-affinity receptor that is specific to CSF2 [181, 182]. CSF2RA is capable of binding to CSF2 with a dissociation constant of 2-8 nM [182]. There are indications that CSF2 can signal through interactions with CSFRA alone [183, 184] but the typical signal transduction involves recruitment of the CSF2RB subunit to the CSF2-CSF2RA complex, which increases the affinity of the receptor to a dissociation constant to 170 pM [181]. The 120-140 kDa CSF2RB subunit
is not specific to CSF2Ra but also serves as part of the receptor complex for IL-3 and IL-5 [181, 182]. In response to CSF2RB binding, CSF2RA expression is downregulated [185]. CSF2RA is expressed in the mouse, human, bovine and porcine embryos [186-189].

Upon binding to CSF2, the CSF2R complex activates Janus kinase (JAK) 2 which in turn causes activation of signal transducer and activator of transcription (STAT) 5 A and B and other Src-homology 2 domain containing proteins [181, 190, 191]. JAK2 associates with the CSF2RB subunit where it is activated and subsequently phosphorylates tyrosine sites located on the CSF2RB subunit [190, 192]. Phosphorylation of Y612, Y695 and Y750 of CSF2RB subunit allow for binding of STAT1 and STAT5, with STAT5 being the most critical for CSF2 [191].

**Production of CSF2 by the Reproductive Tract**

CSF2 has been localized immunochemically to the oviduct and endometrium of the human [29], cow [27], pig [193] and mouse [194]. In the mouse, CSF2 protein has been localized to glandular and luminal epithelial cells, while the contribution from the stroma is minimal; intensity of labeling increases following the post-ovulatory increase in estrogen [28], which stimulates the secretion of CSF2 while progesterone inhibits it [194]. Mating can increase the release of CSF2 into uterine fluid through the actions of TGFB contained in the seminal fluid [33]. In addition, expression of CSF2 in the oviduct is also decreased following mating with males whose seminal vesicle gland was surgically impaired [32].

In the human, luminal and glandular epithelium of the endometrium and stroma express CSF2 mRNA and protein [195]. Glandular epithelial cells have the highest level of protein and mRNA expression [29, 180]. Secretion of CSF2 from luminal and
glandular epithelial cells is highest during the mid-secretory phase of the menstrual cycle before decreasing during the proliferative phase [195]. However, CSF2 is present in significantly large quantities in the decidua throughout the first trimester of pregnancy compared to non-pregnant decidua [196].

In the cow, CSF2 can be localized immunochemically to the luminal and glandular epithelium and stroma [27, 197]. The majority of CSF2 is localized to the luminal epithelium [27]. Although not significant, immunoreactive CSF2 in the endometrium during the estrous cycle had low amounts of CSF2 at estrus (when progesterone concentrations are low) compared to days 7-18 of the estrous cycle [27]. Therefore, it appears that CSF2 secretion in the cow could be under the control of progesterone [27].

Another molecule that can modify maternal production of CSF2 in the uterus in ruminant is IFNT. Transcervical infusions of IFNT into the uterine body increased amounts of CSF2 in the luminal epithelium [197]. The level of obesity of the cow also determines CSF2 immunoreactivity and CSF2 mRNA expression in the ampulla: it was suppressed in the oviduct of obese cows compared to lean cows on the second day following ovulation [34].

**Actions on the Preimplantation Embryo**

The first observed action of CSF2 on the preimplantation embryo was an increase in the proportion of cultured embryos that could develop to the blastocyst stage. Such a phenomenon has been observed in the cow [18, 24], pig [189, 198, 199], mouse [26] and human [200]. The mechanism for the increased competence of embryos to develop to the blastocyst stage is not known. However, in both the mouse blastocyst [187] and cow morula [31], CSF2 alters expression of genes in a way that
would block apoptosis. In the mouse, for example, CSF2 decreased expression and abundance of genes involved in stress response and apoptosis, such as *Hspa5*, *Hsp90aa1*, *Hsp90ab1* and *Gas5*, and proteins HSP1A/1B and BAX. In cattle, CSF2 increased transcript abundance of several anti-apoptotic genes, such as *CD73/NT5E*, *PRKAR2B* and *PGR* and decreased the abundance of pro-apoptotic genes such as *MADD*, *RIPK3*, *NOD2* and *CREM* [31]. Furthermore, treatment with CSF2 also decreased the magnitude of apoptosis in the bovine embryo after exposure to heat stress [31]. In addition, treatment with CSF2 prior to freezing improved post-thaw survival of mouse embryos while preventing apoptosis [187, 201]. Thus, CSF2 may increase competence for development to the blastocyst stage through inhibition of apoptosis.

CSF2 could also act by changing expression of genes involved in development. Among the genes whose expression was altered in the bovine morula were genes associated with neurogenesis, muscle formation, mesenchyme formation and multiple signaling pathways [31]. In the pig, CSF2 altered gene expression in cloned embryos with increases in expression of *LIF*, *CDX2*, *POU5F1*, *BCL2*, *DNMT1* and *PCNA* [198, 199]. In cattle, CSF2 can increase the blastocyst yield regardless of whether it is added after fertilization or if addition is delayed until day 5 of development [18, 24]. Therefore, actions of CSF2 probably occur in later cleavage stages and after embryonic gene activation has occurred.

Not only does CSF2 increase blastocyst yield but also embryos cultured with CSF2 have increased likelihood of establishing pregnancy after transfer to recipients. In the mouse, the addition of 2 ng/ml recombinant murine CSF2 to cultured embryos of
had that we subsequently transferred to recipients resulted in increased litter size and more viable progeny compared pregnancies after transfer of control embryos in culture [26]. In the cow, pregnancy rate and calving rate was higher for recipients receiving an in vitro produced embryos cultured with 10 ng/ml CSF2 from day 5-7 of development (43% and 37%, respectively) than cows that received control embryos (34% and 23%) [18]. The effect of CSF2 was only seen when added during days 5-7 of development. Embryos cultured with CSF2 from days 1-7 did not have superior ability to survive transfer (35% vs. 35% for controls). An interesting observation was that CSF2 also reduced the loss of pregnancies occurring after initial pregnancy diagnosis at Day 30-35 of gestation. Pregnancy losses were 22% for control embryos, 11% for embryos treated with CSF2 from Day 5-7 and 0% for embryos cultured from Day 1-7. [18].

Recently, a similar beneficial effect of CSF2 on embryo competence for survival after transfer was seen in humans. Treatment of embryos with 2 ng/ml CSF2 throughout culture increased survival compared to controls at week 7 (23.5% vs. 20%) and 12 of gestation (23% vs. 18.7%) as well as live births (28.9% vs. 24.1%) [25]. The ongoing implantation rate in women that previously had one or more miscarriages was also greater if the embryo transferred had been cultured with CSF2 [25].

The change in the embryo responsible for increased competence to establish pregnancy is not clear. One possibility is that CSF2 increases number of cells in the blastocyst, particularly the ICM, and that these increased numbers make the embryo better able to survive transfer. Culture of mouse embryos with CSF2 resulted in blastocysts that contained 15 more cells than control blastocysts [186]. When 2-cell stage embryos were split and allowed to develop to the blastocyst stage, diameter and
number of cells of the blastocyst were increased by CSF2 [202]. In the pig, as well, CSF2 improved total cell number in blastocysts [199]. In both the human [187] and cow [18], number of cells in the ICM of blastocysts was increased by CSF2. There was also a reduction in the number of cells undergoing apoptosis in human blastocysts treated with CSF2 [187].

There is also evidence that treatment with CSF2 can alter the developmental program of the embryo at a later stage of pregnancy. In one experiment, bovine embryos were treated with CSF2 from days 5-7 of development, transferred into cows and then recovered at day 15 of development. Embryos that had been treated with CSF2 had increased expression of IFNT2 and KRT18, increased IFNT2 secretion into the uterine lumen and tended to have increased length [30].

Not all studies with CSF2 indicate positive effects of the molecule on embryonic development. In the one study, the addition of 2, 5, 10 or 50 ng/ml of ovine CSF2 to cultured bovine embryos did not improve the number of blastocysts that developed at day 7 after fertilization [203]. In a similar experiment, ovine embryos cultured in the presence of CSF2 did not reach the blastocyst stage at a rate higher than that of control embryos. In the mouse, addition of either 5 or 10 ng/ml CSF2 decreased the proportion of embryos that developed to the blastocyst stage and, although not significant, tended to increase the rate of aneuploidy [204].

Characteristics of the CSF2R in the Preimplantation Embryo

Although CSF2 can affect embryonic function in a variety of species, it may exert its actions through a cell signaling mechanism that is distinct from the prototypical signal transduction pathway initiated by the CSF2RA-CSF2RB complex. This is because, as has been shown for human and mouse, the preimplantation embryo expresses
CSF2RA but not CSF2RB [186, 187]. In the pig, embryos at the blastocyst cell stage express CSF2RB, but transcript abundance is very low compared to that for CSF2RA [189].

Other pathways can be activated following the binding of CSF2 to receptor, such as the MAPK and the PI3K pathway [191, 205, 206]. When CSF2 signaling through PI3K occurs, the regulatory subunit of PI3K (p85), which is bound to CSFRA through its C-SH2 domain, is phosphorylated and activates PI3K [183]. CSF2 signaling is blocked in Day 12 porcine TE cells when treated with the PI3K inhibitor LY294002 [206].

One possibility is that CSF2RB protein is present on the plasma membrane of embryonic cells despite lack of mRNA. This could be the case because bovine oocytes contain CSF2RB in trace amounts [188, 189] and some protein that was synthesized by the oocyte could persist after fertilization.

**Goals of the Current Investigation**

The aim of this dissertation is to understand the mechanisms responsible for specific actions of a maternally-derived cytokine, CSF2 on development of the preimplantation bovine embryo. In cattle, CSF2 treatment from day 5-7 of development can increase the proportion of embryos becoming a blastocyst, alter gene expression, decrease apoptosis, increase ICM number, program the embryo to have altered gene expression and increased trophoblast elongation at day 15 of gestation, and increase competence of the embryo to maintain pregnancy to term [18, 24, 30, 31]. This dissertation is focused on understanding key aspects of the biology of some of these effects of CSF2 as follows:

In Chapter 2, experiments will be described that determine whether CSF2 regulates survival and pluripotency of the ICM or the growth potential and differentiation
of the TE. The mechanism by which CSF2 improves the ability of a blastocyst to establish pregnancy is unknown, but evidence exists that CSF2 affects lineage commitment of the ICM and TE of the blastocyst in addition to altering expression of pluripotency and differentiation related genes [18, 31]. In Chapter 3, the nature of the developmental programming of CSF2 to alter trophoblast elongation and gene expression at day 15 [30] will be elucidated. In particular, it will be tested whether CSF2 from day 5-7 alters the transcriptome and methylome at day 15. In addition, an unexpected finding will be described that indicates the nature of developmental programming by CSF2 depends upon sex, i.e., CSF2 causes different and often opposite effects in female embryos than male embryos. In Chapter 4, the developmental pattern of DNA methylation in the preimplantation embryo will be described as well as possible alterations in the global methylome by CSF2 and sex that could form the basis for sexual dimorphism in response to CSF2 seen in Chapter 3.
CHAPTER 2
REGULATION OF PLURIPOTENCY OF INNER CELL MASS AND GROWTH AND DIFFERENTIATION OF TROPHECTODERM OF THE BOVINE EMBRYO BY COLONY STIMULATING FACTOR 2

Introduction

Communication between the preimplantation mammalian embryo and reproductive tract of the mother is important for ability of the embryo to develop into a blastocyst with the ability to develop to term. Even though embryos can become blastocysts in vitro, they have reduced competence for establishing pregnancy when transferred to recipient females than embryos that developed in vivo [26, 207, 208]. Growth factors and cytokines are important components of the embryotrophic milieu of the reproductive tract. Addition of specific growth factors present in the reproductive tract to culture medium can improve the proportion of embryos that develop to the blastocyst stage. Examples in the cow include activin A, EGF, FGF2, IGF1, IGF2, IL1B, and LIF [20, 22, 23, 209-211]. Moreover, competence of in-vitro produced embryos to establish pregnancy after transfer into females can be improved by LIF and TGFB in the mouse [212-214] and IGF1 in the cow (when recipients were heat-stressed) [129, 215].

Perhaps the best studied maternally-derived cytokine that regulates embryonic development is CSF2. Secreted by the oviduct and endometrium [27, 29, 34], CSF2 can increase the proportion of embryos developing to the blastocyst stage in the cow [18, 24], pig [189, 198, 199], mouse [26] and human [200]. Moreover, CSF2 increased the proportion of in vitro produced embryos that established pregnancy after transfer into mice [26], cows [18] and humans [25]. That variations in CSF2 signaling might be a cause of female infertility is suggested by the recent observation that CSF2 synthesis in the oviduct is decreased in obese cows [34].
The mechanism by which CSF2 improves the ability of a blastocyst to establish pregnancy is not known but there is evidence for the hypothesis that CSF2 affects lineage commitment in both the ICM and TE of the blastocyst. Treatment of embryos with CSF2 at Day 5 post-insemination altered expression of several genes involved in pluripotency and differentiation [31]. In addition, CSF2 increased the number of ICM cells in the bovine blastocyst at Day 7 post-insemination without affecting number of TE cells [18]. At Day 15, the trophoblast of conceptuses formed following treatment with CSF2 from Day 5-7 post-insemination had higher expression of the maternal signaling gene IFNT2 and tended to be numerically greater in length than control embryos [30]. Some actions of CSF2 on embryo survival could be the result of regulation of apoptosis pathways because CSF2 affects expression of genes involved in apoptosis in both cattle [31] and mice [216] and can block the induction of apoptosis caused by heat shock in cattle [31].

In most cells, CSF2 signaling is accomplished through a cell surface receptor composed of a low-affinity α subunit (CSF2RA) and a high-affinity β subunit (CSF2RB) [217]. CSF2RA is a low-affinity receptor specific for CSF2 while CSF2RB, which is involved in signaling for several cytokines, confers increased affinity of the receptor complex to the ligand [181]. CSF2RA is expressed in preimplantation mouse, pig and human embryos but expression of CSF2RB is undetectable or nearly so [186, 187, 189]. These observations suggest that CSF2 signaling in the embryo involves an unknown pathway that is independent of CSF2RB.

The major objective of this study was to determine whether CSF2 could regulate survival and pluripotency of the ICM and growth potential and differentiation of TE.
examine pluripotency, we utilized a model to evaluate ability of isolated ICM to remain pluripotent while being cultured and passaged on a layer of fetal fibroblasts in the presence of MAPK1 and GSKB inhibitors [218]. It was also tested whether CSF2 regulated expression of genes involved in pluripotency (NANOG and SOX2 [46, 219]), differentiation of TE (CDX2 [46]) and formation of hypoblast (GATA6 [46]) in isolated ICM. TE growth and differentiation was assessed in a model of TE outgrowth on Matrigel-coated plates [220]. It was also determined whether both CSF2RA and CSF2RB were expressed by the preimplantation embryo from the zygote to blastocyst stage.

Materials and Methods

Embryo Production

IVP of bovine embryos was performed as previously described [22] unless otherwise noted. Briefly, COCs were obtained by cutting the surface of slaughterhouse-derived ovaries with a scalpel and vigorously rinsing the ovary through a bath of oocyte collection medium [tissue culture medium-199 with Earle’s salts without phenol red (HyClone, Logan, UT, USA), 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin and 1mM glutamine]. Groups of 10 COCs were matured in 50 μl droplets of oocyte maturation medium [tissue culture medium-199 with Earle’s salts (Invitrogen, Carlsbad, CA, USA), 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Bioniche Life Sciences, Belleville, Ontario, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate and 1 mM glutamine] covered with mineral oil for 20 hours at 38.5°C and in a humidified atmosphere of 5% (v/v) CO₂. Up to 300 matured oocytes
were fertilized with Percoll-purified sperm (1.0 x 10^6/ml) for 8 hours at 38.5ºC in 1.7 ml of synthetic oviductal fluid-fertilization (SOF-FERT) [221]. Cumulus cells were denuded after fertilization by vortexing in 600 µl HEPES-TALP containing 1,000 U/ml hyaluronidase. Putative zygotes were then cultured in 50 µl microdrops of synthetic oviduct fluid - bovine embryo 1 (SOF-BE1) [22] covered with mineral oil at 38.5ºC in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with the balance N₂. Cleavage rate was assessed at Day 3 post-insemination. Blastocysts were harvested at Day 7 or 8 post-insemination.

Effect of CSF2 on Survival of Isolated Inner Cell Mass

Bovine fetal fibroblast feeder cells

Culture on bovine fetal fibroblasts prevent isolated ICM from losing pluripotency and undergoing differentiation [218]. Bovine fetal fibroblast (BFF) cells were created from fetal calf skin (~2-3 months old). Pieces of fetal calf skin (~1 cm³) were cut from the mediolateral region of the body and transferred into 15 ml tubes of Dulbecco’s phosphate buffered saline (DPBS) with 1% (v/v) antibiotic-antimycotic [10000 U/ml penicillin G, 10000 µg/ml streptomycin sulfate and 25 μg/ml amphotericin B] (ABAM) (Invitrogen). Rinsed fetal calf skin was then placed onto 60 mm petri dishes (BD Falcon, Franklin Lake, NJ, USA), cut into 5 mm x 5 mm square pieces, and placed onto scored areas of a new 60 mm petri dish containing 10 ml of a medium consisting of a modified Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) containing 10% (v/v) fetal bovine serum [FBS] (Atlanta Biologicals, Norcross, GA, USA) and 1% (v/v) ABAM. On Day 4 after culture, skin pieces were removed from the plate using tweezers. Cells were passaged by removal of medium by aspiration, washing twice with DPBS containing 1% (v/v) ABAM, and trypsinization by addition of 5 ml 0.05% (v/v) trypsin with
0.38 mg/ml ethylenediamine tetraacetic acid (EDTA) (Invitrogen) and incubation at 38.5°C and 5% (v/v) CO₂ in air for 3 min. Trypsin was inhibited by addition of 1.5 ml FBS. Cells were centrifuged for 5 min at 250 x g, supernatant removed and cells resuspended with 25 ml modified DMEM. Cells were then transferred into a 175 cm² flask and cultured until confluent. Medium was changed every two days until 90-100% confluence was reached. Cells were then trypsinized and passaged at a 1:4 dilution.

For culture of ICM, BFF cells at 90% confluence were treated twice, for 3 hours each, with 10 µg/ml mitomycin C (Invitrogen) at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ in air. After three washes in medium, cells were cultured overnight, washed with DPBS with 1% (v/v) ABAM, trypsinized as described above, washed, collected by centrifugation for 5 min at 250 x g, and resuspended to 1.5 x 10⁶ cells/ml in modified DMEM that also included 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). Cells were frozen in liquid nitrogen in 1 ml aliquots until further use.

Isolation of ICM by lysis of trophectoderm using antibody and complement via immunosurgery

Procedures were modified from those described by Ozawa et al. [218]. Blastocysts were washed three times in DPBS containing 1% (w/v) polyvinylpyrrolidone (PVP) (Kodak, Rochester, NY, USA) (DPBS-PVP), treated with 0.1% (w/v) proteinase solution (proteinase from Streptomyces griseus) in DPBS to remove the zona pellucida, washed three times in DMEM (Invitrogen) with 1% (v/v) ABAM, washed twice in DMEM with 1% (v/v) ABAM and 10% (v/v) rabbit anti-bovine serum (Sigma-Aldrich) and then incubated for 1.5 hours at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with the balance N₂. Blastocysts were then washed three times in DMEM with
1% (v/v) ABAM, washed twice in DMEM with 1% (v/v) ABAM and 20% (v/v) guinea pig complement (Rockland Immunochemicals Inc., Gilbertsville, PA, USA), and then incubated in the same medium for 1.5 hours at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with the balance N₂ to lyse TE. Those ICM that were free of visible TE cells were washed three times in embryonic stem cell medium (ESM) consisting of knockout-DMEM (Invitrogen) with 15% (v/v) knockout serum replacement (Invitrogen), 1 mM Glutamax (Sigma-Aldrich), 1% (v/v) ABAM, 0.1 mM minimal essential medium nonessential amino acids, and 0.1 mM β-mercaptoethanol. Isolated ICM were then individually transferred into a single well of a 12-well plate (BD Falcon) coated 24 hours earlier with 1% (w/v) gelatin (BioRad, Hercules, CA, USA) and 2 x 10⁴ cells/cm² mitomycin C treated fibroblasts in 1 ml of ESM+2i medium [ESM containing 3 μM GSK3B inhibitor CHIR99021 (Stemgent, San Diego, CA, USA) and 1.2 μM MEK1 inhibitor PD0325901 (Stemgent)]. These conditions were used because they promote maintenance of ICM in a pluripotent state [218].

**Culture of isolated ICM**

Individual clusters of ICM cells (each from a single blastocyst) were cultured at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with the balance N₂. On Day 7 following ICM isolation, ICM colonies were passaged using a finely drawn glass needle to separate the ICM from any surrounding TE that remained and to dislodge the ICM outgrowth from the BFF layer. Individual ICM outgrowths were then transferred into a well of a new 12-well plate prepared with gelatin and mitomycin C inactivated BFF cells as previously described and cultured in 1 ml ESM+2i. Medium was changed and cells were passaged according to the same procedure above. For some IVP replicates (i.e., a group of embryos produced on a given day), culture was
terminated 7 days after passage 1 while, in others, cells were passaged three times and cultures stopped at the end of passage 3.

**Experiments**

In the first experiment, all embryos were moved at Day 6 post-insemination in groups of 30 to 50 µl microdrops of either 1) 45 µl SOF-BE1 and 5 µl recombinant bovine CSF2 [Novartis, Basle, Switzerland; 100 ng/ml in a 90:10 (v/v) mixture of SOF-BE1 and DPBS containing 1% (w/v) bovine serum albumin (BSA); final concentration of CSF2 after dilution in the drops = 10 ng/ml], 2) 45 µl SOF-BE1 and 5 µl vehicle [90:10 SOF-BE1: DPBS containing 1% (w/v) BSA; control treatment] or 3) 50 µl ESM+2i. The concentration of CSF2 was chosen based on its effectiveness for increasing embryonic survival after transfer into recipient females [18]. The ESM-2i medium was used as a positive control for survival of ICM colonies because it had previously been reported to enhance survival of ICM colonies when used to culture embryos from Day 6-8 post-insemination [218]. Embryos were harvested at Day 8 post-insemination and ICM isolated and used for culture as described above. The experiment was replicated on 5 occasions with a total of 12-16 isolated ICM per treatment.

In a second experiment, procedures were similar except all embryos were harvested on Day 5 post-insemination and cultured with treatments from Day 5 to 7 post-insemination before ICM isolation. The experiment was replicated on 6 occasions with total experiments of 8-11 isolated ICM per treatment.

**Analysis of ICM Colonies Surviving Passage**

One colony derived from CSF2-treated Day 7 blastocysts was harvested 7 days after passage 3, placed in extraction buffer (Arcturus PicoPure RNA isolation kit, Life...
Technologies, Grand Island, NY, USA) and measured for steady-state amounts of NANOG, CDX2 and GAPDH by quantitative PCR (qPCR) as described below.

**Expression of Pluripotency Genes in ICM**

All embryos were moved at Day 6 post-insemination in groups of 30 to 50 µl microdrops of either 45 µl SOF-BE1 and 5 µl CSF2 (prepared as described above to yield a final concentration of 10 ng/ml) or 45 µl SOF-BE1 and 5 µl vehicle (control treatment). Blastocysts were harvested at Day 8 and ICM were isolated by immunosurgery on Day 8 post-insemination as described above. ICM were immediately placed in extraction buffer (Life Technologies) and stored at -80°C until ready for RNA extraction. RNA was extracted and used to measure steady-state amounts of NANOG, SOX2, CDX2, GATA6 and GAPDH. For each replicate, a total of 3-5 ICM were pooled for an individual treatment. The experiment was replicated using a total of 7-9 pools per procedure.

**Actions of CSF2 on Competence of Trophectoderm to Form Outgrowths**

**Establishment of outgrowths**

The procedure for analysis of TE outgrowth was modified from that of Yang et al. [222]. On Day 5 or 6 post-insemination, all embryos in groups of 30 were moved to 50 µl microdrops of either 45 µl SOF-BE1 and 5 µl CSF2 (prepared as described above to a final concentration of 10 ng/ml) or 45 µl SOF-BE1 and 5 µl vehicle (control treatment). After culture for 48 hours at 38.5°C in a humidified atmosphere of 5% (v/v) O2 and 5% (v/v) CO2 with the balance N2, blastocysts were collected and transferred individually to wells of 96-well plates (BD Biosciences, Bedford, MA USA) that had been coated with Matrigel (Matrigel basement membrane matrix growth factor reduced; BD Biosciences). Embryos were cultured in 100 µl of DMEM (Invitrogen) modified to contain 10% (v/v)
FBS (Atlanta Biologicals), 1% (v/v) ABAM, 0.1 mM MEM nonessential amino acids and 0.1 mM β-mercaptoethanol at 38.5°C in a humidified atmosphere of 5% (v/v) O<sub>2</sub> and 5% (v/v) CO<sub>2</sub> with the balance N<sub>2</sub>. After 5-6 d, medium was removed and replenished with fresh medium. At the end of culture, outgrowths were examined for size, labeling with anti-CDX2, and gene expression.

**Immunolabeling of CDX2**

All labeling steps were performed at room temperature unless otherwise stated and in a volume of 100 µl in the wells in which outgrowths were established. Attached TE outgrowths were washed in DPBS-PVP and fixed in 4% (w/v) paraformaldehyde in DPBS-PVP for 15 min. The fixed outgrowths were washed three times in DPBS-PVP, permeabilized for 20 min in DPBS containing 0.25% Triton X-100 and washed three times in 0.1% (v/v) Tween 20 with wash buffer [DPBS containing 10 mg/ml fraction V BSA (Sigma-Aldrich)] followed by three washes with wash buffer. For immunolabeling, nonspecific binding sites were blocked by incubation with DPBS containing 5 mg/ml BSA for 1 h. Outgrowths were incubated with affinity-purified mouse monoclonal antibody against CDX2 (ready to use solution; BioGenex, San Ramon, CA, USA). As a negative control, anti-CDX2 was replaced with an irrelevant mouse IgG antibody (Sigma-Aldrich, St. Louis, MO, USA). After 1 h, outgrowths were washed three times in wash buffer and transferred to 1 µg/ml fluorescein isothiocyanate conjugated anti-mouse IgG (Abcam, Cambridge, MA, USA). After three washes, nuclei were labeled by incubation in 5 µg/ml Hoechst 33342 (Sigma-Aldrich) in DPBS-PVP for 15 min. Outgrowths were washed three times and mounted using ProLong Gold Anti-Fade mounting medium (Invitrogen) and observed under a Zeiss Axioplan epifluorescence microscope (Zeiss, Göttingen, Germany). Images were acquired using a 40x objective.
and green and blue filters. The exposure times were constant for all outgrowths analyzed in an individual replicate.

**Assessment of establishment and growth of trophectoderm outgrowths**

The ability of embryos to attach to the Matrigel-coated well was determined for each IVP replicate as the fraction and percent of embryos that remained attached to the well after aspiration of medium at Day 13 or 15 post-insemination. Similarly, the capacity for formation of TE outgrowths was calculated within IVP replicate as the percent of blastocysts in which outgrowth could be observed microscopically at either Day 13 or 15 post-insemination. Outgrowth surface area was determined with the aid of a microscope graticule at 40x magnification.

**Gene expression**

At Day 15 post-insemination, steady state expression of *CDX2* and *GATA6* was determined for a subset of TE outgrowths. Medium was removed, cells washed three times with DPBS-PVP and the outgrowth removed by adding 100 μl of extraction buffer (Applied Biosystems, Invitrogen) and pipetting multiple times. For each replicate, pools of 2-10 TE outgrowths were prepared. The total number of replicates varied from 6-7 depending on the experiment. RNA extraction, DNase treatment and reverse transcription were completed as described below.

**Antiviral assay**

The ability of samples of conditioned medium to inhibit vesicular stomatitis virus-induced death of Madin-Darby bovine kidney cells by 50% was compared with the activity of recombinant human IFNA standard (3.84×10^8 IU antiviral activity/mg protein; Millipore, Billerica, MA, USA) using a previously-described assay [222]. Assay sensitivity was 2.6 pg of IFNA standard. The antiviral activity of conditioned medium
was adjusted based on this standard to represent the IU activity per mm² of cell outgrowth.

Experiments

A total of 4 experiments were conducted. In two experiments, embryos were treated with 10 ng/ml CSF2 or control from Day 6 to 8 post-insemination. This time interval was chosen because CSF2 treatment during this time increased capacity of the ICM to survive culture after isolation from the TE (See Results). In two experiments, embryos were cultured from Day 5 to 7 post-insemination. This time interval was chosen because CSF2 treatment during this time increased competence of embryos to survive after transfer to females [18]. For each treatment period, one experiment was conducted in which outgrowths were examined at Day 13 post-insemination (i.e., 5-6 days after culture on Matrigel) and one experiment was conducted in which outgrowths were examined at Day 15 post-insemination (i.e., 7-8 days after culture). We examined two separate culture periods because of the possibility that effects of CSF2 on TE characteristics would depend on duration of culture. Each experiment was performed with 7-15 IVP replicates containing 2-26 embryos per treatment for each replicate. The replication is described in Table 2-1.

Expression of CSF2 Receptor Subunit Genes

Analysis of gene expression was performed by qPCR using five pools of RNA extracted from 25-30 embryos/pool at the zygote (0 hpi), 2 cell (32-40 hpi), 3-4 cell, 5-8 cell (48 hpi), 9-16 cell (72 hpi), morula (120 hpi) and blastocyst (168 hpi) stages. Zona pellucidae were removed and oocytes and embryos processed for RNA extraction, DNase treatment and reverse transcription as described below. To verify effectiveness
of primers, total RNA was also extracted from leukocytes collected from a saphenous vessel of a Holstein cow.

**Quantitative PCR**

Total RNA was extracted using the PicoPure RNA isolation kit (Life Technologies), treated with 2 U of DNase (New England Biolabs, Ipswich, MA, USA) at 37°C for 30 min to remove DNA, and then incubated at 75°C for 15 min to denature DNase. DNase-treated RNA was then reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen). Reverse transcription occurred using random hexamer primers and involved incubation at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was stored at -20°C until further use. Negative controls for real-time PCR were also performed by incubation without reverse transcriptase.

cDNA was utilized for real-time PCR analysis. For detection of transcript levels, a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA) was utilized with SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). Each reaction contained 1 μl forward primer (0.5 μM), 1 μl reverse primer (0.5 μM), 10 μl EvaGreen Supermix (Bio-Rad), 6.8 μl H2O and 1.2 μl cDNA sample (0.6 embryo equivalent). Amplification conditions were: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 60°C for 5 sec, and 1 cycle of melt curve analysis at 65-95°C in increments of 0.5°C every 2 sec. Primers for **NANOG**, **SOX2**, **CDX2**, **GATA6**, **SDHA**, and **YWHAZ** were described and validated previously [218, 223]. Primers for **CSF2RA** (F: 5’-ACGCGGCGCTAAATGTGAAGTTTG-3’, R: 5’-ACGTGCACTGACACTCCTGTCTT-3’, XM_002686598.2) and **CSF2RB** (F: 5’-TTCCCAAGAGCTGATGACATGGGT-3’, R: 5’-AGATGATGCAGCTGATGACATGGGT-3’, NM_001192664.1) were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) and validated by
evaluation of melt curves, demonstration of standard curves that exhibited a slope of between -3.0 to -3.5, analysis of amplicon size by agarose gel electrophoresis, and Sanger sequencing of amplicons to confirm identity. The $\Delta C_T$ value was determined by subtracting the $C_T$ value of the sample by the $C_T$ for GAPDH, or for the experiment on CSF2RA and CSF2RB expression, by subtracting the $C_T$ value of sample by the geometric mean of the $C_T$ for three housekeeping genes, SDHA, GAPDH and YWHAZ [224].

**Statistical Analysis**

Most data were analyzed by least-squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS) version 9.3 (SAS Institute Inc., Cary, NC, USA). The exception was for data on survival of ICM after passage, which was analyzed by the GLIMMIX procedure of SAS using the Binomial feature. Depending on the experiment, main effects included IVP replicate, stage, treatment and day of treatment. Interactions were also included. The IVP replicate was considered random. For real-time PCR results, treatment effects were tested using $\Delta C_T$ values and graphed as either fold-change ($2^{-\Delta \Delta C_t}$) relative to control or as the inverse of $\Delta C_T$. Data are shown as least-squares means ± standard error of the means.

For the experiments, a total of 62 IVP replicates were performed in which embryos were treated with CSF2 from Day 5-7 and 36 IVP replicates in which embryos were treated from Day 6-8. Statistical analysis was performed to determine whether response to CSF2 depended on the competence of control embryos in an individual IVP replicate to develop to the blastocyst stage. Each IVP replicate was classified as to whether blastocyst development in the control group was within the lowest, middle or
highest tercile of percent blastocyst. For Day 5-7, percent of oocytes that became blastocysts in the control group varied between 10.6-24.9% for the lowest tercile, 25.0-30.4% for the middle tercile and 30.6-46.8% for the highest tercile. For Day 6-8, ranges were 12.5-22.7%, 22.9-35.7% and 36.4-60% for the three terciles, respectively. Data were analyzed as described in the previous paragraph with the inclusion of blastocyst development class (low, middle and high) and interactions with development class.

**Results**

**Blastocyst Development**

A total of 62 embryo culture procedures were performed in which embryos were cultured in the presence or absence of 10 ng/ml CSF2 from Day 5 to 7. There was no significant difference between treatments in the proportion of oocytes (i.e., putative zygotes) that became blastocysts at Day 7 post-insemination (26.9% ± 0.7 for CSF2 vs. 28.3% ± 0.7 for control, P=0.16). However, the effect of CSF2 varied depending on the percent of oocytes that became blastocysts in the control group. In particular, when procedures were ranked according to percent blastocyst development in controls and classified into low, medium and high terciles, there was an interaction between treatment and development class (P<0.0001). CSF2 increased the proportion of oocytes that became blastocysts in IVP replicates where blastocyst development in the control group was in the lowest tercile, had no effect for IVP replicates in the middle tercile and had negative effects on development when IVP replicates were in the highest tercile (Figure 2-1A). Moreover, the correlation between the percent of oocytes that became blastocysts in the control group and the deviation in percent blastocyst caused by CSF2 was -0.53 (P<0.0001).
A total of 36 embryo culture procedures were performed in which embryos were cultured ± 10 ng/ml CSF2 from Day 6 to 8. There was no significant difference between treatments in the percent of oocytes that became blastocysts at Day 8 post-insemination (30.8% ± 1.3 for CSF2 vs. 30.1% ± 1.3 for control, P=0.69) and no interaction between CSF2 treatment and development class. Nonetheless, there was a correlation of -0.37 between the percent of oocytes that became blastocysts in the control group and the deviation in percent blastocyst caused by CSF2 (P=0.026) (Figure 2-1B).

**Effect of CSF2 on Survival of Isolated ICM**

Previously, it was shown that a proportion of ICM isolated from blastocysts could be maintained in a pluripotent state when cultured on mitomycin-C treated fetal fibroblasts in the presence of the GSKB inhibitor CHIR99021 and the MEK1 inhibitor PD0325901 [218]. Here we tested whether exposure of embryos during the 32-cell to blastocyst stages of development to CSF2 could improve the rate at which isolated ICM persisted in a pluripotent state after repeated passage. In the first experiment, embryos were treated from Day 6 to 8 post-insemination with either control, CSF2 or ESM+2i. The lattermost treatment was previously reported to increase competence of ICM to remain pluripotent when used for culture from Day 6 to 8 [218]. The proportion of ICM colonies that survived until the end of passage 1 was greater for ICM derived from blastocysts produced in the presence of CSF2 than ICM from control blastocysts (P=0.002) (Figure 2-2A). In contrast, ICM from blastocysts cultured in ESM+2i had survival that was not different from controls. Of the ICM that were passaged, a higher proportion derived from CSF2-treated blastocysts survived to passage 2 (43% vs 7%; P=0.016) and passage 3 (36% vs 4%; P=0.025) than ICM from control or ESM+2i
treated blastocysts. One colony from a CSF2-treated blastocyst that survived to the end of passage 3 was examined for expression of *NANOG* and *CDX2*. The colony expressed *NANOG* but not *CDX2* (Figure 2-2B).

In the second experiment, treatments were applied from Day 5 to 7 post-insemination. This timing was chosen because exposure of embryos to CSF2 from Day 5 to 7 improves competence to establish and maintain pregnancy after transfer into recipients [18]. The microscopic appearance of ICM that survived passage 1 is shown in Figure 2-3 and the results are summarized in Figure 2-2C. Although not significant, the percent of Day 7 ICM in the control group that survived passage 1 was higher than in the previous study using Day 8 ICM (64 vs 33%, P=0.17). While also not significant, there was a tendency for survival to the end of passage 1 to be numerically greater for ICM from CSF2-treated blastocysts than control blastocysts.

Data on ICM survival were also analyzed across both experiments. The percent of established colonies that survived to the end of passage 1 was greater (P=0.031) for ICM from CSF2 treated embryos than ICM from control embryos and there was no interaction between treatment and timing of treatment (i.e., CSF2 increased survival regardless of whether treatment was from Days 5 to 7 or 6 to 8 post-insemination).

Data on ICM survival were scrutinized to determine whether effectiveness of CSF2 for improving survival of ICM depended on the percent of oocytes that became blastocysts in the control groups. Effects of CSF2 did not depend on development class at either Day 7 or 8 post-insemination (results not shown).

**Expression of Genes Involved in Pluripotency and Differentiation**

To determine whether CSF2 increases survival of ICM because it regulates genes involved in pluripotency and differentiation, we evaluated the effect of CSF2 from
Day 6-8 post-insemination on expression of genes involved in pluripotency (*NANOG* and *SOX2*) and differentiation of TE (*CDX2*) and hypoblast (*GATA6*) in isolated ICM. There was no effect of treatment on steady-state amounts of mRNA for any of the genes analyzed (Figure 2-4). There was also no interaction between CSF2 and development class.

**TE Outgrowth**

When blastocysts are cultured on Matrigel, TE cells spread onto the surrounding substratum and proliferate [220]. Moreover, exposure to appropriate growth factors (FGF2) causes accumulation of hypoblast cells in the outgrowths [222]. Since the trophoblast of Day 15 conceptuses formed following treatment with CSF2 from Day 5-7 post-insemination had higher expression of the maternal signaling gene *IFNT2* and tended to be numerically greater in length than control embryos [30], it was hypothesized that CSF2 would increase the spread of TE cells, reinforce commitment to the TE lineage by enhancing expression of *CDX2*, decrease expression of the hypoblast marker *GATA6*, and increase IFN T2 secretion (as measured by antiviral activity in the medium). Experiments were conducted by culturing embryos with CSF2 or control from Day 5 to 7 or 6 to 8 post-insemination and culturing the resultant blastocysts individually until Day 13 or 15 post-insemination. Results are in Table 2-1.

There was no effect of CSF2 on the percent of blastocysts that attached, the percent of blastocysts or attached blastocysts that experienced TE outgrowth or the percent of cells in the outgrowth that were positive for CDX2. Similarly, there was no effect of CSF2 on secretion of antiviral activity into the culture medium.
The effect of CSF2 on size of outgrowths depended on the timing of treatment. CSF2 decreased the size of the outgrowth when treatment was from Day 5 to 7 post-insemination but had no effect on outgrowth size when treatment was from Day 6 to 8.

Expression of CDX2 was higher for outgrowths of CSF2-treated blastocysts when treatment was from Day 6 to 8 but not when treatment was from Days 5 to 7 (Table 2-1). There was no effect of CSF2 at either time on expression of GATA6 (Table 2-1).

Effects of CSF2 on characteristics of TE outgrowth were not affected by the overall development to the blastocyst stage in controls, i.e., there were no CSF2 x development class interactions at either Day 7 or 8 post-insemination.

Expression of CSF2 Receptor Subunit Genes

Expression of CSF2RA and CSF2RB was examined at the zygote, 2 cell, 3-4 cell, 5-8 cell, 9-16 cell, morula and blastocyst stages of development. CSF2RA was expressed at all stages. Expression for CSF2RA increased between the zygote to the 9-16 cell stage before declining to the blastocyst stage (P<0.05) (Figure 2-5A). In contrast, CSF2RB was not detected in any sample of embryos regardless of stage of development (Figure 2-5B). CSF2RB was expressed in leukocytes, however (Figure 2-5B), indicating the effectiveness of the PCR procedure.

Discussion

Exposure of bovine embryos from the morula to blastocyst stage of development with CSF2 increases survival of the embryo after transfer into a recipient female in cows [18] as well as in mice [26] and humans [25]. These results implicate CSF2 as an important maternal signal controlling embryonic development. Experiments presented here suggest two potential mechanisms by which CSF2 could enhance the competence
of a blastocyst to establish and maintain pregnancy, namely by regulating pluripotency and survival of cells derived from the ICM and alteration of TE function. Similar to the preimplantation mouse, human and pig embryo [186, 187, 189], actions of CSF2 on the bovine embryo are likely mediated by a signaling mechanism that is distinct from that in most cells because the CSF2RB subunit was not detected in any sample of embryos throughout the period culminating in formation of the blastocyst.

Actions of CSF2 to promote survival of isolated ICM in a pluripotent state are consistent with earlier observations indicating CSF2 regulates ICM formation and embryo differentiation status. In particular, CSF2 increased the number of cells in the ICM of the blastocyst [18] and down regulated expression of several genes involved in differentiation in the Day 6 morula [31]. Actions of CSF2 on the ICM are likely to reflect an important mechanism for enhancing embryo survival. Inadequate formation or survival of the ICM appears to be an important cause of pregnancy loss. At Day 14-17 of pregnancy, about 25-33% of conceptuses generated following embryo transfer in cattle are missing the embryonic disk derived from the ICM [18, 225, 226]. In humans, blastocysts with larger ICM were more likely to implant after transfer than blastocysts with smaller ICM [227].

There are several possible mechanisms by which CSF2 could affect the ability of the ICM to survive in culture in a pluripotent state. The ICM is not a homogenous group of cells, especially by Day 8 when specific subpopulations can be identified based on labeling for NANOG and GATA6 [47]. Perhaps CSF2 changes the relative numbers of cell types in the ICM in a way that promotes survival in a pluripotent state. In the absence of CSF2, ICM from Day 7 blastocysts tended to be better able to survive in
culture than ICM from Day 8. The former is less differentiated [47] and it may be that actions of CSF2 to prevent differentiation is responsible for its actions on ICM survival. There was no effect of CSF2 on expression of NANOG or SOX2 in ICM but regulation of other pluripotency or differentiation factors by CSF2 could be involved. Also, CSF2 might improve survival of ICM by preventing apoptosis because CSF2 can inhibit apoptosis and induce expression of anti-apoptotic genes in bovine [31] and mouse [216] embryos. It is also possible that CSF2 induces changes to cells of the ICM that affect their interaction with the underlying fibroblast layer, for example by affecting cell adhesion or cell signaling.

The reason for the failure of the ESM+2i treatment to improve survival of ICM, despite beneficial effects seem earlier [218], is not clear although the low degree of replication may have obscured small improvements in survival.

One implication of the finding that CSF2 promotes survival of ICM in a pluripotent state is that the cytokine might prove useful in generation of embryonic stem cells (ESC) or induced pluripotent stem cells in the cow. There is a need for growth factors that promote pluripotency in the cow because growth factors that are involved in maintenance of ESCs in the human and mouse (FGF2 [228] and LIF [229], respectively) are not effective in the cow [230].

The effects of CSF2 on characteristics of TE outgrowth were small, dependent upon the timing of treatment and did not involve changes in secretion of antiviral activity that represents the trophoblast-derived interferon, IFNT2. CSF2 decreased the size of TE outgrowths when embryos were exposed from Day 5 to 7 but not when embryos were exposed from Day 6 to 8. In addition, CSF2 increased expression of the
transcription factor \textit{CDX2} slightly when embryos were treated from Day 6 to 8 but not when treated from Day 5 to 7. Like the ICM, the TE of the bovine undergoes important changes between Days 7 and 8. The TE is not yet committed to the TE lineage at Day 7 but rather can give rise to ICM when aggregated with cleavage-stage embryos [94]. Perhaps, the TE is more differentiated at Day 8 than Day 7 and one consequence is the action of CSF2 on TE function changes. Consistent with a change in differentiation status of the TE between Day 7 and 8 is the fact that GATA6 is present in both TE and ICM at Day 7 but becomes localized to the ICM by Day 8 [47]. An increase in \textit{CDX2} expression in outgrowths of TE from Day 8 blastocysts caused by CSF2 could reflect actions of \textit{CDX2} to promote TE function. Indeed, embryos treated with CSF2 from Day 5-7 and transferred into cows had higher expression of the trophoblast gene \textit{IFNT2} at Day 15 [30].

There are many reports that CSF2 can increase the proportion of cultured embryos that develop to the blastocyst stage, including observations in the cow [24], human [200], pig [189, 198, 199] and mouse [26]. In other experiments, however, CSF2 either did not affect development [203] or was detrimental for development of embryos to the blastocyst stage [231]. Present results indicate that the effect of CSF2 on development to the blastocyst state depended on the overall competence of embryos for development. In particular, CSF2 increased the percent of embryos becoming blastocysts when the development rate in control blastocysts was low. In contrast, CSF2 decreased development when blastocyst development rates in controls were high. This finding is reminiscent of the first report indicating CSF2 improved blastocyst yield [24]. In that experiment, CSF2 improved the percent of bovine embryos becoming
blastocysts when serum was not present in the medium and development was low but there was no effect of CSF2 when serum was present and development was high. It appears, therefore, that CSF2 enhances developmental competence of embryos with characteristics that limit ability for development while reducing the prospects for an embryo becoming a blastocyst for embryos that have characteristics that allow development to proceed to the blastocyst stage. Perhaps, the anti-apoptotic effects of CSF2 [31] increase the ability of a poor embryo to become a blastocyst. If in fact CSF2 promotes pluripotency rather than differentiation [18, 30], it might delay but not prevent the differentiation of the TE in an embryo with a high developmental capacity. Such an explanation is consistent with the effects of CSF2 from Days 5-7 on TE outgrowth as well as the finding that the negative effects of CSF2 on blastocyst development were lower at Day 8 than Day 7.

As found earlier for the mouse and human [186, 187], the preimplantation bovine embryo expresses only one of the two subunits of the CSF2R. Typically, CSF2 signaling occurs through a cell surface receptor consisting of CSF2RA and CSF2RB subunits [181, 217]. Binding leads to activation of JAK2 followed by phosphorylation of signal transducer and activation of the STAT5 pathway [190, 232]. CSF2RA binds to CSF2 with low affinity ($K_d = 2.7$ nM) while recruitment of CSF2RB causes an increase in affinity to 170 pM [181]. It is possible to achieve signal transduction in the absence of CSF2RB. In *Xenopus* oocytes, CSF2 binding to CSF2RA in the absence of CSF2RB can signal through activation of phosphatidylinositol 3-kinase [183]. Thus, it seems likely that CSF2 signals through a pathway independent of CSF2RB, even though CSF2 can affect the embryo at concentrations as low as 2 ng/ml (i.e., 140 pM) in humans [25].
Nonetheless, the conclusion that CSF2 signals independent of CSF2RB must be tempered by two recent observations. Very low levels of mRNA for \textit{CSF2RB} were detected in pig embryos at the blastocyst stage [189]. In addition, both CSF2RA and CSF2RB could be immunolocalized to bovine oocytes [188] and it is possible that the embryo uses CSF2RB that was inherited from the oocyte. However, immunolabeling for CSFR2B was much lower than for CSFRA [188] and it seems likely that little CSF2RB is present even in the oocyte.

In conclusion, CSF2 acts on the bovine preimplantation embryo to alter the ICM so it is better able to survive and remain pluripotent while cultured on BFF. Accordingly, it is hypothesized that one mechanism by which CSF2 improves competence of blastocysts to establish pregnancy involves regulation of ICM survival and differentiation status. There were also day-of-development specific effects of CSF2 on TE outgrowths, indicating that CSF2 regulates the function of cells of the TE lineage as well. The nature of this regulation and its implications for subsequent development remain to be established. Transcripts for \textit{CSF2RB} were not detected in embryos in the period culminating in blastocyst formation, indicating the likelihood that CSF2 signals only through a CSF2RB-independent mechanism.
Table 2-1. Effects of CSF2 from Day 5 to 7 or 6 to 8 post-insemination on characteristics of TE outgrowths.

<table>
<thead>
<tr>
<th>Analysis at Day 13 post insemination</th>
<th>Day 5 to 7</th>
<th>Day 6 to 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n^b Vehicle</td>
</tr>
<tr>
<td>Blastocysts that attached (%)</td>
<td>13</td>
<td>66.2 ± 5.8</td>
</tr>
<tr>
<td>Blastocysts with outgrowth (%)</td>
<td>13</td>
<td>36.6 ± 5.1</td>
</tr>
<tr>
<td>Attached blastocysts with outgrowth (%)</td>
<td>13</td>
<td>54.3 ± 6.6</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Analysis at Day 15 post insemination</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocysts that attached (%)</td>
<td>13</td>
<td>67.6 ± 4.3</td>
</tr>
<tr>
<td>Blastocysts with outgrowth (%)</td>
<td>13</td>
<td>48.2 ± 5.1</td>
</tr>
<tr>
<td>Attached blastocysts with outgrowth (%)</td>
<td>13</td>
<td>69.2 ± 6.0</td>
</tr>
<tr>
<td>Size of outgrowth (mm^2)</td>
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<td>3.0 ± 0.4^c</td>
</tr>
<tr>
<td>CDX positive cells (%)</td>
<td>3</td>
<td>94.4 ± 1.2</td>
</tr>
<tr>
<td>CDX2 gene expression (fold change)</td>
<td>7</td>
<td>1.0</td>
</tr>
<tr>
<td>GATA6 gene expression (fold change)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>[IFNT] (IU/mm^2)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

^a Data are least-squares means ± SEM
^b n is equal to the number of IVP replicates in which the experiment was performed. For each replicate, 2 to 26 blastocysts were examined for each treatment.
^cd Vehicle vs CSF2, P=0.034
^ef Vehicle vs CSF2, P=0.047
Figure 2-1. The effect of CSF2 on the percent of oocytes that became a blastocyst depends on the overall competence of embryos to develop to the blastocyst stage. Embryos were cultured + CSF2 from either Day 5-7 (panel A) or 6-8 post-insemination (panel B) and the percent of oocytes becoming blastocysts determined. The total number of IVP replicates was 62 for Day 5-7 and 36 for Day 6-8. The IVP replicates were divided into lowest, middle and highest terciles based on the percent of oocytes that became blastocysts in the control group. For treatment at Day 5-7, the effect of CSF2 depended upon tercile (interaction; P<0.0001) with CSF2 increasing the percent of oocytes that became blastocysts when development was low, having no effect when development was in the middle tercile, and having negative effects when development was high. As shown in the inset graph, there was a negative correlation between the percent of control embryos that became blastocysts and the deviation in percent blastocyst due to CSF2 (-0.53, P<0.0001). At Day 6-8, there was no significant effect of CSF2 or CSF2 x development class but there was a negative correlation between the percent of control embryos that became blastocysts and the deviation in percent blastocyst due to CSF2 (-0.37, P=0.026) (see inset graph in B).
Figure 2-2. Treatment with CSF2 increases ability of isolated ICM to survive following passage. Numbers above each bar represent the number of colonies that survived for 7 days after a passage divided by the total number of colonies that were passaged. In the first experiment (Panel A), embryos were cultured from Day 6 to 8 post-insemination with control medium, CSF2 or ESM containing a MAPK and GSK inhibitor (ESM+2i). Survival of isolated ICM cultured on fetal fibroblasts was determined at Day 7 of passages 1, 2 and 3. Expression of NANOG and CDX2 in one ICM colony from CSF2 treated blastocysts that survived to the end of passage 3 is shown as an insert within panel A. In the second experiment, embryos were cultured from Day 5 to 7 after insemination with or without CSF2 (panel B). While not significant, survival to Day 7 of passage 1 was numerically greatest for ICM from CSF2-treated embryos.
Figure 2-3. Representative appearance of colonies of cells derived from ICM at Day 7 passage 1. Colonies were derived from blastocysts that were produced in control medium with control, ESM containing a MAPK and GSK inhibitor (ESM+2i) or medium with CSF2. The scale bar represents 50 μm.
Figure 2-4. Lack of effect of CSF2 on expression of genes related to pluripotency and differentiation in ICM isolated from Day 8 blastocysts. Data for mRNA are represented as the inverse of the ΔCT least-squares means ±SEM of results from 7-9 pools of 3-5 isolated ICM per treatment. Black and white bars represent control and CSF2 treated ICMs, respectively. There was no significant effect of treatment on gene expression.
Figure 2-5. Developmental changes in steady-state mRNA for CSF2RA and CSF2RB. Panel A: Developmental changes in transcript abundance for CSF2RA expressed as fold-change relative to amount for oocytes. Data are the least-squares means ± SEM of results from pools of 25-30 embryos replicated 5 times. Bars with different superscripts differ (P<0.05). Panel B: Representative electrophoretogram of PCR products for CSF2RA (A), CSF2RB (B), GAPDH, and SDHA. Abbreviations are as follows: blast=blastocyst, leuk.=leukocytes, GAP.=GAPDH. Arrows represent the amplicon location of CSF2RA (A) and CSF2RB (B).
CHAPTER 3
SEXUAL DIMORPHISM IN DEVELOPMENTAL PROGRAMMING OF THE BOVINE PREIMPLANTATION EMBRYO CAUSED BY COLONY STIMULATING FACTOR 2

Introduction

Physiological function and health of the adult is first established during prenatal life: alterations in the maternal environment can lead to changes in the adult that include susceptibility to hypertension, diabetes, cardiovascular disease, and various cancers [233-236]. Programming of development occurs as early as the preimplantation period. Feeding pregnant female mice or rats a low protein diet for a limited period corresponding to preimplantation development caused changes in the offspring that persisted into adult life [13-15]. Similarly, postnatal physiology and immune function were altered in sheep derived from mothers fed a diet deficient in cobalt and sulfur for 8 weeks before and 6 days after mating [16]. Furthermore, embryos that develop in vitro give rise to neonates that have increased likelihood for a variety of abnormalities as shown in humans [237, 238], cows [239, 240], sheep [240] and mice [241, 242].

One of the characteristics of developmental programming in the preimplantation period is that female embryos are programmed differently than male embryos [243]. In mice, for example, feeding a low protein diet during the first 3.5 days following conception affected females differently than males. Postnatal growth and body size was affected by protein diet in female offspring but not male offspring [13, 14]. At adulthood, females from mothers fed low-protein diets had altered behavioral scores [13], increased blood pressure [14] and reduced heart to body weight ratio [13] as compared to animals born from control females. These traits were not affected in male offspring but males from low-protein mothers had elevated lung angiotensin-converting enzyme [17]. In rats, feeding a methyl-deficient diet with no folic acid for 3 weeks prior to mating
and 5 days after gestation increased the concentration of insulin in males but not female offspring [169].

Developmental plasticity in response to maternal environment has been proposed as an evolutionary strategy to allow individuals to adapt to transient changes in environment while preserving genotype [236]. Sexual dimorphism in developmental programming may reflect the fact that relative value of female and males for reproductive fitness varies with environment [244].

The mechanism by which a change in maternal environment alters the developmental program of a female embryo differently than of a male embryo is not known. One possibility is that female embryos respond differently to maternally-derived molecules that regulate embryonic development (hereafter termed embryokines) than do male embryos. If so, alteration in secretion of maternal embryokines in response to environmental change could result in a sex-specific deviation in the developmental program. The possibility of differential responsiveness to maternal embryokines is strengthened by the observations that gene expression is different between female and male embryos as early as the blastocyst stage [245, 246].

Colony stimulating factor 2 is a good candidate for an embryokine responsible for sex-specific programming. This cytokine is produced by the oviductal and endometrial epithelium of a wide range of mammals including mice [28], cattle [27] and humans [29]. Treatment of preimplantation embryos with CSF2 leads to an increase in number and pluripotency of cells of the ICM [18, 247, 248], altered gene expression [30] and, increased resistance to apoptosis [31]. That CSF2 is capable of altering the developmental program of the embryo is indicated by findings that bovine [18], mouse
[26], and human [25] embryos treated with CSF2 have increased likelihood to survive to term when transferred into females. Endometrial secretion of CSF2 also changes in response to maternal environment, as indicated by experiments showing reduced expression of CSF2 in oviduct of obese cows [34] and increased secretion of CSF2 after induction of inflammation [33].

Culture of bovine embryos with CSF2 from day 5-7 of development resulted in alterations in characteristics of the embryo at a later time point [18, 30]. In particular, embryos treated with CSF2 tended to be longer and produce more of the anti-luteolytic signal, IFNT, at day 15 of development than control embryos. For the experiment reported here, we used this model of CSF2-mediated developmental programming to explore whether such changes in the trajectory of development depend upon sex. Day 15 is an important point in time for the survival of the bovine embryo. At this time, the extraembryonic membranes (EEM) of the embryo are in the process of extensive elongation so that the embryo increases ~50 fold in length between day 13 and day 16 [35]. Coincident with elongation of the EEM is activation of trophoblast transcription of IFNT, which encodes for a type I interferon that acts on the endometrium to block secretion of prostaglandin F$_{2\alpha}$ and thereby ensure survival of the corpus luteum and continued progesterone secretion [55]. The results presented here indicate that the consequences of exposure to CSF2 from day 5-7 of development are fundamentally different for the female and male embryo at day 15 with respect to embryo elongation, IFNT secretion, and characteristics of the transcriptome and methylome. These results support the contention that sexual dimorphism in response to maternal embryokines is
an important mechanism for establishment of sex differences in developmental programming.

**Materials and Methods**

**Embryo Production**

In vitro production of bovine embryos was performed as previously described [22] unless otherwise noted. Briefly, COC from Holstein females were purchased from either Sexing Technologies (Navasota, TX, USA) or Ovitra (Midway, TX, USA). The COC were shipped overnight at 39°C in a portable incubator while cultured in a proprietary maturation medium. Once arrived, groups of 20-35 matured oocytes were fertilized with Percoll-purified sperm (1.0 x 10^6/ml) from a single Holstein bull for 8 hours at 38.5°C in 1.7 ml of SOF-FERT [221]. Cumulus cells were denuded after fertilization by vortexing in 600 μl HEPES-TALP containing 1,000 U/ml hyaluronidase. Putative zygotes were then cultured in groups of 30 in 45 μl microdrops of SOF-BE1 [22] covered with mineral oil at 38.5°C in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2 with the balance N_2. Cleavage rate was assessed at day 3 post-insemination. On day 5 post-insemination, drops were supplemented with either 5 μl recombinant bovine CSF2 [Novartis, Basle, Switzerland; 100 ng/ml in DPBS and 1% (w/v) BSA]; final concentration of CSF2 after dilution in the drops = 10 ng/ml] or 5 μl of the vehicle used to dilute CSF2 (control treatment). The concentration of CSF2 was chosen based on its effectiveness for increasing embryonic survival after transfer into recipient females [18].

**Embryo Transfer**

Grade 1 expanded blastocysts [249] were harvested from culture drops at day 7 of development and loaded into 0.25 ml straws in 250 μl HEPES-TALP [250] supplemented with 10% (v/v) fetal calf serum and 50 μM dithiothreitol (Sigma-Aldrich).
Each straw contained one embryo and was inserted horizontally into a portable incubator (AI gun warmer, Rusk, TX, USA) at 38.5°C and transported to the Dairy Unit at the University of Florida (Hague, FL, USA).

For each replicate, ovulation was synchronized using a modified OvSynch 56 protocol [251]. Cows received intramuscular injections of 100 μg gonadotrophin releasing hormone, (GnRH) (Cystorelin®, Merial, Duluth, GA, USA) on the morning of day -8 (day 0 = expected day of ovulation), 25 mg prostaglandin F$_{2α}$, (Lutalyse®, Zoetis, Madison, NJ, USA) on the morning and afternoon of day -2 and 100 μg GnRH on the afternoon of Day -1. Cows having a detectable corpus luteum on day 7, as determined by ultrasonography using a Hitachi Aloka 500 ultrasound with 5 MHz linear array transducer (Hitachi Aloka, Tokyo, Japan), were randomly assigned to receive a CSF2-treated or control embryo. The embryo was transferred transcervically to the uterine horn ipsilateral to the ovary containing the corpus luteum using an embryo transfer pipette after application of a local epidural block using 2% (w/v) lidocaine.

**Embryo Recovery**

Embryos were recovered at day 15 of development by flushing the uterine horn ipsilateral to the corpus luteum with DPBS containing 1% (v/v) polyvinyl alcohol (PVA) (DPBS-PVA). Flushing medium was introduced into the uterus by means of a 20 French Foley catheter inserted transcervically using a stainless steel blunt stylet placed inside the catheter. After placement, the stylet was withdrawn and the catheter held in place by inflation of a cuff at the end of the catheter. The flushing procedure, which involved introduction of 60 ml into the uterus and recovery of fluid through the catheter, was repeated four times or until an embryo was recovered.
Embryos recovered by flushing were assessed by light microscopy for stage of development, length, presence of an embryonic disc (ED), and whether the embryo was recovered intact or in pieces. All recovered embryos were at the filamentous stage of development. If found, the ED was dissected from the EEM using a sterile scalpel and tweezers. The EEM was snap frozen in liquid N\textsubscript{2} in a minimal amount of DPBS-PVA and stored at -80°C until extraction of gDNA and total RNA. The fluid from the first uterine flush was centrifuged for 10 min at 1000 x g and stored at -20°C.

The total number of day 15 embryos collected using the procedures described above were 16. An additional 14 day 15 embryos that had been treated with CSF2 or vehicle were used for the analysis of sex and CSF2 effects on embryo length and IFNT accumulation in the uterus. Details of production of these embryos were reported by Loureiro et al. (2009). To determine whether sex effects on length and IFNT in embryos occur earlier in development, we analyzed effect of sex on 10 day 14 control embryos (i.e., not treated with growth factors during culture). Details of the procedures for production of these embryos are reported elsewhere [225].

**IFNT Assay**

Amount of biologically active IFNT in uterine flushings was determined by measuring the inhibition of vesicular stomatitis virus (VSV) induced lysis of Madin-Darby bovine kidney (MDBK) cells as described elsewhere [222]. Antiviral activity was converted to units of IFN (IU/ml) by comparing inhibition to that achieved with a human IFNA standard (Millipore, Billerica, MA, USA).

Data on embryo length and uterine IFNT were analyzed by least-squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS).
version 9.3 (SAS Institute Inc., Cary, NC, USA) with effects of sex, treatment and sex x treatment.

**Extraction of gDNA and Total RNA**

gDNA and total RNA were extracted using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA, USA). Embryos were disrupted with Buffer RLT Plus containing β-mercaptoethanol, vortexed for 30 seconds and homogenized through QIAshredder columns (Qiagen). Homogenized lysate was then separated into gDNA and total RNA following manufacturer’s guidelines. Samples were eluted in 100 µl for gDNA and 60 µl for total RNA. Both gDNA and total RNA were stored at -80ºC until processing. Total RNA quantity, quality and integrity were determined using an Agilent Bioanalyzer 2100 (Santa Clara, CA, USA). All samples had an RNA integrity >7.5. There was an absence of DNA contamination of RNA samples as assessed by the Bioanalyzer. Concentration of gDNA was quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with all samples being >76 ng/µl.

**Sexing by PCR**

Sex for each embryo was determined by PCR with primers specific for the Y chromosome (forward: 5´-GAT CAC TAT ACA TAC ACC ACT-3´, reverse: 5´-GCT ATG CTA ACA CAA ATT CTG-3´) and an autosomal satellite DNA sequence (forward: 5´-TGG AAG CAA AGA ACC CCG CT-3´, reverse: 5´-TCG TCA GAA ACC GCA CAC TG-3´) [252]. Master-mix was composed of 10X PCR buffer, 2.5 mM dNTP, 50 mM MgCl₂, 10 µM of the aforementioned primers and 0.2 µl of Taq DNA polymerase (5 U/µl) (Invitrogen). A total of 2 µl of DNA sample was combined with the 10.2 µl master mix and subjected to PCR for 7 min at 95ºC, 40 cycles of 30 sec at 95ºC, 30 sec at 57 ºC and 30 sec at 72 ºC followed by a final extension at 72ºC for 7 min. Each sample (5 µl)
was analyzed on a 1.5% (w/v) agarose gel with 5 µl ethidium bromide (10 mg/ml) using a 100 bp ladder (New England Biolabs, Ipswich, MA, USA). A male control consisted of fibroblast or sperm DNA and a female control consisted of DNA extracted from an ovary.

**Overview of Analysis of Transcriptome**

Total RNA for each sample was extracted, amplified and labeled before proceeding to analysis of expression by microarray. The Agilent microarray was specifically designed by EmbryoGene with probes specific for bovine embryo development. Microarray data were analyzed and 6 DEG were verified by qPCR. Raw and normalized data are available on the gene expression omnibus website using the accession number GSE55364.

**RNA Amplification**

Total RNA from 3 female control embryos, 3 female CSF2-treated embryos, 2 male control embryos and 4 male CSF2-treated embryos were subjected to T7 RNA amplification using the RiboAmp HSPlus RNA amplification kit as described by the manufacturer (Life Technologies, Carlsbad, CA, USA). A total of 5 ng RNA was reversed transcribed using an mRNA-specific first primer and first strand synthesis. Single-stranded cDNA was made into double-stranded cDNA using second strand synthesis with an exogenous second primer. aRNA was transcribed in vitro from the purified cDNA second strand synthesis followed by aRNA purification. Round one purified aRNA was then re-converted to double-stranded cDNA, purified and subjected to a second round of in vitro transcription. The aRNA was then purified using PicoPure RNA isolation kit (Life Technologies), quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific) and stored at -80ºC until labeling.
RNA labeling

The aRNA was labeled using the Universal Linkage System (ULS) (Kreatech Biotechnology, Apeldoorn Gelderland, Netherlands) as described by the manufacturer with either ULS-Cy3 or ULS-Cy5. Samples were then incubated at 85°C for 30 min followed by incubation on ice for 3 min. aRNA was briefly centrifuged at 16,000 x g and purified using PicoPure RNA isolation kit (Life Technologies) and eluted in 11 µl elution buffer. Yield and labeling efficiency was determined using the Nanodrop 1000 spectrophotometer.

RNA microarray hybridization and scanning

Microarray analysis of labeled aRNA was performed using an Agilent 4x44,000 slide microarray (Agilent, Santa Clara, CA, USA) designed by EmbryoGENE to over represent genes expressed by the bovine preimplantation embryo. The probes for this array were chosen from one-million high-quality reads from Roche 454 Titanium deep sequencing datasets from in vitro and in vivo produced bovine embryos from the germinal vesicle stage, 2-cell, 4-cell, 8-cell, 16-cell, morula, blastocyst and hatched blastocyst stage [253]. The array contains 42,242 probes which represent 21,139 known reference genes, 9,322 probes for novel transcribed regions, 3,677 alternatively spliced exons, 3,353 3’-tiling probes and 3,723 controls [253]. For hybridization, 825 ng of one Cy3 labeled aRNA sample and 825 ng of one Cy5 labeled aRNA sample were combined with 2.75 µl of 0.01X Agilent spike, 11 µl of 10X blocking agent and 2.2 µl of 25X fragmentation buffer (all from Agilent) and brought to 55 µl with nuclease-free water. One sample that was CSF2 treated (either Cy3 or Cy5 labeled) was always combined with a sample that was a control (either Cy5 or Cy3 labeled) and of the same sex. The mixture was heated at 60°C for 15 min to fragment the RNA, placed on ice,
allowed to cool for 1 min and then mixed with an equal volume of 2X GEx hybridization buffer HI-RPM (Agilent) to stop RNA fragmentation. The solution was centrifuged for 1 min at 16,000 x g and placed on ice.

For each array, 100 μl aRNA solution was added into one of the four chambers. Hybridization proceeded for 17 hours at 65°C while rotating at 10 rpm. Following hybridization, array slides were washed as described by the manufacturer, and scanned with a Tecan PowerScanner microarray scanner (Tecan Group Ltd., Männedorf, Switzerland).

Analysis of transcriptome microarray data

Microarray images were submitted to BioDiscovery Inc. (Hawthrone, CA, USA) to generate separate datasets for each color (Cy3 and Cy5). In addition, spots were aligned to the corresponding GenePix array list, which describes the size, position and label of each spot on the array, and examined for removal if spots on the array that were larger than the spot or contained a blemish. The mean intensity for each spot was corrected by subtracting the mean intensity of background values for each sample to remove background noise. Mean intensity was then transformed by log2 and analyzed by using JMP Genomics (SAS, Cary, NC, USA). Data were normalized with loess normalization for dye-dye correction followed by quantile normalization for between-array correction. Genes that were differentially expressed were determined by analysis of variance with a model that included effects of treatment, sex and treatment x sex. P-values for significant genes were then adjusted for multiple testing by SGofF+ estimation, as described by Carvajal-Rodriguez and Uña-Alvarez [254]. All genes that had an adjusted P-value of <0.05 and where the difference in expression was ≥ 1.5 or ≤ 0.67 were considered differentially expressed.
Ingenuity pathway analysis software (Ingenuity Systems, Redwood City, CA, USA) was used to determine gene ontologies and pathways and to identify pathways with predicted gene activation or inhibition. Canonical pathways were considered significant when the –log of the p-value for each pathway was greater than 1.30. Only pathways with 3 or more differently expressed genes were used for analysis. DAVID was used to determine gene ontologies and functions in which differentially expressed genes were overrepresented at P< 0.05 [255]. iPath 2.0 was used to identify KEGG metabolic pathways that contained overrepresentation of differentially expressed genes [256].

Validation of transcriptome microarray by PCR

Validation of microarray gene expression was accomplished by quantitative reverse transcription PCR of 12 samples of embryo RNA. Samples were treated with 1 µl (2 U) of DNase I enzyme (New England Biolabs, Ipswich, MA, USA) in a total volume of 20 µl volume at 37°C for 30 min to remove DNA, and then incubated at 75°C for 15 min to denature DNase. DNase-treated RNA was then reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Reverse transcription occurred using random hexamer primers and involved incubation at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was stored at -20°C until further use. Negative controls for real-time PCR were also performed by incubation without reverse transcriptase.

cDNA was utilized for real-time PCR analysis. For detection of transcript levels, a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA) utilizing SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) was employed. Each reaction contained 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), 10 µl EvaGreen
Supermix (Bio-Rad), 6.8 µl H₂O and 1.2 µl cDNA sample. Amplification conditions were 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 60°C for 5 sec, and 1 cycle of melt curve analysis at 65-95°C in increments of 0.5°C every 2 sec. The ΔCₜ value was determined by subtracting the Cₜ value of the sample by the geometric mean [224] of the Cₜ for three housekeeping genes, GAPDH, SDHA and YWHAZ, which were utilized because expression is stable over preimplantation development [31, 257]. Primer sequences are in Table 3-1. The primers for XIST [258], GAPDH²⁸, SDHA⁵⁸ and YWHAZ⁵⁸ were described elsewhere and other primers were designed using PrimerQuest (IDT DNA, Coralville, IA, USA). The accuracy of results from microarray analysis was determined by calculating the Pearson’s correlation coefficient between the ΔCₜ value as determined by PCR with the normalized intensity as determined by microarray analysis.

**Overview of Analysis of Methylome**

To analyze the methylome, gDNA was extracted, digested by MseI and digested with methylation-sensitive enzymes HpaII, HinpII and AciI. Products were then amplified, labeled, and then labeled gDNA was subjected to microarray analysis using an Agilent array designed by EmbryoGene. Raw and normalized data are available on the gene expression omnibus website using the accession number GSE55873.

**Fragmentation and amplification of gDNA**

The use of restriction enzymes that are sensitive to 5-methylcytosine was employed. These restriction enzymes, HpaII, HinpII and AciI, digest when the CpG dinucleotide is not methylated but leave DNA intact if the CpG is methylated. Concentrated gDNA from 3 female control embryos, 3 female CSF2-treated embryos, 2 male control embryos and 2 male CSF2-treated embryos were diluted to 10 ng in 15 µl
and spiked with a 1.5 µl mix of specific DNA sequences sensitive to the restriction enzymes to serve as a control for subsequent qPCR. gDNA was digested with 0.5 µl (5 U) Msel in a total volume of 50 µl for 16 hours at 37ºC followed by inactivation for 20 min at 65ºC. Msel-digested gDNA was precipitated using 1 µl of 3 M sodium acetate and linear acrylamide (Life Technologies). The gDNA pellet was reconstituted in 5 µl H₂O. Msel designed linkers were ligated to the 5’ and 3’ end of the digested gDNA using 1.0 µl (5 U) T4 DNA ligase and 10 mM ATP at 15ºC for 16 h. Linker-ligated gDNA was digested by adding 0.5 µl (5 U) HpaII and 0.5 µl (5 U) HinpII at 37 ºC for 12 h. After 12 hours of incubation, an additional restriction enzyme, 0.5 µl (5 U) of Acil, was added to each sample and incubated at 37 ºC for 4 hours followed by heat inactivation at 80ºC for 10 min. Digestion efficiency was verified using qPCR on a Roche lightcycler 2.0 with HpaII, Acil and HinpII specific primers. A difference of a least 5 cycle thresholds was required to proceed to DNA precipitation. If the results were below 5 threshold cycles, the gDNA was subjected to one more round of enzyme digestion with the aforementioned enzymes for 4 hours at 37 ºC.

Digested DNA was precipitated as previously mentioned and amplified by ligation-mediated PCR. Ligation-mediated PCR was accomplished using Msel primers that bind to the Msel ligated linkers. A second PCR was performed on the amplified digested gDNA using only the 3’ Msel primer followed by PCR purification using PCR purification kit (Qiagen). DNA was eluted in 43.5 µl of elution buffer. Msel linkers were removed using 0.5 µl Msel (10 U/µl) at 37ºC for 16 hours followed by heat inactivation at 65ºC for 20 min. gDNA was then re-purified using PCR purification kit (Qiagen) and eluted in 43.5 µl.
DNA labeling

Digested gDNA was labeled as described for the procedure to label RNA. gDNA was purified using QIAquick PCR purification kit (Qiagen) and eluted in 23.5 µl elution buffer. Yield and labeling efficiency of gDNA was determined using the Nanodrop 1000 spectrophotometer. The degree of labeling of gDNA with Cy3 was between 1.75% and 3.5% while labeling with Cy5 was between 0.75% and 2.5%.

DNA microarray hybridization and scanning

The degree of methylation was determined by measuring hybridization of restriction-digested, amplified, and labeled DNA to probes for DMR in the bovine genome using an Agilent 2 x 444,000 slide designed by EmbryoGENE. The microarray probes were designed based on a methylation-specific deep sequencing analysis of the bovine genome which generated 1.2 million reads from genomic libraries from day 7 blastocysts and day 12 elongated embryos [259]. The microarray containing 414,566 probes surveying 20,355 genes as well as 34,379 CpG islands. For each slide, 1.0 µg of Cy3 labeled DNA from one sample (either CSF2 or control treated embryo for the same sex) and 1.0 µg of Cy5 DNA from a second sample (either control of CSF2 treated embryo for the same sex) were combined with 25 µl of bovine Hybloc DNA (1 mg/ml) (Applied Genetics, Melbourne, FL, USA), 130 µl of 2X Hi-RPM (Agilent) and 100X blocking agent (Agilent); the final volume was brought to 195 µl with nuclease-free water. This solution was heated for 3 min at 95°C and 30 min at 37°C. Samples were then centrifuged at 10,000 x g and mixed with 65 µl Agilent-CGH Block solution (Agilent). A total of 245 µl of each sample was placed into the slide and hybridized at 65°C while rotated at 10 rpm for 40 h. Following incubation, slides were washed according to manufacturer’s procedure and scanned as described previously.
Analysis of methylome array

DNA microarrays were analyzed using the limma package of Bioconductor [260]. Raw data were normalized using within-array loess normalization followed by between-array quantile normalization. Normalized data were then fit to a linear model before being subjected to an empirical Bayes correction. Mean intensity of each spot on the array was transformed by log2 while p-values were determined using a moderated t-statistic [260]. Regions with an estimated absolute fold-change above 1.5 or below 0.67 and a p-value < 0.05 were considered as differentially methylated. Development of visualization tools was designed to analyze the quality of both overall genomic and spiked-in gDNA control digestion. DMR were categorized based on CpG island density [261], length [262] and distance [263], location of the DMR in the genome and types of repeated elements [264].

Regions of each chromosome that had a frequency of DMR caused by CSF2 greater than expected were determined by chi-square analysis. For each chromosome, 5 Mbp segments of the genome were examined for observed number of differentially expressed probes. The expected frequency for chi-square analysis was calculated as the number of probes in the 5 Mbp segment times the overall percent of probes that were differentially methylated. The analysis was performed using sliding windows of 5 Mbp that shifted by 500 Kbp at a time until reaching the end of the chromosome. Analysis was performed separately for female and male embryos.

Results

Embryo Length and IFNT Accumulation in the Uterus

Sex of each embryo was determined by PCR (Figure 3-1A). Embryo length and amount of IFNT in uterine flushings at day 15 were affected by a treatment x sex
interaction (Figure 3-1B, P<0.05). For embryos not treated with CSF2, female embryos were longer than male embryos and there was greater accumulation of IFNT in the uterus than for male embryos. In females, CSF2 decreased embryo size and IFNT accumulation while, in males, CSF2 increased embryo size and IFNT accumulation. As a result, male embryos treated with CSF2 were larger and secreted more IFNT than female embryos treated with CSF2.

Another set of embryos collected at day 14 of gestation were examined for sex differences. In this case, involving embryos not treated with growth factors, there was no difference in length between female and male embryos (19.4 mm ±11.1 and 23.6 mm ± 11.1 respectively, n=5 for each sex). Thus, it is likely that differences in length between sexes in control embryos at day 15 represent earlier onset of elongation in female embryos rather than an inherently-larger size in females.

**Differentially Expressed Genes**

A Venn diagram illustrating numbers of DEG is presented in Figure 3-2. Among control embryos, a total of 514 genes differed in expression between female and male embryos. This represents 1.5% of the probes on the array. Using DAVID (Database for Annotation, Visualization and Integrated Discovery), DEG genes were overrepresented in 38 gene ontology terms (P<0.05), with terms related to mitochondrial function, protein ubiquitination, electron transport and translation being prominent. KEGG pathway functional annotation terms that were overrepresented in the set of DEG were those for oxidative phosphorylation (Figure 3-3, P<0.02) and ribosome (P<0.05). Using IPA software, three canonical pathways having a minimum of three DEG were identified: EIF2 signaling (P<0.0002), mitochondrial dysfunction (P<0.0003) and fatty acid β-
oxidation 1 (Figure 3-4A, P<0.007). This finding was reiterated when the list of DEG was analyzed by iPATH 2.0 [256].

The biofunction analysis feature of IPA was used to predict cellular processes that would experience a change in activation as a result of changes in gene expression between female and male embryos. It predicted that synthesis of protein (z-score: -1.723), translation (z-score: -1.673) and expression of protein (z-score: -1.230) would be lower for female embryos than male embryos (Figure 3-4B-D).

CSF2 caused differential expression of 54 genes in females and 93 genes in males. A large proportion of genes regulated by CSF2 in both females and males were among the set of genes whose expression differed between male and female control embryos. In particular, 31 of 54 DEG genes in females (57%) and 48 of 93 DEG in males (52%) were also regulated by sex in control embryos. Only 7 DEG were regulated by CSF2 in both females and males and regulation was always in opposite directions. For example, expression of HSP90B1 was increased by CSF2 in females and decreased in males (Figure 3-2).

Among the genes regulated by CSF2 in females, only 25 of 54 were annotated. These genes were overrepresented in one DAVID gene ontology, generation of precursor metabolites and energy (P<0.05), one KEGG pathway, oxidative phosphorylation (P<0.01). There were no significant IPA canonical pathways or cellular processes identified by the biofunction analysis feature of IPA. In males, only 60 of 93 were annotated. There were no significant DAVID gene ontology, KEGG pathways or IPA canonical pathways containing these DEG. However, the biofunction analysis
feature of IPA indicated CSF2 increased metabolism of protein and decreased invasion of cells in male embryos (Figure 3-5).

The accuracy of microarray results was evaluated by determining the correlation between intensity of hybridization signal in microarray analysis with the $\Delta C_T$ value from qPCR for nine genes significantly affected by either CSF2 within sex or between sexes with control treated samples (Table 3-2). For 7 of 9 genes, an increase in hybridization signal was negatively correlated with $\Delta C_T$ (i.e., positively correlated with amount of mRNA). The correlation was significant for 6 of 9 genes.

**Methylome**

A Venn diagram illustrating numbers of DMR is presented in Figure 3-6.

For control embryos, there were 31,706 DMR between females and males. This represents 7.6% of the probes on the array. Of these DMR, 20,658 (65%) were in open sea (>4 Kbp from CpG island), 7,824 (24.6%) were in shelf (2-4 Kbp from CpG island), 2,463 (7.7%) in shore (1-2 Kbp from CpG island) and 731 (2.3%) were located in CpG islands. A total of 30 DMR were not classified.

Treatment with CSF2 from day 5-7 caused hypermethylation at day 15 of 6,227 probes in females and 9,842 probes in males while causing hypomethylation of 3,292 probes in females and 9,322 probes in males (Figure 3-5). In the female, 6,102 (64%) of these DMR were located in the open sea, 2,452 (25.7%) in the shelf, 787 (8.2%) in the shore and 181 (1.9%) within a CpG island, while 2 DMR were not classified. In the male, 11,931 (62%) DMR were located in the open sea, 4,790 (25%) in the shelf, 1,641 (8.5%) in the shore and 792 (4.1%) within a CpG island, while 10 DMR were not classified.
Of the 9519 DMR regulated by CSF2 in females, 2766 (29.1%) were DMR whose methylation status in controls differed between females and males. Similarly, of the 19164 DMR regulated by CSF2 in males, 6597 (34.4%) were regions that were differentially methylated between females and males in control embryos. Thus, a greater percentage of methylation sites regulated by CSF2 are also modified by sex than is the case for the entire genome (7.6%). A total of 1,186 DMR were affected by CSF2 in both females and males. Of these, 773 (65.2%) were regulated in the opposite direction (hypermethylation in one sex and hypomethylation in the other sex).

Chi-square analysis was used to identify whether 1) spatial distribution of DMR on regions of each chromosome was non-random (as would be expected if DMR were under regulation) and 2) whether the chromosomal regions that were differentially methylated by CSF2 in females were distinct from those regions in males. For each chromosome, there were regions that contained hyper- and/or hypomethylation that was more frequent than would be expected due to chance if DMR were randomly distributed across the chromosome. Moreover, the regions of differential methylation caused by CSF2 were usually different for females than for males, as is illustrated for BTA 1 in Figure 3-7.

**Discussion**

It is well known that alterations in the maternal environment during prenatal life can have long-term effects on the physiology and health of the offspring and that the nature of these effects differs between females and males [14-16, 169]. From an evolutionary perspective, sexual dimorphism in developmental programming probably reflects the differential value of males and females for sexual propagation [244]. While the phenomenon has been well described, the mechanism by which sexual dimorphism
in developmental programming is achieved has hitherto been unknown. Current findings lead to the hypothesis that sexual dimorphism results because female and male embryos respond differently to environment-driven alterations in secretion of maternally derived embryokines. In this model, shown schematically in Figure 3-8, the reproductive tract uses altered secretion of embryokines as a signal transducer to transmit information about the maternal environment to the embryo. For certain signals, as shown here for CSF2, male embryos respond differently than do female embryos so that the deviation in the developmental trajectory in response to environmental change varies between sexes.

The evidence for this idea comes from the present experiment that exposure of bovine preimplantation embryos to CSF2 for a two-day period from day 5-7 of development, results in changes in embryonic growth, gene expression and DNA methylation in the EEM of embryos at a later time point in pregnancy, day 15 of development. It is at this stage of pregnancy that the bovine embryo first signals its presence to the mother through the secretion of the antiluteolytic molecule, IFNT [55]. Secretion of IFNT is coupled to a process of embryo elongation where the embryo undergoes a rapid increase in length from about 2-5 mm in length on day 14 to about 150 mm in length on day 16 [265]. Elongation is a prerequisite for the upregulation of IFNT secretion in the ruminant [56].

Present results suggest that, in the absence of prior exposure to CSF2, the female embryo is larger and secretes more IFNT into the uterus than the male embryo. The larger size of female embryos probably reflects earlier onset of elongation rather than an inherently larger size because there was no difference in embryo length.
between female and male embryos at day 14, around the time when elongation is being initiated. Similar lack of difference between female and male embryos in length were observed for in vitro produced embryos [266] and, in one report, for embryos produced in vivo [267]. In another report, female in vivo embryos tended to be larger than male embryos at day 14, although not significantly different [266]. Moreover, IFNT secretion in vitro was greater for female embryos produced in vivo than male embryos at day 14 [267]. By day 16, male embryos have been reported to be longer than female embryos, with differences greater for embryos produced in vivo than for those produced in vitro and subsequently transferred to recipients.

Despite its larger size, analysis of the transcriptome reveals that the female embryo is less active with respect to oxidative phosphorylation, with NDUFA11, NDUFS7, UQCRC1, COX6A1 and ATP6V1B2 being downregulated in females and only NDUFB1 being upregulated. Gene expression in female embryos also indicated decreased translation and metabolism. The decreased metabolism of female embryos could reflect the earlier onset of elongation and reduced metabolism as the rate of growth decreases at the end of the elongation period. However, female embryos have less mtDNA copies as early as the blastocyst stage [245, 268] and may simply be less metabolically active than males.

Exposure to CSF2 from day 5-7 reverses differences between female and male embryos by decreasing length and IFNT release in female embryos while increasing it in males. Concomitantly, CSF2 programmed gene expression of female embryos differently than for male embryos. In most cases, genes whose expression at day 15 was altered by prior exposure to CSF2 from day 5-7 were different for female embryos
than for male embryos. In the few cases where the same gene was altered in expression by CSF2 in females and males, regulation was in opposite directions. A similar phenomenon was observed when examining characteristics of the methylome in that DMR tended to be different for females and males and when the same, were most often regulated in opposite directions.

CSF2 is a good candidate for transmitting information about the maternal environment to the embryo. Produced by the luminal and glandular epithelium of the endometrium [27], expression of CSF2 is sensitive to perturbation in response to the maternal environment. In particular, expression of CSF2 in the bovine oviduct was reduced in obese cows [34] while accumulation of CSF2 or steady-stage amounts of CSF2 mRNA in the endometrium was increased by deposition of semen in human, pig and mice [33, 193, 269]. One component of semen responsible for increased expression of CSF2 in the mouse is Tgfb [33].

Other embryokines exist that could potentially be involved in developmental programming. Among the embryokines in the cow are IGF1 [270], TGFB [20], LIF [20] and FGF2 [22]. While each of these molecules can affect embryonic development, it is not known whether their actions on the embryo also depend upon sex.

It is also not clear how the sex-dependent changes in embryo function seen here affect the survival of the embryo or the physiology of the resultant neonate. In female embryos, treatment with CSF2 from day 5-7 increases the proportion of embryos that establish and maintain pregnancy after transfer to recipient females [18]. Thus, the reduction in embryo length and IFNT secretion at day 15 is not inimical to embryo survival. Among the genes upregulated by CSF2 in females were several candidates...
related to oxidative phosphorylation and it may be that increased capacity of the EEM to metabolize energy substrates contributes to the embryo’s survival. There is no comparable information about the effects of CSF2 on subsequent survival of male embryos. CSF2 altered gene expression in male embryos in a manner suggesting decreased metabolism of protein and increased invasion of cells in male embryos and these actions could potentially limit embryo survival.

There is also no information regarding the consequences of CSF2 treatment from day 5-7 on characteristics of the resultant calves. Data that CSF2 causes sex-specific changes in the methylome suggest the possibility of long-term changes that could extend into fetal or postnatal life.

An important unresolved question is the molecular basis for the differential response of female and male embryos to CSF2. One facet of this response may involve the nature of genes regulated by CSF2, which include an overrepresentation of genes and CpGs that are also regulated by sex. Over 50% of the DEG and 35% of the DMR regulated by CSF2 were among the small pool of genes and DMR that were regulated by sex. One possibility is that CSF2 regulates transcription factors and other signaling systems responsible for sex-regulated differences in gene expression. Alternatively, a large fraction of genes regulated by sex have transcription factors or regulatory elements also regulated by CSF2.

In conclusion, we present data to indicate a mechanism for sexual dimorphism in developmental programming. In particular, treatment of preimplantation embryos with the maternally-derived cytokine, CSF2, from Day 5-7 of development changes subsequent development so that, at a later stage of pregnancy, Day 15, embryonic
development is altered with respect to length, secretion of the pregnancy signal, IFNT, the transcriptome, and the methylome. Alterations in these characteristics caused by CSF2 were different between female and male embryos, with effects being often in the opposite direction. We conclude that alterations in secretion of maternal regulatory factors caused by change in maternal environment could modify the developmental program of the female embryo differently than for the male embryo.
Table 3-1. List of primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Forward</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>HSP90B1</td>
<td>NM_174700.2</td>
<td>5'-CAGGAACAGACGAGGAAGAAGA-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CTCTCCACACAGCATCCAAA-3'</td>
<td></td>
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<tr>
<td>PAGE4</td>
<td>NM_001081580.2</td>
<td>5'-CATGGAAACCTGGACAAGAGAAG-3'</td>
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<tr>
<td></td>
<td></td>
<td>5'-CAAGCTCACCTCAACCTTT-3'</td>
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<tr>
<td>NDUFB1</td>
<td>NM_175809.2</td>
<td>5'-TTGTTCCGGAAGGAGTTAG-3'</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>5'-TTGACTTGCTCCTATGGGAATTTG-3'</td>
<td></td>
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<tr>
<td>PAG2</td>
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<td>5'-CCCAAGGATCCAGTTTAGAG-3'</td>
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<tr>
<td>EIF3C</td>
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<td>5'-CATACCGGTGTCGGAAGAAGAAG-3'</td>
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<td>5'-GGTTATACACTCCAGCAGAC-3'</td>
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<tr>
<td>XIST</td>
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<td>NM_174178</td>
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Table 3-2. Validation of microarray results by determination of the correlation between fluorescent intensity on the array with C\textsubscript{T} value in qPCR.

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<tr>
<th>Gene</th>
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<th>P</th>
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<td>&lt;0.05</td>
</tr>
<tr>
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<td>&lt;0.05</td>
</tr>
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<td>PAG2</td>
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</tr>
<tr>
<td>EIF3C</td>
<td>-0.47</td>
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</tr>
<tr>
<td>PEX10</td>
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<td>NS</td>
</tr>
<tr>
<td>XIST</td>
<td>-0.89</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PCMTD1</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td>AMACR</td>
<td>-0.73</td>
<td>&lt;0.05</td>
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</table>
Figure 3-1. Sex by CSF2 interaction affecting length of the embryo and accumulation of IFNT in uterine flushings at day 15 of pregnancy. Panel A depicts a representative electrophoretogram of results of the PCR procedure used to sex embryos. Shown are results for three embryos as well as control male and female DNA. The band located at 216 bp represents an amplicon from an autosomal satellite DNA sequence while the band at 141 bp represents an amplicon from the Y-chromosome. Interactions between CSF2 treatment from day 5-7 of development and sex on embryo length and concentration of IFNT in uterine flushing are shown in Panel B. Data are the least-squares means ± SEM of results from 7-8 males and 6-8 females per treatment. The treatment x sex interaction was significant (P<0.05) for both endpoints.
Figure 3-2. Venn diagram illustrating differentially-expressed transcripts at day 15 of pregnancy in male and female embryos treated with CSF2 or control medium from day 5-7 of development. Gene symbols represent differentially expressed genes that were annotated. Genes displayed in red increased in expression (female > male or CSF2 > control) while genes displayed in green decreased in expression.
Figure 3-3. Diagram illustrating genes encoding for proteins in the electron transport chain that were differentially expressed between control female and male embryos at day 15 of pregnancy. Genes upregulated in females are in red and genes upregulated in males are in green.
Figure 3-4. Biological processes predicted to vary with sex in control embryos. Panel A shows the canonical pathways containing an overrepresented number of differentially expressed genes. Only pathways with 3 or more genes are shown. Gene symbols represent differentially expressed genes for each pathway; genes in red were upregulated in females and genes in green are upregulated in males. Panels B-D display analyses of predicted differences in cellular function between female and male embryos. Genes in red were upregulated in females while genes in green were upregulated in males (we need to change this). Blue lines represent genes in which the observed change in gene expression are predicted to inhibit the biological function, yellow lines represent genes where the effect of differential expression is inconsistent with the predicted stage of the process and grey lines represent relationships that are not predicted. Biological processes in blue are predicted to be inhibited. Analysis indicates that female embryos were likely to be less active in expression of protein, translation, and translation of protein (panel B), metabolism of protein (panel C) and transport of lipid (panel D) than male embryos.
Figure 3-5. Predicted changes in cellular function in male embryos at day 15 of pregnancy caused by CSF2 treatment between day 5-7 of development. Genes labeled in red were upregulated by CSF2 while genes labeled in green were downregulated by CSF2. Orange lines represent genes where the change in expression is predicted to activate the biological function while blue lines represent genes in which the observed change in gene expression is predicted to inhibit the biological function. Yellow lines represent genes where the effect of differential expression is inconsistent with the predicted state of the process and the gray line represents a relationship that is not predicted. Biological processes in blue are predicted to be inhibited. Analysis indicates that embryos treated with CSF2 were likely to experience decreased metabolism of protein and increased invasion of cells.
Figure 3-6. Venn diagram illustrating differentially-methylated probes at day 15 of pregnancy in male and female embryos treated with CSF2 or control medium from day 5-7 of development.
Figure 3-7. Non-random clustering of differentially methylated probes – example from bovine chromosome 1. The probability that the actual number of differentially-methylated probes (DMR) in a 5 Mbp segment was greater than due to chance was determined by chi-square analysis after calculating the expected frequency of DMR if location of DMR was randomly distributed throughout the genome. The analysis was performed using sliding windows of 5 Mbp that shifted by 500 Kbp at a time until reaching the end of the chromosome. The Figure shows the chi-square value for 5 Mbp regions where the number of DMR was greater than expected due to chance. Bars of orange and red represent regions where for female embryos the number of hyper- and hypomethylated DMRs was greater. Blue and green bars represent regions for male embryos where the number of hyper- and hypomethylated DMRs was greater than expected due to chance.
Figure 3-8. Model illustrating a mechanism for achievement of sex-specific developmental programming in response to a change in maternal environment. In this model, a change in maternal environment alters secretion of uterine embryokines such as CSF2. The resultant alteration in the trajectory of development is different for female embryos than male embryos so that subsequent characteristics of the embryo and neonate are modified differently for each sex. In the particular example of CSF2, exposure to CSF2 from days 5-7 of development causes different and often opposite actions on development reflected in characteristics of the embryo at day 15 of development.
CHAPTER 4
DYNAMICS OF DNA METHYLATION DURING EARLY DEVELOPMENT OF THE PREIMPLANTATION BOVINE EMBRYO

Introduction

Following fertilization, the embryo remains in a state of transcriptional quiescence that is maintained until a species-specific stage (8-16 cell stage in the cow, 2-cell stage in the mouse, 4-cell stage in the pig and 4-8 cell stage in the human) when transcription is resumed through a process referred to as EGA [271]. In the mouse, activation of transcription is preceded by a decrease in global methylation of DNA as a result of active and passive demethylation that begins at the zygote stage and persists through the morula stage [73, 74]. Demethylation is followed by a wave of DNA methylation beginning at the blastocyst stage [84] that is mediated by de novo methyltransferases Dnmt3A and Dnmt3b [82, 84]. Overall levels of methylation in the blastocyst are greater for ICM than TE [84].

Epigenetic remodeling is important for EGA because mouse embryos in which the chromatin remodeling gene, Brg1, was knocked out exhibited arrest at the 2-cell stage and decreased transcription [272]. Moreover, injection of siRNA for the H3K27me3 demethyltransferase JMJ3 decreased competence of bovine embryos to develop to the blastocyst stage [273]. Nonetheless, developmental patterns of de novo methylation in early development are not conserved across mammalian species. The demethylation occurring during early cleavage-stages in the mouse also occurs in sheep [85] but not in the pig [86] or rabbit [274]. By the blastocyst stage, the ICM is more methylated than the TE in the sheep [85] and pig [86] while, in the rabbit, the ICM is less methylated than TE [274]. De novo methylation is not as clearly understood in other species. In the sheep, overall DNA methylation declines from the two-cell stage...
until the blastocyst stage and the ICM is more methylated than the TE [85]. The ICM is also more methylated than the TE in the pig blastocyst, but unlike the mouse and sheep, there is no apparent loss of DNA methylation from the two-cell to morula stages of development [86].

Results are unclear in the bovine embryo, with one report indicating widespread demethylation occurs through the 8-cell stage before methylation increases at the 16-cell stage of development [87] while another report indicates that demethylation persists through the morula stage [88]. The difference in methylation between the ICM and TE of the bovine blastocyst is also unclear, with one report indicating higher methylation in the ICM [87] and another indicating higher methylation in TE [88].

Developmental changes in the embryonic methylome are also likely to be modified by genetics of the embryo and the environment in which it resides. Genetic sex can have profound effects on development of the embryo as early as the blastocyst stage when, for example, total transcript abundance in cattle is higher in female blastocysts than male counterparts [245]. Expression of de novo methyltransferases is also differentially expressed between the two sexes, with female blastocysts in the cow having lower expression of DNMT3A and DNMT3B compared to male blastocysts [268]. Signals derived from the mother can also affect embryonic development in a way that improves competence for survival after transfer into females, as has been demonstrated in the cow for CSF2 [18], insulin-like growth factor-1 [129] and hyaluronan [21]. The molecular basis for improvement in competence for long-term development is not known but could include remodeling of the epigenome. Indirect evidence for epigenetic programming during early development is the observation that treatment of embryos
with CSF2 from Day 5-7 of pregnancy improves late embryonic and fetal survival much later in pregnancy (after Day 30-35 of gestation) [18].

In this study, we characterized dynamics of DNA methylation during early development in the cow and determined whether degree of methylation depends upon cell lineage (ICM vs TE), sex, and exposure to CSF2. In addition, developmental changes in expression and methylation of *DNMT3B* were assessed to determine whether developmental changes in DNA methylation could conceivably be due to differences in activity of this methylase.

**Experimental Procedures**

**Embryo Production**

*In vitro* production of bovine embryos was performed as previously described [22] unless otherwise noted. COC were obtained with permission by cutting the surface of slaughterhouse (Central Beef Packing Co. (Center Hill, FL, USA)-derived ovaries with a scalpel and vigorously rinsing the ovary through a bath of oocyte collection medium [tissue culture medium-199 with Earle’s salts without phenol red (Hyclone, Logan UT), 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin and 1mM glutamine]. Groups of 10 COC were matured in 50 μl droplets of oocyte maturation medium [tissue culture medium-199 with Earle’s salts (Invitrogen, Carlsbad, CA), 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Bioniche Life Sciences, Belleville, Ontario, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate and 1 mM glutamine] covered with mineral oil. Maturation proceeded for 20 hours at 38.5°C and in a humidified atmosphere of 5% (v/v) CO₂. Up to 300 matured oocytes were fertilized
with Percoll-purified sperm (1 x 10^6) for 8 hours in 1.7 ml of SOF-FERT [221]. Cumulus cells were denuded after fertilization for 4 min by vortexing in 600 μl HEPES-TALP [250] containing 1,000 U/ml hyaluronidase. Embryos were then cultured in 25 μl microdrops of SOF-BE1 [22] covered with mineral oil at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with the balance N₂. Cleavage rate was assessed at Day 3 post-insemination (pi) and embryos were cultured until Day 7 pi.

**Immunofluorescent Labeling for 5-Methylcytosine**

All labeling steps were performed at room temperature unless otherwise stated and in a volume of 100 μl (either in microdrops covered with oil or in wells of a 96-well plate). Embryos were harvested from culture drops, washed in DPBS containing 1% (w/v) polyvinylpyrrolidone (PVP) (Kodak, Rochester, NY, USA) and fixed in 4% (w/v) paraformaldehyde in DPBS-PVP for 15 min. The fixed embryos were washed three times in DPBS-PVP, permeabilized for 20 min in 0.25% Triton X-100 (Fisher Scientific, Waltham, MA, USA) diluted in DPBS, and washed three times in 0.1% (v/v) Tween 20 with wash buffer [DPBS containing 10 mg/ml fraction V BSA (Sigma-Aldrich, St. Louis, MO, USA)]. Embryos were then incubated for 1 hour in 50 μg/ml RNase A (Qiagen, Valencia, CA, USA) diluted in DPBS-PVP at 37°C, washed three times in wash buffer and then incubated at 37°C for 30 min with 3 M HCl/ 0.1% (w/v) PVP. The pH was neutralized by incubation of embryos for 10 min with 100 mM Tris-HCl, pH 8.5 containing 1% (w/v) PVP followed by three washes with wash buffer.

Nonspecific binding sites were blocked by incubation with DPBS containing 5 mg/ml BSA for 1 h. Embryos were then transferred to a solution of 1 μg/ml anti-5-methylcytosine (affinity-purified mouse monoclonal antibody; (Calbiochem, Darmstadt, Germany) diluted in DPBS containing 0.05% (v/v) Tween 20 and 0.01% (w/v) BSA. As
a negative control, anti-5-methylcytosine was replaced with an irrelevant mouse IgG1 antibody (Sigma-Aldrich, St. Louis, MO, USA). After 1 h, embryos were washed three times in wash buffer and transferred to 1 μg/ml fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Abcam, Cambridge, MA, USA). After three washes, nuclei were labeled by incubation in 50 μg/ml propidium iodide (PI) diluted in DPBS-PVP for 15 min. Embryos were washed three times and mounted with coverslips on slides using ProLong Gold Anti-Fade mounting medium (Invitrogen, Carlsbad, CA, USA) and observed under a Zeiss Axioplan epifluorescence microscope (Zeiss, Göttingen, Germany). Images were acquired using a 40x objective and FITC, blue and rhodamine filters. The exposure times were constant for all embryos analyzed in an individual replicate.

**Immunofluorescent Labeling for 5-Methylcytosine and CDX2**

In one experiment, labeling with antibody to 5-methylcytosine was determined for ICM and TE using anti-CDX2 to label TE [46]. Embryos were labeled for anti-methylcytosine as described above, washed six times and transferred to a solution of antibody against CDX2 (affinity purified mouse monoclonal antibody against CDX-2, ready to use solution; BioGenex, San Ramon, CA, USA) for 1 h. Embryos were washed three times and transferred to 10 μg/ml Alexa Fluor 350 labeled goat anti-mouse IgG diluted in DPBS containing 0.05% (v/v) Tween 20 and 0.01% (w/v) BSA for 1 h. Embryos were then washed, labeled with PI, mounted on slides and examined as described above.

**Image Analysis Using ImageJ**

Immunofluorescent intensity was quantified using ImageJ software (version 1.60_41, NIH, Washington DC, USA). For two color images (green for 5-methylcytosine
and red for nuclei), individual nuclei were identified by PI labeling and outlined using the free-hand tool on imageJ. Mean gray intensity of the green and red images were determined separately. The ratio of intensity for green and red (5-methylcytosine/DNA) was calculated for each nucleus. Values for all analyzed nuclei from a single embryo were averaged to obtain the average degree of methylation for that embryo. A similar process was used for three-color images except that staining for CDX2 was used to distinguish between TE (blue nuclei) and ICM (no blue labeling) and values were averaged separately for TE and ICM.

**Separation of TE and ICM by Magnetic-Activated Cell Sorting**

Magnetic-activated cell sorting was performed as previously described with modifications [223]. Blastocysts at Day 7 were harvested and incubated in acidic Tyrode’s solution (Millipore, Billerica, MA, USA) to remove zona pellucidae. Zona pellucida free blastocysts were washed three times in MACS buffer [DPBS with 0.5% (w/v) BSA and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2]. Blastocysts were then incubated for 10 min in concanavalin A conjugated with FITC (Sigma-Aldrich, St. Louis, MO, USA; ConA-FITC, 1 mg/ml in MACS buffer). Following three washes in MACS buffer, blastocysts were incubated with 1 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) in MACS buffer for 3 min. Hoechst 33342 labeled blastocysts were then washed three times in MACS buffer and incubated in DPBS containing 1 mM EDTA for 5 min followed by incubation in 0.05% (w/v) trypsin-0.53 mM EDTA solution (Invitrogen Carlsbad, CA, USA) for 10 min at 38.5°C. Groups of 20-30 blastocysts were then disaggregated into single blastomeres by vortexing three times for 10 sec each. Disaggregated blastomeres were transferred into 500 μl DPBS containing 1 mM EDTA and 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA) followed by
three washes in MACS buffer by centrifugation at 500 x g for 5 min. Large blastomere clusters were eliminated by passing the solution through a 0.2 μm cell strainer (BD Biosciences, San Jose, CA, USA). Single blastomeres that passed through the strainer were collected, centrifuged at 500 x g for 5 min and resuspended in 110 μl of MACS buffer. The re-suspended solution was incubated with 10 μl magnetic microbeads conjugated to mouse anti-FITC (Miltenyi Biotec, Auburn, CA, USA) for 15 min on ice. Following three washes by centrifugation at 500 x g for 5 min, the magnetic bead solution was resuspended in 500 μl MACS buffer and passed through MACS separation columns (Miltenyi biotec, Auburn, CA, USA) attached to a magnetic board (Spherotech, Lake Forest, IL, USA). The FITC-negative fraction (ICM) was eluted by three 500 μl MACS buffer washes followed by FITC positive (TE) elution by removing the MACS separation column from the magnetic board and washing three times with 500 μl MACS buffer. Purity of eluted ICM and TE using this technique is >91% [223].

**Reverse Transcription and Quantitative PCR**

Analysis of gene expression was accomplished by quantitative reverse transcription PCR. Pools of embryos were treated with 0.1% (w/v) proteinase from *Streptomyces griseus* to remove the zona pellucida, washed three times in 50 μl droplets of DPBS with 1% (w/v) PVP, placed in 100 μl extraction buffer from the PicoPure RNA isolation kit (Applied Biosystems, Carlsbad, CA, USA) and heated at 42°C for 30 min. Total RNA was extracted using the PicoPure RNA isolation kit (Applied Biosystems), treated with 2 U of DNase (New England Biolabs, Ipswich, MA, USA) at 37°C for 30 min to remove DNA, and then incubated at 75°C for 15 min to denature DNase. DNase-treated RNA was then reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription
occurred using random hexamer primers and involved incubation at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was stored at -20°C until further use. Negative controls for real-time PCR were also performed by incubation without reverse transcriptase.

CDNA was utilized for real-time PCR analysis. For detection of transcript levels, a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA) utilizing SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). Each reaction contained 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), 10 µl Evagreen Supermix (Bio-Rad), 6.8 µl H2O and 1.2 µl of cDNA sample (0.6 embryo equivalents). Amplification conditions were: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 60°C for 5 sec, and 1 cycle of melt curve analysis at 65-95°C in increments of 0.5°C every 2 sec. The ΔC_T value was determined by subtracting the C_T value of the sample by the geometric mean of the C_T for three housekeeping genes, SDHA, GAPDH and YWHAZ [224, 257]. The primer sequence for DNMT3B (Genbank accession number: AY244711) was 5’-GGGAAGGAGTTTGGAATAGGAG-3’ and 5’-CGGAGAACTTGCCATCAC-3’ [275].

High Resolution Melting Analysis

The MS-HRM (methylation specific high resolution melting analysis) procedure was based on Wojdacz and Dobrovic [276]. MS-HRM is a procedure that uses an intercalating dye and sensitive thermocycler to detect single nucleotide changes within an amplicon based on the temperature at which that dsDNA sequence denatures [276, 277]. In the case of methylation analysis, nucleotide changes are induced by bisulfite conversion of cytosines (but not methylcytosine) to uracil and, following DNA replication, thymidine.
As a preliminary step, 0% and 100% methylated standards were generated for use in standard curve generation. For the 0% methylation control, bovine embryonic fibroblast cells derived from skin cells of a fetus at 2-3 months of gestation were cultured for 1 week in a culture medium composed of 89% (v/v) Dulbecco’s Modified Eagle’s medium (Invitrogen), 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 1% (v/v) of a 100 IU/mL penicillin, 100 μg/ml streptomycin sulfate and 250 ng/ml amphotericin B solution (Invitrogen) and 12 μM 5-azacytidine (to inhibit DNA methylation). The concentration of 5-azacytidine used was chosen because it was the highest concentration that did not cause complete loss of cell growth. Other cells were cultured without 5-azacytidine. Cells were collected, stored at -80°C until extraction of DNA and RNA extraction using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA, USA). gDNA and total RNA were stored at -80°C in extraction buffer and RNase-free water, respectively, until ready for use. A sample of 100% methylated DNA was prepared by treating gDNA from fibroblasts cultured in the absence of 12.5 μM 5-azacytidine with SssI methylase (New England Biolabs, Ipswich, MA, USA) for 1 hour at 37°C. gDNA was then treated with bisulfite using the Imprint DNA modification kit (Sigma-Aldrich, St. Louis, MO, USA) and frozen at -20°C until further use. Samples of 0 and 100% methylated gDNA were mixed together to create samples of 0, 5, 25, 50, 75 and 100%, methylated gDNA.

High resolution melting analysis was performed using a BioRad C1000 thermal cycler with CFX96 Real-time system and Precision Melt analysis software (BioRad, Hercules, CA, USA). PCR conditions were set to the following: 95°C for 2 min, 45 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec, and heteroduplex formation
at 95°C for 30 sec and 60°C for 1 min. Subsequently, high resolution melting was performed at 0.2°C increments from 65°C to 95°C. Primers were designed using Methyl Primer Express 1.0 (Applied Biosystems, Carlsbad, CA, USA) with settings to include at least one nucleotide toward the 5’ end that could bind to both bisulfite-converted and non-bisulfite converted sequences to decrease bias [278]. Primers were designed to amplify an intronic 81 bp sequence containing 5 CpG. This region of DNMT3B was chosen based on the estimation of a CpG island as determined by Methyl Primer Express (Applied Biosystems, Carlsbad, CA, USA). The primer sequence for HRM DNMT3B (Genbank accession number NC_007311) were as follows: 5’-GGAYGGGTGGTGGGTATT-3’ and 5’-CAAACCCCCRCAAAATAATTCT-3’. Primers were generated by Integrated DNA Technologies (Coralville, Iowa, USA). A standard curve was run for each PCR procedure using gDNA of 0-100% methylation and prepared as described above. The difference relative fluorescence units (DRFU) were recorded at the melting peak for each sample. All samples were analyzed in duplicate. The relationship between methylation percent and DRFU was exponential. The standard curve was calculated and used to obtain the degree of methylation for samples.

**Design of Experiments**

The first experiment was conducted to determine developmental changes in DNA methylation. Embryos were fertilized with X-sorted spermatozoa (Genex Cooperative, Shawano, WI, USA, Accelerated Genetics, Baraboo, WI, USA and Select Sires, Plain City, OH, USA) from three separate bulls and then placed into culture with SOF-BE1. Separate drops of embryos were cultured to allow harvest of 2-cell embryos [28-32 hours post-insemination (hpi)], 4-cell embryos (52-56 hpi), 6-8 cell embryos (76-80 hpi),
9-25 cell embryos (104 hpi), 32 cell-morula stage embryos (148-152 hpi) and blastocyst-stage embryos (176 hpi). Embryos were analyzed for DNA methylation by labeling with anti-5-methylcytosine and PI as described above. The experiment was replicated on 6 occasions and a total of 11-24 embryos per stage were subjected to labeling for anti-5-methylcytosine.

The effect of sex on DNA methylation was determined by fertilizing oocytes with either X-sorted spermatozoa or Y-sorted spermatozoa (Accelerated Genetics, Baraboo, WI, USA) from the same bull, using three separate bulls for each replicate. Embryos were then cultured and harvested from separate drops at either the 6-8 cell stage (76-80 hpi) or blastocyst stage (176 hpi) for analysis of labeling with anti-5-methylcytosine. The experiment was replicated on 3 occasions and a total of 11-21 embryos per stage were subjected to labeling for anti-5-methylcytosine.

Effects of CSF2 and cell lineage (ICM and TE) on DNA methylation in the blastocyst were tested as follows. Oocytes were fertilized with either X-sorted spermatozoa or Y-sorted spermatozoa (Accelerated Genetics, Baraboo, WI, USA) from the same bull, using three separate bulls for each replicate. Embryos were then placed in 45 µl drops of SOF-BE1 and cultured for 7 d. At Day 5 pi, 5 µl of 100 ng/ml recombinant bovine CSF2 (Novartis) in vehicle [DPBS and 1% (w/v) BSA] or vehicle alone was added. The concentration of CSF2 was chosen based on its effectiveness for increasing development to the blastocyst stage and embryonic survival after transfer into recipient females [24]. Blastocysts were harvested at Day 7 pi and subjected to labeling with anti-5-methylcytosine, anti-CDX2 and PI. The experiment was replicated
on 3 occasions and a total of 14-26 embryos per stage were subjected to labeling for anti-5-methylcytosine.

Developmental changes in expression of *DNMT3B* were determined in another experiment. Embryos were fertilized with conventional semen (Androgenics, Oakdale, CA, USA and Nebraska Bull Service, McCook, NE, USA) using three different bulls for each replicate and then placed into culture with SOF-BE1. Separate drops of embryos were cultured to allow collection of groups of 30 zygotes (0 hpi), 2-cell (28-32 hpi), 3-4 cell (48 hpi), 5-8 cell (56-60 hpi), 9-16 cell (72 hpi), >16 cell (120 hpi) and blastocyst stage (168 hpi) embryos. RNA was extracted as described above and used for RT-PCR. The experiment was replicated on 5 occasions.

Methylation of an intronic region of *DNMT3B* was determined in two experiments. In the first experiment, embryos were collected at the 6-8 cell stage (76-80 hpi) and at the blastocyst stage (176 hpi). In the second, blastocysts were harvested at 176 hpi and ICM and TE isolated using MACS. In both experiments, embryos were fertilized with conventional semen (Androgenics, Oakdale, CA, USA and Nebraska Bull Service, McCook, NE, USA) using three different bulls for each replicate and then placed into culture with SOF-BE1. In the first experiment, embryos were collected in groups of 18-48, treated to remove the zona pellucida and snap-frozen in liquid N$_2$ before storage at -80°C until ready for use in MS-HRM analysis. A total of 3 pools of embryos were collected at each stage of development. In the second experiment, groups of 20-40 blastocysts were subjected to the MACS procedure to isolate ICM and TE and cells frozen in 20 µl of MACS buffer (DPBS with 0.5% (w/v) BSA and 2 mM EDTA, pH 7.2) at
-80°C until ready for MS-HRM. The experiment was replicated with 8 pools of ICM and TE.

**Statistical Analysis**

Data were analyzed by least-squares analysis of variance using the GLM procedure of the Statistical Analysis System version 9.3 (SAS Institute Inc., Cary, NC, USA). Depending on the experiment, sources of variation included replicate, stage, sex, cell type (ICM and TE), treatment (CSF2 or vehicle) as well as all interactions. Replicate was considered a fixed effect. For real-time PCR results, treatment effects were tested using ΔC_T values and graphed as fold-change differences relative to values for oocytes. All data are shown as least-squares means ± standard error of the means.

**Results**

**Changes in DNA Methylation during Development to the Blastocyst Stage**

Representative images of labeling for 5-methylcytosine from the 2-cell stage through blastocyst development are presented in Figure 4-1 while results from quantitative analysis are presented in Figure 4-2. There was an effect of stage of development on immunoreactive 5-methylcytosine (P<0.0001), with amounts decreasing from a peak at the 2-cell stage to a nadir at the 6-8 cell stage. Thereafter, immunoreactivity increased to the blastocyst stage.

**Effect of Sex on DNA Methylation**

Embryos were fertilized separately with X and Y sorted sperm from the same bull to produce either female or male embryos. DNA methylation was examined at either the 6-8 cell or blastocyst stages of development by evaluating labeling with anti-5-methylcytosine. Results are shown in Figure 4-3. DNA methylation was greater for embryos at the blastocyst stage than at the 6-8 cell stage (stage of development;
P<0.0001). There were, however, effects of sex (P=0.0029) and sex x stage (P=0.0007). The interaction occurred because female embryos had more intense labeling for 5-methylcytosine than male embryos at the 6-8 cell stage but lower labeling than male embryos at the blastocyst stage.

**DNA Methylation in Blastocysts as Modulated by Sex, CSF2 and Cell Differentiation**

Use of CDX2 to distinguish between ICM and TE revealed that fluorescent activity for immunoreactive 5-methylcytosine was lower in ICM than TE. A representative blastocyst is shown in Figure 4 and quantitative analysis is summarized in Figure 5. As seen in the previous experiment, DNA was less methylated in female embryos than male embryos (P<0.02) and this was apparent in both the ICM and TE compared to the TE (Figure 4-5). There was no effect of CSF2 or interactions of CSF2 on degree of methylation in either the ICM (0.53 ± 0.09 vs. 0.46 ± 0.07 for CSF2 and vehicle, respectively) or TE (1.17 ± 0.09 vs. 1.18 ± 0.07 for CSF2 and vehicle, respectively).

**Developmental Changes in DNMT3B Gene Expression**

To determine whether developmental changes in DNA methylation could conceivably be caused by changes in DNMT3B activity, changes in expression of *DNMT3B* from the zygote to the blastocyst stage were determined (Figure 4-6). Stage of development affected (P<0.0001) steady-stage amounts of DNMT3B mRNA. Expression increased between the zygote stage and 2-cell stage and then declined to a nadir for embryos at the >16 cell stage of development. Thereafter, steady-state concentrations of *DNMT3B* increased at the blastocyst stage.
Developmental Changes in Methylation of DNMT3B

The possible role of methylation in controlling developmental changes in expression of DNMT3B was evaluated by determining changes in the degree of methylation in an 81 bp CpG rich intronic region of DNMT3B (Figure 4-7A). A representative standard curve is shown in Figure 4-7B and representative results in Figure 4-8A. Methylation in the analyzed region, which contains 5 CpGs, was higher (P<0.0001) for 6-8 cell embryos than for embryos at the blastocyst stage (Figure 4-8B). When ICM and TE were separated by MACS, there was no significant difference (p=0.25) in methylation of DNMT3B between ICM (70.2 ± 9.8%) or TE (56.3 ± 8.6%).

Discussion

Epigenetic control of gene expression is an important aspect of early embryonic development. Using the preimplantation bovine embryo as a model, it was observed that dynamic changes in DNA methylation occur throughout development in a manner that depends on sex and cell type. Changes in DNA methylation were associated with similar changes in expression and methylation of DNMT3B, indicating that, as for the mouse [82], this gene may play an important role in modification of the embryonic methylome.

There is a steady decrease in overall level of DNA methylation as the mouse embryo undergoes successive cell division [75]. A similar loss occurs in the developing sheep embryo [85] and as, shown here, the bovine embryo (Figure 4-2). In contrast, there appears to be no widespread demethylation during early cleavage divisions in the pig [86] or rabbit [274]. In the mouse, the loss of methylation during early development occurs as a result of both passive [279] and active [280] demethylation. Passive demethylation occurs when DNA replication occurs without the presence of DNMT1 to
methylate the newly replicated strand [281] while active DNA demethylation is thought to occur by base excision repair mechanisms utilizing glycolysis or deamination of 5-methylcytosine although the exact enzymes responsible are still unknown in mammals [282].

The bovine embryo is also similar to the mouse embryo in that the period of demethylation is followed by a period of de novo methylation (Figure 4-2). In the cow, methylation begins following the 6-8 cell stage and is coincident with activation of the embryonic genome [283]. Re-establishment of methylation marks on DNA in the mouse embryo is accomplished by the de novo methyltransferases Dnmt3a and Dnmt3b [82]. Knockout of Dnmt3b caused incomplete methylation of pluripotent genes in the mouse embryo [82, 284]. Current results implicate DNMT3B as involved in de novo DNA methylation in the cow based on the observation that steady-state amounts of mRNA for DNMT3B parallels the pattern of methylation (Figures 4-2 and 4-6). In fact, DNMT3B itself might be under the control of methylation because methylation of an intronic region within DNMT3B was lower at the blastocyst stage than at the 6-8 cell stage (Figure 4-8B). The role of methylation in DNMT3B expression can be further clarified through experiments to detail the methylation landscape of DNMT3B more fully as well as the consequences of changes in methylation on DNMT3B expression. It is also likely that there is regulation of DNMT3B transcription independent of DNA methylation.

Developmental patterns of methylation in the preimplantation embryo diverge between the mouse and cow at the blastocyst stage of development. In the mouse, the ICM is more highly methylated than the TE [76] while current results indicate the ICM is less methylated than TE in the cow (Figure 4-4). Indeed, there is much divergence
between species in overall levels of methylation in the ICM and TE, with the sheep and pig showing a pattern similar to the mouse [85, 86] and with the ICM less methylated than the TE in the rabbit [274, 285]. One implication of the species variability in DNA methylation patterns in the blastocyst is that there is likely to be significant differences between species in the genes that are differentially regulated between ICM and TE. One example is OCT4 (i.e, POU5F1). In the mouse, Oct4 is strictly expressed in the ICM while OCT4 in cattle is expressed in both ICM and TE [94]. A genome-wide study of the bovine ICM and TE revealed genes that exhibited different patterns of differential expression than is the case in mice or humans [286].

Sex of the bovine embryo is an important factor that affects development as early as the first cleavage, when the sex ratio is skewed towards males [50]. SRY is expressed as early as the 8-cell stage of development [287], more male embryos than female embryos develop past the 8-cell stage of development [288], and male embryos become blastocysts at a more rapid rate than females [50, 288, 289]. At the blastocyst stage, gene expression is affected by sex, with the overall level of transcription being greater for female embryos [245]. Methylation is likely to play a role in establishing sex patterns of transcription. Earlier studies reported differences in degree of methylation of specific genes at the blastocyst stage [8, 268, 290]. Here we show that the overall level of DNA methylation at the blastocyst stage was lower for female embryos than male blastocysts (Figure 4-3). Such a sex difference would be expected if DNA methylation is important for the higher transcript abundance for female blastocysts [245]. Earlier in development, at the 6-8 cell stage, DNA is more methylated for female embryos (Figure 4-3), probably because the rate of demethylation was less for female embryos.
DNA methylation can be altered by development in vitro [8]. Thus, it will be necessary to determine the extent to which changes in DNA methylation caused by stage of development, cell lineage and sex seen here also occur for embryos developing in vivo. Moreover, it is possible that regulatory molecules such as CSF2 [18], insulin-like growth factor-1 [129] and hyaluronan [21] that improve competence of the in vitro produced embryo to survive after transfer into recipients could exert their action, at least in part, by regulating DNA methylation. This hypothesis was tested in the present experiment for the maternally derived cytokine CSF2 [27]. There was no effect of CSF2 treatment on the overall level of DNA methylation at the blastocyst stage (Figure 4-3C) so actions of CSF2 on the embryo that enhance its survival probably do not depend upon broad changes in DNA methylation. Nonetheless, it is possible that CSF2 causes changes in methylation of a more narrow set of genes. There are long-term consequences of embryonic treatment with CSF2 (decreased pregnancy loss after Day 35 of gestation [18]) that could represent epigenetic programming.

In conclusion, preimplantation development of the bovine embryo is characterized by dynamic changes to DNA methylation that are dependent upon sex and cell lineage. In particular, global methylation declines to a nadir at the 6-8 cell stage and increases thereafter. Methylation is lower for female embryos than male embryos at the blastocyst stage and lower for the ICM than TE. The developmental pattern of DNA methylation in the cow is partially representative of events in the mouse, with the major difference being in the relative degree of methylation in ICM and TE. Like the mouse, changes in expression of DNMT3B may be responsible for developmental
changes in DNA methylation because levels of methylation are related to expression of

*DNMT3B.*
Figure 4-1. Representative images of embryos labeled for immunoreactive 5-methylcytosine at the 2 cell, 4 cell, 6–8 cell, 9–25 cell, 32 cell-morula and blastocyst stages of development. The scale bar represents 26.75 µm.
Figure 4-2. Developmental changes in labeling of immunoreactive 5-methylcytosine from the 2-cell to blastocyst stages of development. Data represent the ratio of fluorescent intensity for anti-5-methylcytosine to that for propidium iodide. There was an overall effect of stage on labeling intensity ($P<0.0001$). Means with different superscripts differ ($P<0.05$). Data are least-squares means±SEM of results from 11–24 embryos per stage.
Figure 4-3. Effects of sex on immunoreactive 5-methylcytosine in embryos at the 6–8 cell and blastocyst stages of development. Data represent the ratio of fluorescent intensity for anti-5-methylcytosine to that for propidium iodide. The solid line represents female embryos while the hashed line represents male embryos. Data are least-squares means±SEM of results from 11–21 embryos per stage. Amounts of DNA methylation were affected by sex ($P = 0.0029$), stage of development ($P<0.0001$) and the stage × sex interaction ($P = 0.0007$).
Figure 4-4. Representative images of an embryo in which labeling with CDX2 (blue) was used to evaluate immunoreactive 5-methylcytosine (green) in ICM (CDX2-negative) and TE (CDX2-positive). Nuclei of all cells were labeled with propidium iodide (red). Panels represent merged images for all three fluorescent labels (A), 5-methylcytosine and PI (B), CDX2 and PI (C), CDX2 and 5-methylcytosine (D) and control antibodies for anti-5methylcytosine and anti-CDX2 as well as PI (E). The scale bar is 20 µm.
Figure 4-5. Effects of sex and cell type [ICM and TE] on immunoreactive 5-methylcytosine in blastocysts. Data represent the ratio of fluorescent intensity for anti-5-methylcytosine to that for propidium iodide. Data are least-squares means±SEM of results from 14–26 embryos per sex. Amounts of DNA methylation were affected by sex (P<0.02), and cell type (P<0.0001) but not the sex × cell type interaction (P<0.25).
Figure 4-6. Developmental changes in steady-state mRNA for DNMT3B. Data for mRNA are expressed as fold-change relative to amounts for zygotes and represent the least-squares means±SEM of results from 5 pools of 30 embryos per stage. Means with different superscripts differ (P<0.05).
Figure 4-7. Characteristics of the high resolution melt analysis for methylation of an intronic region of DNMT3B. Panel A shows the location of the intronic region between exons 7 and 8 that was analyzed. Note the presence of five CpG eligible for methylation in the 81 bp region amplified by PCR. Panel B is a representative result for the standard curve generated by analysis of a mix of control DNA at 0% and 100% methylation. The graph is a plot of the difference relative fluorescence units (RFU) as a function of shifted temperature (X-axis normalization to reduce variation between wells).
Figure 4-8. Developmental changes in DNA methylation for DNMT3B. Panel A is a representative result of analysis of samples from 6–8 cell embryos (lime green) and blastocysts (red). Panel B shows the degree of methylation between the two stages. Data represent least-squares means±SEM of results from 3 pools of 23–48 embryos per stage. Methylation percent was higher at the 6–8 cell stage than at the blastocyst stage (P<0.0001).
CHAPTER 5
GENERAL DISCUSSION

The maternal environment is a critical component for the successful development of the embryo. Alterations in this microenvironment during preimplantation development can alter the developmental fate of the fetus, with potential consequences that extend into post-natal life [13, 15, 99, 291]. It was the assumption of this dissertation that effects of the maternal environment on the developmental trajectory of the preimplantation embryo are mediated, at least in part, by altered secretion of regulatory molecules from the endometrium that affect development. Indeed, a variety of molecules produced by the endometrium have been identified that can affect development to the blastocyst stage and competence of the embryo to establish pregnancy. In the cow, these embryokines, include IGF1 [292], IL1 [23], hyaluronan [21], TGFB [20], LIF [20] and FGF2 [22]. The most well studied embryokine is CSF2, which has been shown to have a variety of effects on the preimplantation embryo of cattle [24], mice [26], pigs [189], and humans [25] including improved ability to develop to the blastocyst stage [24, 26, 189, 200], increased ICM numbers [18], resistance to apoptosis [31, 187], altered gene expression [30] and improved competence to establish pregnancy when embryos are transferred to females [18, 25, 26]. The purpose of the research described in this dissertation, was to identify mechanisms by which CSF2 increases the competence of the bovine blastocyst to establish pregnancy.

The conclusions derived from the dissertation are summarized in Figure 5-1. It was demonstrated in Chapter 4 that early preimplantation development is characterized by a loss in DNA methylation to a nadir at the 8-16 cell stage. At this time, the female embryo maintains a higher level of methylation than the male. Subsequently,
methylation is increased but in a pattern where, at the blastocyst stage, the TE is more methylated than the ICM. At this stage, DNA is less methylated for the female than the male. Sex effects on methylation may be important for actions of CSF2, which occur in a sex-specific manner. At the blastocyst stage, CSF2 affects characteristics of the ICM so that its ability to survive passage and remain pluripotent when isolated from TE is increased (Chapter 2). The mechanisms are unclear but, by the blastocyst stage, the program of the embryo has been altered by CSF2 differently in female embryos than in male embryos (Chapter 3). Differential programming is apparent when examining development of embryos after transfer to recipients. There is no size difference between female and male embryos at Day 14 of development but, by Day 15, control female embryos are larger and secrete more IFNT than male embryos. This phenomenon probably represents earlier elongation for females. Moreover, CSF2 alters the differences between sexes by decreasing size and IFNT secretion for females but increasing these characteristics for males. In addition, CSF2 programs the transcriptome and methylome at day 15 in a sex-specific manner. This observation leads to the possibility that sex-specific developmental programming [14-17, 169] occurs because alterations in the maternal milieu during the preimplantation period in response to changes in environment cause sex-specific effects on the embryo.

While several molecules have been shown to increase the competence of an embryo to establish pregnancy after transfer, including CSF2 [18], IGF1 [292] and hyaluronan [21], we know nothing of the mechanism by which embryo competence is increased. Several genes have been identified whose expression at the blastocyst stage differs between embryos that subsequently establish pregnancy versus those that
do not [293], and it may be that certain embryokines alter gene expression in a manner than improves embryo competence. However, none of the genes that were differentially expressed between embryos establishing pregnancy or not were found to be regulated by CSF2 at the morula stage [31]. Results from Chapter 2 suggest that one possible way CSF2 improves embryo competence for survival is regulation of pluripotency and capacity for survival of the ICM. Such an action, if exerted by CSF2, could potentially have a large effect on embryonic survival because 20-25% of in vitro produced embryos lose the ED derived from the ICM by Day 14-15 of gestation [18, 225, 226]. Perhaps CSF2 increases pregnancy rate in embryo transfer recipients by reducing loss of the ED. The evidence for CSF2 regulation of the ICM is based on experiments showing that CSF2 increases ICM cell number [18], and that isolated ICM cultured on mitomycin C treated fibroblasts were more likely to survive passage while maintaining pluripotency if embryos were exposed to CSF2 from Day 5-7 or 6-8 of development (Chapter 2).

The mechanism by which CSF2 affects the ICM is not known. One possibility is that CSF2 reduced induction of apoptosis. CSF2 can blunt induction of apoptosis by heat shock in the bovine embryo [31]. Another possibility is that CSF2 regulates expression of genes associated with pluripotency. Expression for pluripotency related genes NANOG and SOX2 in isolated ICM were not affected by CSF2, however (Chapter 2). However, morula-stage embryos collected after 24 hours of CSF2 treatment did contain differentially expressed genes related to pluripotency [31].

Another possible mechanism by which CSF2 could increase embryo competence to establish pregnancy is through alteration of the methylome of the embryo. As shown in Chapter 3, some effects of CSF2 treatment from Day 5-7 of development are not
realized until several days later (Day 15 in this case) and such effects are consistent with epigenetic modification. Earlier, Loureiro et al (2011) found a similar long-term effect of CSF2 since embryos that established pregnancy were less likely to be lost before term if treated with CSF2 from Day 5-7. Even though there was no effect of CSF2 on the overall level of methylation in the embryo at the blastocyst stage of development (Chapter 4), the technique used to assess methylation (labeling with anti-5-methylcytosine) is crude and it is possible that specific regions of DNA undergo differential methylation in response to CSF2. Use of the methylome array described in Chapter 3 could prove effective at examining whether specific CpG undergo differential methylation at the blastocyst stage in response to CSF2.

One implication of the findings that CSF2 increases survival of isolated ICM is that CSF2 might be a useful additive to culture media for production of ESCs in the cow. ESCs from the cow are difficult to keep in the pluripotent state. Regulatory molecules utilized to maintain pluripotency of mouse [229] or human [228] ESC cultures are ineffective for that purpose in cow [230]. It is possible that the addition of CSF2 to culture media could improve the survival of bovine stem cells in an undifferentiated state.

As has been found earlier for the human [187], the bovine preimplantation embryo expresses CSF2RA while not expressing CSF2RB. CSF2 binds to CSF2RA, which combines with the CSF2RB subunit to signal through JAK2/STAT5ab [181]. CSF2RA is capable of signaling in the absence of the CSF2RB subunit, but does so at much lower affinity [182]. Given that CSF2 can alter the properties of the bovine embryo despite the absence of CSF2RB mRNA, it is likely that CSF2 signals through a separate
pathway other than the JAK-STAT signaling pathway regulated by the CSF2RA-CSF2RB dimer. The lack of the CSF2RB subunit implies that the embryo does not have high affinity signaling. However, the dosage of 10 ng/ml of CSF2 used in these experiments, is below the $K_d$ of the low affinity CSF2RA. In the absence of the CSF2RB subunit, human myeloid cells are still able to signal through the PI3K pathway [183]. Perhaps a similar pathway is operative in blastomeres of the preimplantation embryo.

As has been alluded to many times in this dissertation, one important rationale for studying regulation of embryonic development is that the embryo produced in vitro is abnormal in many respects [3-5, 7, 8] and is less capable of establishing pregnancy when transferred to recipients [99]. Identification of maternal-derived molecules that enhance embryonic competence could be useful to improve effectiveness of embryo transfer using in vitro produced embryos. Few laboratories that study embryonic development in the cow have the opportunity to transfer embryos to recipients and, instead use in vitro measures of development, such as the percent of oocytes becoming blastocysts, as an indicator of the adequacy of culture systems. Results in this dissertation point out the inadequacy of this approach. There was no consistent effect of CSF2 on the percent of embryos that progressed to the blastocyst stage of development. Nonetheless, CSF2 can improve the pregnancy rate after transfer to recipients in cattle [18], mice [26] and humans [200]. This change in pregnancy rate after transfer without affecting the percent of embryos that develop to the blastocyst stage is also true for other molecules such as IGF1 [129] and hyaluronan [21]. In consequence, optimization of culture systems for production of embryos in vitro will
require transfer of embryos to recipient females or development of other in vitro indices of blastocyst quality more predictive of embryo survival than percent blastocyst.

Perhaps the most important finding of this dissertation was the unexpected observation that exposure of the morula and blastocyst stage embryo to CSF2 alters the developmental program in a sex-specific manner (Chapter 3). One implication is that sexual differentiation occurs as early as Day 5-7. This is to be expected because gene expression differs significantly between male and female embryos at the blastocyst stage [245] and other results from this dissertation show differences in global DNA methylation between female and male embryos as early as the 8-cell stage (Chapter 4).

One possibility is that CSF2 uses signal transduction pathways that differ in important respects between males and females. While the signaling pathway for CSF2 in the embryo has not been identified, and is likely different than the classical JAK-STAT pathway (Chapter 2), it is possible that there is more than one pathway and that the relative activity of one or more varies between male and female embryos. Experiments to elucidate signaling pathways for CSF2 in the embryo should be conducted to determine whether there are sex differences. Another possible reason for sex differences in response to CSF2 would be differences in transcriptional repression of specific regions of DNA by epigenetic modification. At the blastocyst stage, the male embryo is methylated at a higher intensity than the female (Chapter 4) while the female is transcriptionally more active than males at the same stage [245]. Interestingly, over 50% of the genes regulated by CSF2 are also regulated by sex. Thus, CSF2 acts preferentially on genes that are controlled directly or indirectly by transcription factors involved in sexual differentiation. There may also be sex-specific methylation of genes
encoding transcription factors or enhancers. At least in part, genes regulated by sex may have transcription factors or regulatory elements that are also regulated by CSF2.

The observation on sex differences in response to CSF2 is important because it provides a mechanism for the phenomenon by which developmental programming affects female embryos differently than male embryos. Perhaps changes in nutrition or stress that the mother is subjected to in her environment lead to alterations in the embryokine secretion in the uterus, thus preparing the embryo for post-natal life in a sex specific manner. In addition, this observation implies that much of early development much be examined in a sex-specific context.

For the phenomenon of sex-specific response to CSF2 to be generally relevant to preimplantation developmental programming, it should be that the phenomenon occurs for more than one embryokine and in species other than the cow. Other embryokines, such as IGF1 [292], IL1B [23], hyaluronan [21], TGFB [20], LIF [20] and FGF2 [22], have been shown to improve the development of the embryo in the cow, but it has not been examined whether response of the embryo to these molecules varies with sex. We do not know whether sex bias responses to embryokines occurs in other species but at least the embryokine examined, CSF2, causes changes in development in a wide variety of species, including mice [26], pigs [189] and humans [200].

CSF2 is a good candidate for mediating certain environmental effects on developmental programming because its expression in the uterus depends on diet and inflammatory status. In particular, obese cows had lower CSF2 protein and mRNA present in the oviduct than non-obese cows [34]. Uterine inflammation caused by the presence of semen, increases CSF2 protein secretion due to TGFB within the seminal
plasma [32, 33]. Understanding how CSF2 is regulated by diet may prove useful for minimizing the effects of undernutrition on fertility [294] as well as on the characteristics of the resulting offspring.

One implication of the finding that CSF2 affected female embryos differently than male embryos is that research on regulation of preimplantation development should now consider the possibility of differences between female and male embryos. Heretofore, there has been little consideration of embryo sex when trying to understand preimplantation development, which should change.

Findings from this dissertation provide the groundwork for understanding 1) the signal transduction system for CSF2 in the preimplantation embryo, 2) mechanisms by which CSF2 affects ICM survival and pluripotency, 3) and how developmental programming occurs in a sex-specific manner during preimplantation development. Among the promising areas of future research would be to address the mechanism by which CSF2 programs the embryo in a sex specific manner (whether due to sex effects on signal transduction, transcription factor usage or epigenetic modification) as well as the mechanisms for actions of CSF2 on survival and pluripotency of the ICM.
Figure 5-1. Schematic illustration of conclusions derived from the dissertation including developmental changes in methylation and sex-specific actions of CSF2 on embryonic development. Early preimplantation development is characterized by a loss in DNA methylation to a nadir at the 8-16 cell stage. At this time, the female embryo maintains a higher level of methylation than the male. Subsequently, methylation is increased but in a pattern where, at the blastocyst stage, the TE is more methylated than the ICM. At this stage, DNA is less methylated for the female than the male. Sex effects on methylation may be important for actions of CSF2 which occur in a sex-specific manner. At the blastocyst stage, CSF2 affects characteristics of the ICM so that its ability to survive passage and remain pluripotent when isolated from TE is increased. The mechanisms are unclear but, by the blastocyst stage, the program of the embryo has been altered by CSF2 differently in female embryos than in male embryos. Differential programming is apparent when examining development of embryos after transfer to recipients. There is no size difference between female and male embryos at Day 14 of development but, by Day 15, control female embryos are larger and secrete more IFNT than male embryos. This phenomenon probably represents earlier elongation for females. Moreover, CSF2 alters the differences between sexes by decreasing size and IFNT secretion for females but increasing these characteristics for males. In addition, CSF2 programs the transcriptome and methylome at day 15 in a sex-specific manner.
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BIOGRAPHICAL SKETCH

Kyle Dobbs was born and raised in Webster Groves, MO, a suburb of St. Louis, to Kathy and Brad Dobbs. He is the older brother of Kory. His father spurred his love of science and animals at a young age. He attended high school at Lutheran High School South in Afton, MO where he graduated in 2004. Following graduation, he moved to Columbia, MO to begin his collegiate career at the University of Missouri where he graduated in 2008 with his Bachelor of Science degree in biological sciences and a minor in chemistry. During his undergraduate studies, he worked as a researcher in the laboratory of Dr. Randall Prather. Following graduation, he began his graduate career in Dr. Prather’s laboratory, where he studied polyadenylation of porcine transcripts during early embryogenesis. After graduation in 2010 with his Master of Science degree, he married his high school sweetheart, Lauren (Kienstra) and immediately moved the next day to Gainesville, FL to begin work on his doctorate with Dr. Peter J. Hansen in the Animal Molecular and Cellular Biology Graduate Program. While at Florida, he was supported by a Graduate Fellowship.

One of the experiments for his dissertation was recognized as the outstanding student poster presentation at the 40th Annual Conference of the International Embryo Transfer Society held January 11-14, 2014 in Reno NV.

After completing his Doctor of Philosophy degree, Kyle and Lauren will move to Boston, MA where he will continue his career as a post-doctoral researcher at Northeastern University in the laboratory of Dr. Jonathan Tilly.