Manipulating Embryonic Development and Endometrial Function in Ruminants

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Science

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March 16, 2018
Blacksburg, VA

Keywords: Primitive Endoderm, Conceptus, Endometrium, Maternal Obesity

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ABSTRACT

Early embryogenesis is highlighted by the emergence of several embryonic end extraembryonic lineages. One such lineage is the primitive endoderm, which will eventually give rise to the yolk sac. Once believed to be a vestigial structure, the yolk sac is now believed to play a more prominent role in embryogenesis as it provides nutrients to the preimplantation embryo. The endoderm may also interact with the trophectoderm lineage, as they develop in close contact within the embryo. The efficiency of developing primitive endoderm in vitro is considerably low, leading to a lapse in our understanding of its development and function in cattle and other ruminants. The goal of the first study was to establish a protocol for developing primitive endoderm cultures and characterizing these cells. Bovine embryos were produced *in vitro*, and primitive endoderm outgrowths were created with fibroblast growth factor 2 (FGF2) supplementation. These cells can be produced in culture with 80.3 ± 5.6% efficiency. Furthermore, outgrowths can be maintained in culture for 6-8 weeks before reaching a quiescent state. A true bovine primitive endoderm cell line does not currently exist, however, these cells hold potential in improving the current understanding of early lineage specification in cattle.

A second set of studies was performed to examine the effects of maternal obesity on the preimplantation conceptus and endometrium. Exposure to maternal obesity *in utero* affects offspring development at the postnatal, adolescent, and adult stages of development; however, its impacts on early embryogenesis are not well studied. This work utilized an obese ewe model. Once the obese phenotype was established, ewes were bred. Conceptus and endometrial tissue were collected at D 14 of pregnancy, and samples were processed for RNA-sequencing analysis. There were no differences in pregnancy rate, ovulation rate, or pregnancies/ovulation between obese and lean animals. At an RPKM threshold of ≥ 0.2, fold-change ≥ 2, and FDR ≤ 0.05, 669 and 21 differentially expressed genes (DEGs) were identified between obese- and lean-derived endometrial samples and conceptus samples, respectively. Likewise, 137 DEGs were identified between male and female conceptuses. The PANTHER GO-Slim Biological Process system identified several biological processes affected by obesity in both the endometrium and conceptus tissue. GO terms do not currently exist for “placenta” and “trophoblast”, so a literature search was conducted to identify DEGs involved in implantation and placentation. This revealed 125 placentation DEGs in the endometrium, and 4 DEGs in conceptuses between obese and lean groups. A follow-up study was conducted to examine the abundance of transcripts with regulatory roles in embryogenesis. Conceptuses exhibited differential expression of DNA methyltransferase 1 (*DNMT1*) based on obesity exposure, fibroblast growth factor receptor 2 (*FGFR2*) in a sex*obesity interaction, and peroxisome proliferator-activated receptor gamma (*PPARG*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) in a sex-specific manner. Collectively these results identify the preimplantation period as a susceptible time to maternal obesity in both conceptus and endometrial tissue.

Together, these studies aim to provide a better understanding of the events controlling early embryogenesis, and insight into the implication of insults during this time. These findings will prove to be beneficial in establishing the link between maternal health, endometrial function, and subsequent offspring outcomes, with the hope of promoting a more viable embryo and thus healthier offspring.

Manipulating Embryonic Development and Endometrial Function in Ruminants

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GENERAL AUDIENCE ABSTRACT

Early embryogenesis in cattle is afflicted with high embryonic loss, costing the dairy and beef industries a fair portion of their profits. The mechanisms behind these losses are not well understood, however, cellular miscommunications during lineage specification are likely to blame. Of particular interest is the endoderm lineage, which gives rise to the embryonic yolk sac. Initially believed to be a transient structure, we now believe the yolk sac is indispensable in embryogenesis as it provides nutrients to the preimplantation embryo. Our current understanding of primitive endoderm and the resulting yolk sac in cattle is severely lacking because few primitive endoderm in vitro models exist. A portion of the following work is focused on developing a protocol for producing primitive endoderm cell lines *in vitro*. This work improved the rate of producing primitive endoderm cells in vitro and characterized those cells. These cells will be used as a tool to better understand the mechanisms involved in early embryogenesis. Furthermore, they may help identify targets for manipulating early development to lessen the high rate of embryonic loss in cattle.

The stage of early embryogenesis may also be particularly susceptible to intrauterine stressors, such as maternal obesity, because of the lineage segregation events occurring at this time. Insults to the earliest lineages can have lasting developmental effects, as these cell types will give rise to the embryo proper, yolk sac, and placenta. The effects of maternal obesity have been extensively studied in the postnatal, adolescent, and adult stages of development, however, insights into the effects on early embryogenesis are missing. The final studies of the following work are focused on the effects of maternal obesity on the preimplantation ovine conceptus and endometrium. This work utilized RNA-sequencing technology as well a qRT-PCR to identify differential gene and transcript expression in conceptus and endometrial samples collected from lean and obese ewes. Following analysis, we identified several crucial biological processes affected by maternal obesity. Of particular interest were those involved in implantation and placentation, indicating developmental programming events during early embryogenesis may be at fault for the abnormal offspring outcomes observed in previous studies. This work highlights the susceptibility of the preimplantation conceptus to maternal obesity and identifies the endometrium as a mediator between maternal nutrition and conceptus development. Additionally, this work identifies alterations in genes involved in placentation in both the conceptus and endometrium, indicating developmental programing events have occurred.

As a whole, this work developed a new tool for examining early embryogenesis and the specification events that occur during that time. It also examines the embryonic impacts of maternal obesity during that critical window of development. These findings will prove to be invaluable in factors involved in early embryo development and function in ruminant species.

ACKNOWLEDGEMENTS

I would first like to thank my parents for teaching me that hard work pays off. You have loved and supported me throughout my entire academic career. I certainly would not be where I am today without you both. I also want to thank my brother, Ethan, thanks for always making me look good. To my fiancé, Will, you certainly deserve a pat on the back for making it through graduate school. You have been my voice of reason for 6 years, and I would not have made it through this wild ride without you. Love you, mean it! Thank you to the rest of my family for all of your love and support, even though I'm pretty sure most of you still think I want to be a veterinarian or MD. To all my girlfriends, thank you for teaching me about the importance of work-life balance. I will always be grateful for your friendships.

I would like to thank the members of the Johnson-Ealy lab and my officemates, past and present. From helping me with sample collections, being my personal thesauruses, letting me bounce ideas off you, or just providing a good laugh, I could not ask for a better group of collogues and friends.

To my committee members, Dr. Kiho Lee, Dr. Will Eyestone, and Dr. Shelly Rhoads, thank you for all of your guidance through the past several years. Your words of wisdom and advice have pushed me to become a better scientist.

Finally, to my mentor Dr. Alan Ealy, thank you for giving me the right amount of guidance and freedom to become the scientist I am today. You have always encouraged me to come up with my own ideas, all while teaching me to not get too carried away with them. I will forever be thankful for your mentorship and friendship, and I hope to one day be a mentor to my own students like you have been to me.

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Chapter 1

Literature Review

Early embryogenesis is recognized as a time of vast cellular remodeling. During this period of development, the embryo must complete two major lineage specification events; trophoblast-inner cell mass segregation and primitive endoderm-epiblast specification. The trophoblast (TE), primitive endoderm (PrE), and epiblast (EPI) will eventually give rise to the placenta, yolk sac, and embryo proper, respectively. Interestingly, the preimplantation period suffers from the highest rate of pregnancy loss of all gestational stages. This high rate of embryonic wastage is observed in many mammalian species, including humans and ruminant species [1-3]. In humans, reports range from 30-70% loss between the time of fertilization to implantation [4]. Dairy and beef cows experience similar embryonic loss, with rates ranging from about 60% and 20-44%, respectively [3, 5]. While the causes of these losses are unknown, irregular lineage specification is likely to blame. Furthermore, exposure to environmental stressors, particularly maternal nutrition, *in utero* is known to impair early development and lead to poor fetal outcomes [6]. Therefore, it is imperative that we gain a better understanding of the events coordinating preimplantation development and how environmental stressors may impact those events. This literature review will provide a report of our current understanding of embryonic lineage specification events and how maternal obesity may affect preimplantation development. This review will also provide insight into current gaps in our knowledge of these processes with the hope of developing new approaches to limit early embryonic losses and counteract poor postnatal development resulting from intrauterine stress.

Pregnancy Loss

Pregnancy loss is not uncommon in mammalian species, though some mammals experience higher rates than others. Of particular interest is the prevalence of pregnancy loss in cattle, as they are a major agricultural resource. In the dairy industry, increases in the incidence of pregnancy loss are highly correlated with the boom in milk production experienced over the past several decades. The two occurrences appear to be inversely correlated, with increases in milk yield coinciding with decreases in fertility [2, 3]. Approximately 60% of all viable pregnancies fail in lactating dairy cattle [4, 5]. Though slightly less severe, reports in beef cattle indicate rates of pregnancy loss averaging 20-44% from fertilization to calving [7]. These losses are typically blamed on aggressive genetic selection practices and the use of various assisted reproductive technologies, however, the timing of these losses suggest developmental miscues during early embryogenesis may be to blame.

Early embryogenesis (day [D] 0-24) has been identified as a prominent period of pregnancy loss in cattle. Utilizing an embryo transfer scheme, data indicate that fewer than 50% of viable embryos are able to progress past early embryogenesis [7]. Late embryogenesis (D 24-42) is not immune to loss, as an average rate of 12.8% of pregnancies fail at this stage. Fetal losses, those occurring after D 42, average 2.5% loss. Pregnancy losses are costly for the dairy industry, amounting to approximately $555/pregnancy based on the cost of re-breeding and loss in milk production [7]. Embryonic and fetal mortality also costs the United States beef industry an estimated $1.2 billion each year [7]. While the timing of these losses suggests developmental miscues during the earliest cellular divisions and specification events, the exact mechanisms involved remain unknown.

Humans experience relatively high rates of pregnancy loss as well, however, mortality rates are highly variable. Reports indicate between 50-90% of all conceptions fail to make it to

term. A majority of these losses occur during the first trimester, and between 30 to 75% of losses are experienced before implantation (D 6-7 post-fertilization) [1]. Early embryonic losses typically go unnoticed by most women, however, it can become a financial burden for those undergoing IVF, which can cost upwards of $12,000/cycle. As in cattle, little is known about the causes of pregnancy loss in humans. Early embryogenesis incurs the highest rates of pregnancy loss, therefore a better understanding of the developmental events occurring at this time will likely provide information to help mitigate these losses.

Early Embryogenesis

Early embryogenesis varies among mammalian species, however some similarities in the mechanisms controlling the earliest cell fate decisions exist. Work in mice, humans, and ruminant species has identified similarities in the signaling mechanisms involved in the earliest cell divisions and lineage specification events. A majority of work has been conducted in mice because of their short gestation length and they can be easily manipulated. Cows have also been extensively studied because of their agricultural importance, as well as the popularity of conducting various assisted reproductive technologies for production purposes. These processes include artificial insemination (AI), *in vitro* fertilization (IVF), and embryo transfer (ET). The similarities in the earliest developmental events among mammals allow researchers to apply findings across several species.

While the mechanisms involved in early embryogenesis are similar among most mammals, the timing of implantation is a phase of development in which species differences become more evident. Unlike the human and mouse, the ruminant embryo will elongate into a filamentous conceptus prior to implantation. The ruminant conceptus also experiences delayed implantation and requires the production of interferon-tau (IFNT) for maternal recognition of

pregnancy. These events will be discussed at length in subsequent sections, and species differences will be identified.

Initial Embryonic Cell Divisions

Mammalian reproduction requires the fusion of two parental haploid cells, the sperm and oocyte, via fertilization. Following oocyte penetration, the sperm nucleus must decondense to allow male and female nuclear material to interact. The process of fertilization concludes with the fusion of the male and female pronuclei into a single diploid nucleus, termed syngamy. Interestingly, this phenomenon is not observed in all species. The first cell cleavage of bovine embryos begins 23-31 hours post-fertilization [8, 9]. Progression to the 4-cell stage occurs at approximately 36-50 hours after fertilization, and the 8-cell stage is reached at 56-64 hours. Viable bovine embryos reach the 16-cell stage by 80 hours post-fertilization [10]. On day 3 of pregnancy, the maternal genome becomes inactivated, and the embryo begins transcribing its own genome. The maternal to embryonic transition is termed embryonic genome activation (EGA) [11]. Prior to EGA, the maternal genome has complete control of embryonic development, from guiding early cellular patterning to directing the first mitotic cellular divisions [12]. The maternal to embryonic transition of genome control is crucial for embryonic survival, as it allows the embryo to respond to environmental stressors that may otherwise impede development.

Soon after EGA, the embryo begins a process termed “compaction”. Embryonic compaction results from increased contact of neighboring blastomeres. The increase in cell-cell contact results in the formation of a dense sphere of cells. This change in cell behavior marks the beginning of the morula stage. The morula must then undergo a process termed “cavitation” prior to the end of the morula stage to form the internal cavity [13]. Cavitation and the formation

an internal blastocoel cavity marks the transition of the embryo from the morula to the blastocyst stage. This process occurs by day 7 post-fertilization in bovine embryogenesis.

The differentiation of the trophectoderm (TE) from the inner cell mass (ICM) is the first cell-fate decision in the developing embryo. This process has been extensively studied in mice, and similar events presumably exist in other mammalian species, including cattle and other ruminants. In mice, the process begins during compaction, when the *PAR* (partitioning-defective) proteins, *EMK* (ELKL motif kinase) and *aPKC* (atypical protein kinase C), are disproportionately expressed throughout the embryo [14, 15]. These factors are known for their roles in asymmetric cellular divisions. Work in the mouse revealed that a member of the *PAR* protein family, *PARD6b* (par-6 family cell polarity regulator beta), and *EMK1* are initially co-localized in the nuclei of all blastomeres. However, following compaction, *PARD6b* travels to the apical pole of blastomeres, while *EMK1* re-distributes along the basolateral area of the embryo. By the 16-cell stage, *aPKC* co-localizes with *PARD6b*, forming a PAR/aPKC complex [16]. This complex is recognized as a mediator of cell polarity, as blastomeres from 4-cell mouse embryos that are artificially forced to down-regulate *aPKC* tend to localize to the inner embryo rather than along the apical pole as they would under normal conditions [15].

Subsequent cellular divisions occur by either symmetric or asymmetric divisions. Symmetric divisions result in two polar daughter cells, both maintaining an outer position within the embryo. Asymmetric divisions produce one polar outer cell and one apolar inner cell [17]. At the completion of these consecutive cellular divisions, two distinct cell populations have formed; outer TE cells and internal ICM cells. Though cell lineage allocation begins at the 8-cell stage in the murine embryo, cells maintain a level of plasticity for a short period of time. This occurrence is observed when excised ICMs conserve their ability to form blastocyst-like structures, containing both ICM and TE cells [18]. This suggests that in early embryogenesis, exposure of

ICM cells to the outer perimeter of the blastocyst is sufficient to cause cells to re-polarize and incorporate into the TE lineage. However, this plasticity is not maintained, but rather is lost by the expanded blastocyst stage, at which point cells are fated to either the ICM or TE lineage [19, 20].

Transcriptional Control of Trophectoderm Differentiation

The molecular control of TE specification in the murine model is extensively studied. Trophectoderm commitment is dependent on the expression of caudal-related homeobox 2 (*Cdx2*) and the simultaneous repression of octamer-4 (*Oct4*), a factor responsible for maintaining ICM cell pluripotency [21]. *Cdx2*-null mouse embryos have been used to highlight the necessity of *Cdx2* in TE specification. These mutants progress normally through the blastocyst stage, however, they fail to implant as a result of inadequate TE development [22]. Furthermore, reduced *Cdx2* expression in embryonic stem cells (ESCs) promotes the maintenance of a pluripotent ICM, while increased expression drives cells toward a TE fate [23]. This suggests that *Cdx2* and *Oct4* interact to maintain homeostatic expression. In mice, *Cdx2* and *Oct4* begin to be restricted to the TE and ICM around the morula stage and achieve complete differential expression at the blastocyst stage [24].

Tead4 (TEA domain family member 4) is also recognized as a mediating factor of TE segregation in the mouse model. *Tead4* plays a role in the development of the blastocoel cavity and acts upstream of *Cdx2*. Reductions in *Tead4* expression result in impaired TE development, and decreased expression of TE-specific genes [25, 26]. Interestingly, *Tead4 is* not lineage-restricted, but rather is expressed in both the ICM and TE cells. However, it is only required for TE differentiation and plays no apparent role in ICM formation in the mouse [25, 27]. *Tead4* acts as regulator of Hippo signaling, the pathway responsible for contact inhibition in proliferating

cells [28]. Cells actively proliferate at low densities, but proliferation ceases as cells become confluent. This contact-dependent proliferation is observed within the developing embryo, where Hippo signaling is active in the inner cells, those with a high level of cell contact and weak in bordering cells [27]. Hippo activation within the inner cells of the embryo has an inverse relationship with Yes-associated protein (Yap) expression. Yap proteins are present in the nuclei of the outer cells of the embryo and are absent from inner cells. When Hippo signaling is artificially suppressed within the embryo, inner cells co-express Yap and Cdx2, patterning that is normally exclusive to the outer cells of embryo [27]. Thus, the outer cells of the embryo, those with limited cell-to-cell contact, experience weak Hippo signaling and increased Yap expression. This cascade of events activates *TEAD4*, thus inducing *Cdx2* expression, and promotes TE-specification thereafter.

Few of these TE specification processes have been explored in bovine embryos, though key components appear to be similar between mice and cattle. TE-specific expression of *CDX2* and *TEAD4* appears in bovine embryos; however, *OCT4* expression differs in cattle, and likely other ruminants. In the mouse model, *Oct4* is co-expressed in ICM- and TE-fated cells until the blastocyst stage. Expression is limited to the mouse ICM thereafter. In bovine embryos, *OCT4* is produced in both ICM and TE cells until day 11 post-fertilization, well beyond blastocyst formation [29]. Subsequently, *OCT4* is localized exclusively within the ICM of bovine embryos. The significance of this discrepancy between species is unclear, though the distinction in *CDX2/OCT4* profiles suggests that the relative importance of *OCT4* in lineage specification in the bovine embryos may be minor compared to that in murine embryogenesis.

Ruminant Conceptus Elongation

Embryonic implantation into the uterus occurs soon after the blastocyst stage in many species, including humans and mice, occurring on days 7-9 and 4, respectively [30]. In cattle and sheep, however, the implantation process is delayed. Following the blastocyst stage, bovine TE cells experience extensive proliferation, resulting in an elongated, filamentous embryo, termed "conceptus". By D 16 of pregnancy, the bovine conceptus reaches 10-30 cm in length. The elongation process continues until the time of uterine attachment, around D 21, at which time the conceptus reaches a length of 50-200 cm [31]. The process of elongation is imperative for the maintenance of pregnancy, as it creates increased surface area for conceptus attachment to the endometrium. It also increases the number of IFNT-producing TE cells, thus increasing the likelihood that maternal recognition of pregnancy will occur. While less studied, these events appear to be similar in the sheep, though they occur 2-3 days earlier than in the cow.

Primitive Endoderm

The second fate-determining step of embryogenesis is the allocation of a population of ICM cells to form the primitive endoderm (END) [32]. Following TE formation, the ICM differentiates into two distinctive cell populations; the END and the epiblast. Once segregated, END cells migrate along the inner TE border until they completely line the blastocoel cavity. The END differentiates further into two sub-types; the visceral endoderm (VE) and parietal endoderm (PE), which give rise to distal and proximal portions of the embryonic yolk sac, respectively. These tissues provide gas, nutrient, and waste exchange between the embryo and the maternal system prior to the establishment of the chorio-allantoic placenta [33-35]. In cattle, the yolk sac reaches maximal development at D 20 of pregnancy. The yolk sac then undergoes a rapid regression because its nutritive function is taken over by the emerging allantois [36, 37].

The yolk sac is present during bovine conceptus elongation and maternal recognition of pregnancy, times of marked embryonic loss. This makes its precursor cells, the END, a particular interest of this lab.

Primitive Endoderm Specification

Early research of the END focused on identifying the timing of END/EPI differentiation in the mouse model. These studies revealed that by the expanded blastocyst stage, cells of the ICM lose some plasticity and the ability to differentiate into the TE lineage; however, their ability to differentiate into both the END and EPI cells is conserved [19, 38]. When the ICMs of early mouse blastocysts are isolated, cells are able to differentiate into each of the primary lineages [39, 40]. Thus, a timeline of END and EPI fate-determining events was established. Unfortunately, the mechanisms controlling the expression patterns of lineage-specific precursors were not well-understood at that time.

By embryonic day 4.5 in the mouse, END and EPI are morphologically distinct, an observation that lead to the initial model for END-EPI segregation [41]. In this paradigm, the ICM was made up of a homogenous cell population, thus differentiation was believed to be dictated by location within the embryo; cells lining the outside of the ICM became END, while cells localized deeper within the structure adopted an EPI fate [42]. This initial model was later challenged when cells of the murine ICM were found to regulate the expression of various transcription factors depending on their fated lineage. This was later described as the "salt and pepper" model. This model described the differential expression of EPI and END markers, *Nanog* and *Gata6*, prior to the emergence of a definitive END layer within the murine embryo [43]. Lineage-tracing experiments provide further support of this model. These studies examined the patterns of individually-labeled ICM cells from early mouse blastocysts and reported that

individual cells contribute to either the END or EPI lineage. They also found that location of these cells within the ICM did not definitively dictate a cell's fate [44]. These data suggest cells of the ICM are fated toward one lineage or the other prior to the appearance of a distinct END layer. This lead to the proposal of a new paradigm referred to as the three-phase model. In phase one, the morula stage, *Gata6* and *Nanog* are co-expressed in the ICM. Expression then becomes highly regulated, and cells experience "salt and pepper" distribution throughout the ICM. The final step is the physical separation of the two cell types to form the END and EPI lineages [45].

Further examination of ICM cell sorting indicates the process is more complicated than the concurrent expression and repression of *Gata6* and *Nanog*. Live-imaging technology paired with the END -specific single-cell resolution fluorescent reporter, $Pdgfra^{H2B\text{-}GFP}$, uncovered highly-regulated cell behaviors involved in the process of differentiating mouse END from the ICM. This work found that cells lining the blastocoel cavity rarely changed their position within the embryo. These cells also appeared to increase GFP, and thus *Pdgfra*, expression. Interestingly, the behaviors of cells deeper within the ICM were more multifaceted. Some GFP-positive cells migrated to the blastocoel border and gave rise to the END. Additionally, a sub-population GFP-positive cells also within the ICM either down regulated GFP expression or underwent apoptosis [45]. These findings revealed the intricacy of the cell sorting process and identified a second cell sorting mechanism in which cells failing to integrate into the END lineage are forced to either undergo apoptosis or revert to the EPI fate.

Molecular Determinants of END and EPI Precursor Cells

The allocation of embryonic cells to the END or EPI lineage is initiated by the highly regulated expression of *Gata4/6* and *Nanog*, two lineage-specific transcription factors. EPI precursors express markers of pluripotency, *Sox2*, *Oct4,* and *Nanog.* However, *Nanog* is the only

known factor to be expressed exclusively by EPI cells, while *Sox2* and *Oct4* are common pluripotency markers found in several different cell types. This finding implicated *Nanog* as the predominant factor driving EPI specification. Similar to EPI precursor cells, END progenitors express *Sox2* and *Oct4* until implantation, though *Gata4/6* is used as their identifying transcription factor. However, the increased expression of lineage-specific transcription factors is not observed until the early blastocyst stage. Prior to the blastocyst stage, the ICM appears to be a homogenous cell population co-expressing *Gata6* and *Nanog* [44, 45].

Following the early blastocyst development, cells within the mouse ICM become a more heterogenous population and begin to express the lineage-specific transcription factors of their fated cell type. A subpopulation of cells down regulates *Nanog* expression, while simultaneously increasing *Gata6* production. This, in turn, results in the increased expression of *Sox17* [46]. Cells experiencing this transcript patterning are fated toward the PrE lineage. The remaining cells of the ICM reduce *Gata6* expression while concurrently increasing *Nanog* levels. This expression behavior produces the EPI lineage.

While the END and EPI lineages differentially express *Gata4/6* and *Nanog*, a cross-talk mechanism appears to be present between the two transcripts. This phenomenon was identified using *Nanog*-mutant mouse embryos, in which *Nanog*-null embryos failed to produce the END marker *Gata4* [47]. This finding opposed the previously accepted idea that *Nanog*-null embryos would show rampant expression of END factors due to the absence of inhibitory action from *Nanog*. Instead, these findings point out the necessity for both *Nanog and Gata6* signaling in END development and necessity of these interactions for successful embryogenesis.

The Requirement of Fibroblast Growth Factors in Primitive Endoderm Development

Fibroblast growth factors (FGFs) are recognized as regulators of mammalian END development. Mouse embryos lacking *Fgf4*, its receptor *Fgfr2*, or the receptor adaptor protein *Grb2* failed to form the END lineage [48-51]. Additionally, the targeted disruption of *Grb2* in mouse embryos inhibits *Gata6* expression and impairs subsequent END development [44]. Moreover, murine embryoid bodies derived from $Fgfr1^{-/-}$ embryonic stem cells experienced a reduction in expression of α-fetoprotein, a END marker and failed to produce viable visceral endoderm (VE), a derivative of the END [52]. Finally, murine germ layer explants were differentiated into the endoderm lineage following exposure to *Fgf4* supplementation [53]. Collectively, these studies implicate FGFs in early END specification and development.

Primitive Endoderm Formation in Ruminant Species

Primitive endoderm markers show a more diverse expression pattern in cattle compared to other mammalian species. Work in this laboratory found that *GATA4/6* mRNA levels increase during blastocyst hatching and expansion [54]. Unlike in mice, however, *GATA4* expression is not lineage-specific and can be detected in both TE and ICM cells, whereas *GATA6* is restricted to the ICM [55]. The significance of these species differences in early *GATA4/6* expression remains unclear. A better understanding of these events may help identify the mechanisms responsible for maintaining early pregnancy in ruminant species.

As in the mouse, FGFs appear to have a role in bovine END development. Work from this laboratory identified FGF2 as a product of the ruminant endometrium during early pregnancy. Studies also identified FGF2, FGF4 and FGF10 as factors produced by the peri-implantation ruminant embryo [56-58]. Additionally, several FGF receptors, have been identified in the peri-implantation bovine embryo. One such receptor is FGFR2, which is required for END

specification in the mouse embryo [58, 59]. Work from this laboratory also determined that FGF2 supplementation in bovine embryo culture medium can promote the development of END cells [54]. This method improved the frequency of END development so drastically that it appears it can be utilized as an *in vitro* tool to study bovine END development. Cell lines for bovine endoderm are noticeably absent, creating a gap in our knowledge of bovine endoderm formation. We have yet to identify the techniques required to maximize END development *in vitro*. Similarly, the importance of END, and resulting yolk sac, in the maintenance of early pregnancy is not well understood.

Epiblast Specification in Cattle

A subpopulation of cells within the blastocyst are maintained in a pluripotent state throughout early development. These cells are referred to as the epiblast, or EPI. The outermost cells lining the EPI, termed "ectoderm", segregate from other EPI cells by forming an elongated ridge along the germinal disc. This ridge is the called neural ectoderm, and will give rise to various nervous system derivatives, including the brain, and spinal cord [60].

By gestational D 10 in cattle, the inner boarder of the TE is completely lined with endoderm cells [31]. Shortly thereafter, a second layer of cells forms between the endoderm and the TE. This layer is referred to as the extraembryonic mesoderm (ExM), and its specification begins on D 14 of development [61]. The mesoderm will eventually develop into connective tissues, vascular systems, bones, and muscle, whereas the ExM will juxtapose the TE and create the chorion [60]. As embryogenesis progresses, the chorion will grow to form amnionic folds which eventually fuse to surround the entire conceptus. The fusion of the amnionic folds creates the amnion [62]. Initially, the chorion and amnion are separated by a relatively large cavity,

however, as embryogenesis continues to progress, the amnionic membrane fuses with the chorion, resulting in the amnio-chorionic membrane [60].

The formation of the allantois from the primitive gut occurs around D 20 in cattle and is concurrent with amnion development [61]. Almost simultaneously, the embryonic yolk sac regresses, although it remains visible and active in cattle until day 50-70 of pregnancy [60, 63]. The allantois serves as a collection site for embryonic waste, and also supports blood vessel formation to ensure sufficient blood flow to the chorionic and amnionic tissues. Eventually, the chorion and allantois fuse to become the chorio-allantoic placenta, thus pulling the fetal blood supply closer to the maternal system to provide efficient nutrient and waste exchange [64].

Maternal Recognition of Pregnancy in Ruminants

Prior to implantation, it is imperative that the maternal system recognizes viable pregnancies. Failure to do so results in the resumption of the estrous cycle and pregnancy loss. The factor responsible for maternal recognition of pregnancy in ruminants is IFNT, which is produced by the TE cells. IFNT is a member of the Type 1 interferon family which are responsible for eliciting an immune response following viral infection. Type 1 IFNs also possess anti-proliferative properties [65]. However, IFNT is unique to other type 1 IFNs, as it has a critical role in maternal recognition of pregnancy in ruminant species.

Expression of *IFNT* is highly regulated and occurs during a distinct window of conceptus development. *IFNT* mRNA and proteins can initially be detected at the morula and early blastocyst stage, D 6-7 of development [66, 67]. While moderately low at the blastocyst stage, bovine *IFNT* levels progressively increase until around D 14 for pregnancy. At this time, conceptuses experience a surge in *IFNT* mRNA production [67, 68]. This exponential increase in *IFNT* mRNA levels is also observed in sheep, though it occurs at D 12 of pregnancy [69, 70].

The sharp increase in *IFNT* production corresponds with substantial conceptus elongation. *IFNT* mRNA levels decline dramatically after day 19 to 21 in cattle and day 16 in sheep, coinciding with conceptus attachment to the uterus [71, 72]. The mechanisms responsible for this sudden reduction in IFNT expression are unknown, though it may be a response to physical contact with the maternal system, resulting in altered conceptus gene expression.

IFNT Mechanism of Action

Currently, IFNT is recognized as the exclusive factor involved in maternal recognition of pregnancy in ruminants. This argument is based on work where uterine profusion of IFNT extended the estrous cycle length in non-pregnant ewes [73, 74]. Similar work was performed in the bovine model and demonstrated that intrauterine IFNT supplementation extended the lifespan of the CL and the inter-estrous interval. IFNT treatment also eliminated the rise in prostaglandin-F2α (PGF2α), a hallmark of the beginning of luteolysis [75]. It is important to note, however, that IFNT is only present until implantation. This suggests the potential for other pregnancy recognition factors later in gestation. IFNT may only be the first in a series of pregnancy recognition factors required to maintain pregnancy.

Interferon-tau plays a multifaceted role in the maintenance of early pregnancy. The primary role of IFNT is to prevent corpus luteum (CL) regression by acting as an anti-luteolytic factor. In non-pregnant animals, uterine pulses of PGF2α regress the CL and cause the resumption of estrous cyclicity. This allows females to prepare for another attempt at a successful breeding. Oxytocin (OT) controls the PGF2α pulses by binding to its receptor (OTR) which stimulates PGF2α production. A feed-forward system ensues. The CL releases OT in response to PGF2α, thus increasing endometrial PGF2α production [71]. IFNT directly prevents CL regression by blocking OTR expression in the endometrium, and therefore inhibits PGF2α

synthesis [62]. IFNT may also act indirectly by inhibiting estrogen receptor expression, as OTR development is stimulated by estrogen. This mode of IFNT action prevents the development of OTRs in the uterine epithelium [76]. These direct and indirect actions of IFNT ensure viable pregnancies are maintained in ruminant species.

Interferon-tau has been implicated in several other activities presumed to aid in pregnancy maintenance. IFNT acts directly on the endometrium to promote the expression of multiple IFN-stimulated genes (ISGs). The temporal expression of ISGs strongly correlate to their proposed roles in conceptus elongation, uterine receptivity, and implantation [72]. Many ISGs, are expressed in the uterine epithelium during pregnancy. Studies indicate these genes increase in expression during conceptus elongation, and their expression is reduced following during uterine attachment. This patterning parallels that of IFNT concentrations, implicating IFNT as a modulator of their expression [77, 78]. IFNT has is implicated as a modulator of the maternal immune response during early pregnancy. This has not been proven, however, as immune function during the peri-implantation period is not currently well-defined [79].

Uterine receptivity and Conceptus Implantation

In mammals, a successful pregnancy requires that the uterus be receptive to the implanting embryo. This receptivity occurs in a short window of time when the uterine environment is ideal for implantation [80]. The timing of implantation varies among species, with rodent embryos implanting on D 4 of pregnancy, and ruminants experiencing “delayed implantation” beginning around D 14-16 post-fertilization [81, 82]. Additionally, rodent, non-human primate, and human embryos implant as spherical blastocysts, while ruminant species undergo rapid elongation prior to implantation. The reason behind these discrepancies is unknown, however, the process of implantation and factors involved in uterine receptivity appear

to be somewhat conserved. Adequate implantation is a prerequisite for placentation and fetal growth throughout the remainder of pregnancy. Insufficient implantation likely plays a role in the high rates of pregnancy losses observed in both humans and cattle following early embryogenesis. The causes of these pregnancy losses are unknown, however, the timing points to inadequate implantation and possible secondary issues resulting from it (i.e. poor placental vasculogenesis, inefficient maternal-embryonic nutrient transport, etc.). The following sections will discuss implantation and placentation events in ruminant species.

Changes to Adhesion Molecules on the Endometrial Surface Prior to Implantation

Uterine receptivity in the sheep appears to be predominantly controlled by progesterone. Progesterone plays a pivotal role in the establishment and maintenance of pregnancy in mammals, and its receptor (PR) undergoes spatial and temporal expression throughout gestation. PR expression is detectable throughout gestation in the endometrial stroma and myometrium through most of pregnancy, while expression in the luminal and germinal epithelium is not detected until D 11 and 13, respectively [83, 84]. This scripted regulation of PR expression is believed to directly regulate downstream determinants of conceptus attachment, including anti-adhesion molecules and various embryotrophic factors.

Prior to implantation, the endometrium contains an abundance of *MUC1*, a large mucin glycoprotein acting to prevent premature TE attachment by preventing cell-cell and cell-extracellular matrix adhesion [85, 86]. The adhesion/implantation cascade in the ewe begins with the loss of PR and down regulation of MUC1 on the uterine epithelium [87]. The reduction in MUC1 exposes other glycoproteins and pro-adhesion factors to initiate the conceptus-endometrium interaction. One such factor is GlyCAM-1. This factor is also a member of the mucin family and is primarily found along the luminal surface of the endometrium [72, 88].

Work in cyclic and pregnant ewes highlights GlyCAM-1 as a probable adhesion factor [89]. In cycling ewes, GlyCAM-1 expression increases in the endometrium between D 1-5 and then decreases between D 11-15. In pregnant ewes, expression is relatively low at D 13, increases on D 15, and is abundant on D 17-19. Interestingly, GlyCAM-1 expression can also be detected on the conceptus on days 13-19. The temporal patterning of GlyCAM-1 expression coincides with the implantation period, thus it is believed to play a role in maternal-conceptus communication and successive implantation in the ewe.

Finally, firm adhesion to the endometrium is mediated by integrin heterodimers and their binding molecules, such as osteopontin (OPN). Integrins are transmembrane glycoprotein receptors with known roles in cell adhesion, and mutations in integrin subunits results in implantation failure [90, 91]. In ewes, *OPN* mRNA expression increases in uterine gland tissue beginning at D 13 [92]. It is also found in uterine flushings, although in decreasing concentrations between D 11-17 [92]. The role of the integrin-OPN interaction has been identified in the ewe. As with other endometrial adhesion factors, OPN induction is associated with a loss in PR in the endometrium [93]. The reduction in MUC1 allows for the uninhibited binding of OPN to its integrin receptors, thus promoting TE binging to the endometrium [87].

Phases of Implantation in the Ovine Model

Conceptus implantation in the ewe occurs over the course of 4 phases [72, 81, 94]. Phase 1 involves shedding of the zona pellucida and occurs between D 8 and 9 of pregnancy. The zona pellucida is the outer-most glycoprotein layer of an early embryo, and acts to protect the blastocyst from damage. It is also suggested the zona pellucida acts to prevent the embryo from premature uterine attachment, making it imperative to successful implantation.

Phase 2 of implantation takes place between D 9 and 14 of gestation [72]. During this time, the embryo rapidly elongates into a filamentous conceptus, as previously reviewed. The conceptus becomes immobilized during this time, though no direct contact with the uterine endometrium is made. Blastocysts fail to elongate *in vitro*, thus the lack of contact makes this phase of implantation attractive to researchers interested in early embryonic development. At D 14, conceptuses contain ample tissue and can easily be collected via uterine flush. The inability to attain conceptus elongation in an *in vitro* culture system highlights the importance of the uterine environment and maternal-embryonic cross-talk during early development.

The third phase, termed "apposition", involves the conceptus trophectoderm becoming loosely associated with the uterine endometrium, and takes place between D 14 and 15 of gestation. During this phase, the endometrium must undergo biochemical changes as well a cellular restructuring events to be receptive to the implanting conceptus. These processes will be discussed in later sections. Conceptus apposition is accomplished by the interdigitation of trophoblast projections and endometrial microvilli, and appears to be a somewhat controlled process, beginning with the TE cells in the nearest the ICM and expanding outward to the extremities [81]. During the early stages of apposition, D 15-18, the TE forms villi that penetrate uterine glands, acting as temporary sites of attachment and direct absorption of uterine secretions [95]. These projections regress at D 20, when firm conceptus-uterine attachment occurs [81].

The final phase of implantation in the ewe is adhesion, as the sheep conceptus does not undergo true endometrial invasion. The conceptus begins to firmly adhere to the endometrium at D 16, and the process is completed by D 22 [72, 81, 96]. During this time, the TE and endometrial epithelium interdigitate to form the conceptus cotyledon and endometrial caruncle [94, 97]. Together, these structures form the maternal-fetal interface, referred to as placentomes, and are responsible for utero-placenta transfer throughout the remainder of gestation.

The Ruminant Placenta

The placenta is a temporary endocrine organ required for embryonic and fetal survival in eutharian mammals. The placenta acts to mediate maternal-fetal nutrient, waste, and gas exchange [98, 99], and also produces and secretes several hormones, growth factors and cytokines to maintain a hospitable environment [100, 101]. These processes are required to promote fetal growth.

Ruminant species have developed a highly specialized synepitheliochorial and cotyledonary placenta. The reasoning for the evolution of the cotyledonary placenta is still not fully understood, however, the timing closely correlates to the evolution of the rumen. The rumen has allowed ruminant species to thrive on high fiber food, though it came at the cost of low glucose availability for resorption. The blood glucose levels are lower in a majority of ruminant species compared to monogastric [102]. Insufficient glucose levels during pregnancy can lead to severe health conditions (i.e. ketosis), however, glucose is necessary for fetal development, so the maternal system cannot overly restrict its transfer to the fetus [103]. Evolutionary biologists postulate that this maternal-fetal conflict lead to the evolution of the less efficient cotyledonary placenta from the highly efficient diffuse placenta of the pig and horse [104].

Cell Types of the Placenta

The ruminant placenta contains two distinct cell types, mononucleate cells (MNCs) and binucleate cells (BNCs). These morphologically and functionally distinct cell types play crucial roles in pregnancy. MNCs are the most abundant cell type of the placenta, contributing to nearly 80% of the trophoblast population [105]. Early identification of MNCs noted the irregular shape of the cells' single nuclei, as well as their resemblance to epithelial cells [105]. The surface of

MNCs form microvilli that interdigitate with similar processes along the surface of endometrial cells, thus initiating maternal-embryonic contact and adhesion [106, 107]. These points of contact are where a majority of maternal-fetal nutrient exchange occurs. MNCs are also responsible for producing IFNT, as described in a previous section.

Ruminant BNCs, sometimes referred to as trophoblast giant cells, are formed by MNC differentiation, just prior to implantation [108]. The BNC population makes up about 20% of all TE cells, however, these numbers drop drastically 1-2 days prior to parturition [109]. The BNCs serve at least two main functions; 1) forming the feto-maternal syncytium by fusing with the maternal epithelium and 2) serving as the major endocrine cells of the placenta [100, 110]. BNCs are highly migratory and can be seen throughout migrating to the apical TE surface throughout pregnancy [111]. Following their migration, BNCs fuse with the maternal epithelium, forming maternal-fetal hybrid cells, or trinucleate cells and create the syncytial plaques of the placenta [110, 112]. Syncytial plaques persist throughout pregnancy and continue to grow because of constant BNC migration [110, 112].

The endocrine function of placenta BNCs cannot be overlooked, as they are responsible for secreting a number of hormones into the uterine environment. One major hormone produced by BNCs is placental lactogen (PL), which also is occasionally referred to as chorionic somatomammotropin hormone 1 (CSH1). PL production begins on D 16 of gestation in the sheep [113]. PL is able to bind to and activate GH/PRL/PL receptors, as it is a member of the growth hormone (GH) and prolactin (PRL) hormone family [114, 115]. PL may play a role in uterine gland function during early pregnancy, and its production is closely associated with gland morphogenesis and histotroph production by the glandular epithelium [84, 116]. PL's role in uterine gland function implicate it as an important facilitator of conceptus development.

A second member of the growth hormone (GH) /PRL family secreted by BNCs is prolactin-related protein (PRP) [117]. Nine PRPs have been identified in the bovine placenta, and 2 novel PRPs have been identified in sheep [118, 119]. As in other ruminant species, ovine PRPs are localized to BNCs in both the placentomal and intercodyledonary tissue [118]. The implications of multiple PRPs is not currently understood, however, their specific temporal patterning throughout pregnancy suggests they play specific roles in conceptus implantation and subsequent fetal and placental growth [118-120].

In addition to PL and PRP, BNCs are responsible for the production of pregnancy associated glycoproteins, or PAGs [121, 122]. PAGs have been identified in a majority of ruminant species, though primarily researched in cows, sheep and goats [122-125]. PAGs can be separated into two classes; ancient and modern, with a majority belonging to the modern PAG class. Both classes are represented throughout pregnancy, however, specific temporal and localization patterning exists [126, 127]. For example, the ancient PAGs -2 and -8, are expressed on MNCs. Conversely the modern PAGs -1, -6, -7, and -9, are found in BNCs [122].

The role of PAGs in pregnancy is unknown, however, their blood plasma concentrations serve as a useful tool for early pregnancy detection as early, as they can be measured by D 28 of pregnancy [128, 129]. In cattle, PAG concentrations gradually increase until D 240 of pregnancy when they experience a drastic increase in concentration [130, 131]. PAG levels remain elevated until parturition, when they decrease as a result of placental expulsion [130]. Finally, plasma PAG concentrations can also serve as measurements of pregnancy viability and placental fitness. Reduced PAG concentrations in pregnant animals is indicative of impending fetal loss [132, 133]. Likewise, abnormal PAG concentrations are observed in the placentas of somatic cell nuclear transfer (SCNT) animals, presumably from less-efficient placentas [134]. While the

function of PAGs is not currently understood, they appear to be directly related to pregnancy viability.

To summarize, early embryogenesis is plagued by high rates of embryonic loss. These losses coincide with lineage specification events, implicating miscommunications in TE-ICM and PrE-EPI segregation for the losses. While the TE has been extensively studied in cattle, an understanding of the formation and function of PrE is currently lacking. A major factor impeding our understanding of PrE development is the absence of a method of promoting PrE development *in vitro*. A portion of the following work is focused on developing a protocol to produce bovine PrE *in vitro*. This work will further our understanding of PrE development and allow us to assess its potential interaction with TE to promote conceptus implantation and placentation.

The Developmental Origins of Adult Health and Disease

Abnormal developmental programming likely impacts early embryogenesis and placentation. Exposure to intrauterine stressors can have lasting impacts on offspring development. As described earlier, the period of early embryogenesis encompasses the phases of initial lineage specification. The earliest lineages eventually develop into the placenta, the yolk sac and the embryo proper, therefore, perturbations in the development of of these cell types likely result in the poor offspring outcomes observed following intrauterine stress. The following sections will review the current understanding of developmental programming and the effects of intrauterine exposure to maternal obesity.

<u>Early Evidence of Developmental Programming in Humans</u>

The impact of environmental factors on an organism's behavior and biology has been extensively studied, as this interaction is the recognized catalyst behind evolution. However, more recent research focuses on elucidating the immediate impacts the environment has on an

organism, and more specifically the effect of the uterine environment on offspring health. The hypothesis that environmental insults early in life can program offspring disease susceptibility was initially described by D. Barker. This phenomenon is often referred to as the Developmental Origins of Adult Health and Disease (DOAHD). Barker's early work demonstrated the relationship between geographical location, dictated by socio-economic status, and the incidence of heart disease and childhood mortality [135]. Subsequent work revealed that individuals with low birth weight had an increased risk of developing heart disease in adulthood [136, 137]. In addition, offspring with the lowest birth weights had a higher risk of developing type 2 diabetes as adults [135]. Results from these studies lead to the development of the thrifty phenotype hypothesis, which states that fetal programming resulting from environmental stressors, under nutrition in particular, is an adaptive mechanism developed to maximize the chances of postnatal survival. However, if the uterine nutrient supply does not match that of the postnatal environment, these fetal adaptations can increase the risk of disease in adulthood.

The link between poor intrauterine environment and resulting postnatal outcomes was well-documented in the Dutch famine cohort study from World War 2. During the famine, food rations fell to 400-800 kcal/day. The famine had profound effects on fetal outcomes, however, the effects appeared to be highly dependent on the stage of gestation during which exposure occurred. For example, males exposed to famine during early gestation were more likely to be obese while those exposed in late gestation were not [138]. Exposure to famine during early gestation was also linked to more atherogenic lipid profiles [139], increased blood pressure [140], a higher risk of coronary heart disease [141], and an increased incidence of breast cancer [142]. These epidemiological studies provided some of the earliest evidence of embryonic plasticity and the susceptibility to nutritional insults during embryogenesis.

Maternal Over Nutrition in Humans

The concept of DOAHD has more recently come to encompass the effects of maternal over nutrition, as the worldwide obesity rates are constantly rising. Approximately one-third of child-bearing age women (20 to 39 years of age) are overweight, and another one-third are obese in the United States [143]. Surprisingly, maternal over nutrition and an increased birth weight can illicit poor health outcomes similar to those observed in low birth weight offspring. Epidemiological studies show an association between maternal BMI and offspring obesity [144, 145]. Maternal obesity is linked to a rise in the in several birth defects including limb abnormalities, cleft lip, and hydrocephalus [146, 147]. Exposure to maternal obesity also affects health outcomes in later life, as it has been linked to an increased incidence of high blood pressure, increased risk of premature mortality from a cardiovascular event, and type 2 diabetes in offspring [148-151]. The exact mechanism behind maternal obesity and offspring outcomes is not well understood, however, various animal models have begun to provide insight into these interactions.

Fetal Programming; Insights from Animal Models

While human epidemiological investigations are valuable tools in understanding the effects of maternal obesity on offspring development, results are often influenced by uncontrollable confounding factors (i.e. maternal age, dietary nutrient levels, or environment). Thus, animal models have become invaluable in establishing the influence of maternal health on offspring outcomes. Rodents are the most common species used to study maternal-fetal programming, and they have been used to study a variety of obesogenic diets. Some of the earliest work in rodents examined the offspring of dams fed high-fat diets found increased adiposity, insulin resistance, and hypertension in those pups [152, 153]. More recently, however,

experimental diets have been changed to include high sugar content to better reflect current human diets. Under this dietary duress, offspring exhibit insulin resistance, hypertension, glucose intolerance, and reduced skeletal muscle mass [154-156]. Furthermore, maintaining these offspring on a high-fat diet post-weaning exacerbates the onset of metabolic syndrome.

Sheep models have been widely used to study maternal obesity and its impacts on offspring development. Dietary induced obesity is shown to increase triglyceride levels in lambs resulting of increased fatty acid transporter expression in the placenta, and also to up regulate inflammatory signaling [157, 158]. Lambs from obese ewes exhibit altered growth and glucose tolerance, and have increased adiposity in adulthood [159]. Furthermore, ewes exposed to an obese environment *in utero* are hyperglycemic, hyperinsulinemic, and show significant increases in pancreatic weight [160]. Unlike the human and rodent models, however, altered birth weight in lambs exposed to maternal obesity has not been reported, though postnatal growth is clearly affected [161, 162]. This suggests that while fetal programming events may be similar across species, differences in placentation, and thus nutrient allocation to the fetus, may impact initial offspring phenotypes.

The ovine model has also been used to demonstrate the effects of over nutrition on adolescent females. Utilizing an overfed adolescent ewe model, researchers showed over nutrition was associated with reduced fetal and placental weights, fewer cotyledons, and a higher rate of spontaneous abortion [163, 164]. These reports oppose findings in adult ewes, which indicate no effect on birth weight. An explanation for this discrepancy is that a growing animal will preferentially allocate nutrients toward growth rather than the fetus, thus resulting in reduced fetal growth during pregnancy.

The Placenta as a Facilitator of Fetal Programming

The placenta's intimate relationship with the maternal-fetal system, and its highly vascularized structure permit it to adapt to its environment in order to optimize its function and promote fetal development [165, 166]. However, this adaptive ability is limited, and placental inefficiency results in altered fetal development. In this context, the placenta is recognized as a mediator of fetal programming and DOAHD [167, 168]. With the increasing prevalence of obesity worldwide, an understanding of the impacts of maternal nutrition on placental development has become more urgent. In the rodent model, females experienced reduced placental and fetal growth when maintained on a high fat diet [169]. Placental growth retardation results in a higher fetal:placental ratio, an indicator of placental dysfunction. These findings support the paradigm that maternal nutrition directly impacts placental growth, which then negatively affects consequent fetal development.

The impact of maternal obesity on placental vasculature has also been assessed, as blood flow is a determinant of placental function and fetal growth. Again, utilizing the sheep model, researchers found comparable results to previous work. Fetal:placental ratios of the overfed group were greater on both day 75 and 135 of gestation [170]. Furthermore, blood vessel diameter was significantly larger in the obese group compared to controls on day 75 of gestation, however diameters were not different at day 135. Control animals were observed to increase blood vessel diameter during later gestation to support the developing fetus, while the obese animals did not experience the same growth. These vascular defects directly impacted fetal development. Fetuses of obese ewes were ~30% greater at day 75, though the weight discrepancy was lost by day 135. These data suggest that while the placenta was highly efficient at transporting the excessive amount of nutrients to the fetus during early gestation, it was not able to maintain that level of efficiency. Additionally, the loss of the weight discrepancy between

D 75 and 135 further suggest the placentas of obese ewes were inefficient at maintaining the fetal growth trajectory of earlier gestation. Finally, the cotyledons of obese ewes had a decreased expression of pro-angiogenic factors compared to lean animals.

Collectively, these data demonstrate that dietary excess during pregnancy negatively impacts placental function, thus implicating the placenta as a facilitator of DOAHD and fetal programming. Animal models have been extensively used to study the mechanisms involved in DOAHD; however, a majority of work has focused on fetal and postnatal outcomes of maternal obesity. There is a current gap in our understanding of the preimplantation implications of maternal obesity exposure. Examination of this critical period of development will provide insight into the effects of maternal obesity on the earliest cell lineages, which will eventually give rise to the placenta and fetus.

Summary and Implications

Preimplantation embryogenesis is hallmarked as a time of drastic cellular restructuring and lineage specification. Major developmental events during this time include TE-ICM and PrE-EPI specification, as reviewed in Figure 1. These cell lineages will eventually form the placenta, embryo proper, and yolk sac of the embryo. This time in development is also recognized as a period of high embryonic loss, likely due to misregulation of lineage specification events. Furthermore, exposure to environmental stressors, such as maternal obesity, during early embryogenesis likely impact lineage specification events, as altered placentation and fetal development have been reported. The following work was focused on developing a method of producing PrE in vitro, and on examining the effects of maternal obesity on the preimplantation conceptus and uterine endometrium.

A major gap currently exists in our understanding of the mechanisms behind mammalian PrE development and function. The identification of FGF2 as a necessary factor in PrE formation was an important discovery in lineage specification events of mice, primates, and ruminants; however, few bovine PrE cell lines exist. This limited availability has severely hindered our understanding of PrE and yolk sac development, and potential interactions with the TE. The following work also will describe the development of a protocol to promote PrE development *in vitro*. These cells will provide us with an *in vitro* model to study the mechanisms underlying PrE development. This work will provide us with information to allowing us to manipulate PrE growth and potentially lessen the high rate of embryonic loss currently observed in cattle.

Finally, these studies examine the impact of maternal obesity exposure on gene expression in the preimplantation conceptus and endometrium. A majority of work in this area has focused on the fetal and postnatal impacts of maternal obesity. The results of these studies are listed in Figure 1. The disregard for the preimplantation impacts of maternal obesity is surprising because placental and fetal precursors are specified during this time. Moreover, these precursors are likely susceptible to the environmental stress of obesity. The current work establishes the preimplantation period as a time of susceptibility to the maternal environment, and identifies several perturbed mechanisms following obesity exposure. These findings highlight specific biological processes with potential roles in DOAHD, and they may provide targets to reduce the impact of maternal obesity on offspring outcomes.

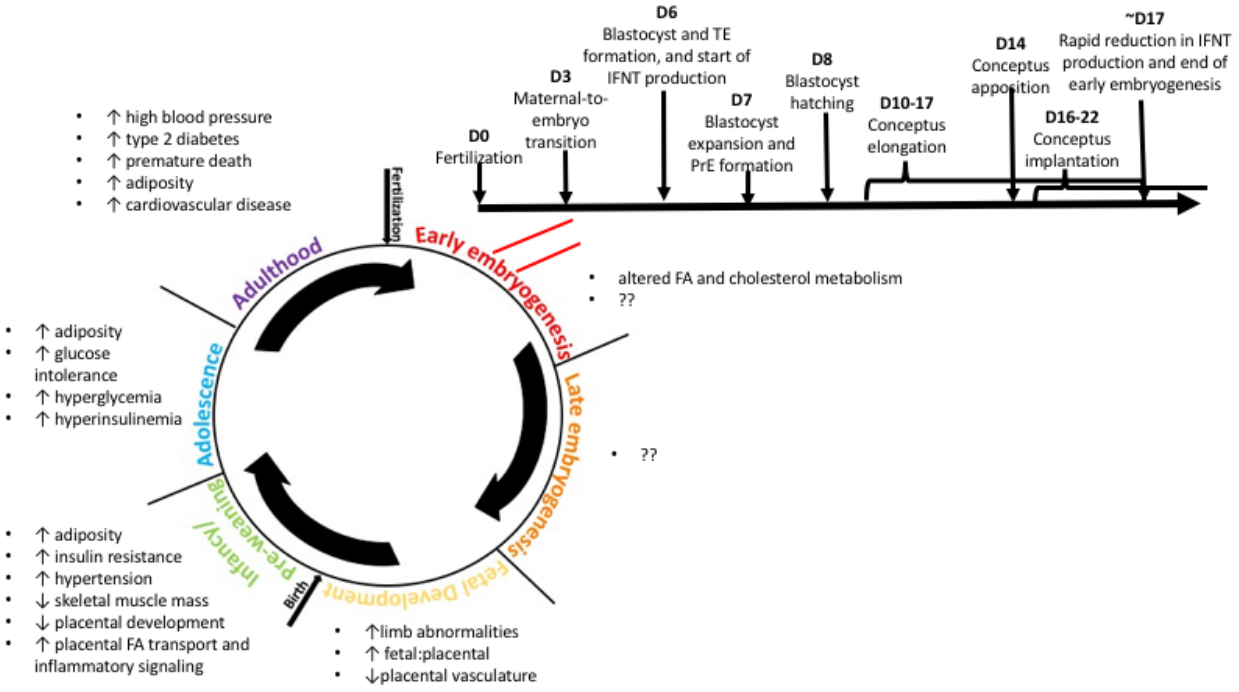


Figure 1-1. A summary figure of offspring outcomes at various stages of development following intrauterine exposure to maternal obesity. Effects of maternal obesity exposure on early embryogenesis are vastly understudied, though this period contains several crucial developmental events.

Chapter 2

Improving the Establishment of Bovine Endoderm Outgrowth Cultures

Introduction

Early embryonic development is distinguished by two cell lineage specification events. The first is segregation of TE and ICM. This process begins during blastomere compaction at the morula stage in cattle (approximately D 5-6 post-fertilization) and is determined primarily by blastomere position utilizing a Hippo-based system that responds to cell-to-cell contacts, or lack thereof [17, 27]. The second cell fate decision during early embryogenesis is the differentiation of the ICM into primitive endoderm (END) and EPI. The EPI will give rise to the embryo proper and the allantoic sac. The END will contribute to both embryonic and extraembryonic lineages. Extraembryonic END will form the yolk sac by migrating along the inner blastocoel border and underlying the TE layer in the blastocyst cavity. Based on position relative to the EPI, END will then differentiate into visceral endoderm (VE) or parietal endoderm (PE) and give rise to distal and proximal portions of the yolk sac, respectively. In cattle, extraembryonic END development begins at day 8 post-fertilization [54]. The resulting yolk sac develops into a large, elongated and vascularized membrane over the next few weeks. After D 40 it begins to recede but remains visible and functional until D 50 to 60 of gestation [63, 171].

A common misconception is that the yolk sac is rudimentary in ruminants and other mammals. Although the mammalian yolk sac does not contain yolk, like in reptiles, avians and monotremes, the yolk sac is indispensable in early pregnancy. In mice, early post-implantation embryonic lethality occurs in the absence of a yolk sac [172-174]. The yolk sac likely is also

vital for pregnancy retention in cattle. Yolk sac abnormalities appear to be an underlying cause for pregnancy failures for *in vitro* produced embryos and embryos generated by somatic cell nuclear cloning [175, 176]. The general population of dairy and beef cattle (*i.e.* those bred naturally or by artificial insemination) also suffer from substantial peri- and post-implantation pregnancy losses during the first 42 days of pregnancy [177, 178]. Placentomes are not well established in bovine pregnancies until on or after D 40 of pregnancy [37, 171], and prior to this time the conceptus must rely on histotroph (uterine gland secretions) and hemotrophe (blood-derived nutrients) for nourishment. The yolk sac is the organ responsible for the transfer of nutrients, vitamins, ions and gases to the embryo at this time in development [35, 179]. It also provides various carbohydrates, lipids and amino acids that act in metabolic signaling pathways to regulate growth, differentiation and cell death [63]. Several yolk sac proteins are also produced. These include transferrin, retinol binding proteins, apolipoproteins, insulin-like growth factors and their binding proteins, and several angiogenic factors [179, 180]. Lastly, the yolk sac is the site of initial blood vessel formation, hematopoiesis, and primordial germ cell migration [179].

Arguably the biggest limitation with learning more about the functions of END and the yolk sac in cattle and other ruminants is the lack of suitable cell culture models in ruminants. Several END cell lines exist for the human and mouse, and these cells have been used extensively to study early embryogenesis in these species [181-183]. However, only a few reports exist describing bovine END cell lines [54, 184, 185]. This has limited the close examination of early embryogenesis and END development in cattle and other ruminants.

Fibroblast growth factor 2 (FGF2) has been implicated as a promoter of END development. It is produced in the bovine endometrium and is secreted into the uterine lumen throughout the per-implantation period [56]. Additionally, the supplementation of FGF2 to

bovine embryo culture medium increases the incidence of END outgrowth production, though culture conditions appear to be sub-optimal with outgrowth rates averaging only 25% [54]. This report describes work aimed at 1) developing a protocol for improved rates of END outgrowth formation from bovine blastocysts, and 2) delineating the various types of END lineages represented in the resulting cultures.

Materials and Methods

In Vitro Embryo Production

In vitro production of bovine embryos was completed as described previously [186, 187]. Cumulus oocyte complexes (COCs) were derived from two sources. In some studies, COCs were collected from ovaries obtained from Brown Packing Co. (Gaffney, SC) and cultured in oocyte maturation medium (TCM199 containing Earle's salts [Thermo Fisher Scientific], 10% FBS, 25 μg/ml bovine FSH [Bioniche Life Sciences, Bellville, ON, CA], 2 μg/ml estradiol [Sigma-Aldrich, St. Louis, MO], 22 μg/ml sodium pyruvate, 1 mM glutamine, and 25 μg/ml gentamicin sulfate [Life Technologies]) at 38.5°C in 5% CO_2 in humidified air. In other studies, COCs were purchased from DeSoto Biosciences (Seymour, TN). These COCs also were derived from Brown Packing and were matured overnight in medium containing all of the components listed above and 50 ng/ml human recombinant EGF (R & D Systems, Minneapolis, MN). Maturation occurred in a battery-operated shipper incubator (Minitube Embryoentransp; Minitube; Delavan, WI) in vials gassed with 5% CO_2 in air before sealing.

After 21-24 h, COCs were fertilized with bovine semen from four Holstein bulls (generously provided by Select Sires, Inc.). Semen was thawed and viable spermatozoa were isolated by gradient purification (BoviPure™, Nidacon, Spectrum Technologies, Healdsburg, CA). After 14-18 h at 38.5°C in 5% CO_2 in humidified air, cumulus was removed by vortexing and

hyaluronidase (10,000 U/ml), exposure (Sigma-Aldrich), and groups of 20-30 presumptive zygotes were placed in 50 µl drops of synthetic oviduct fluid (SOF; Caisson Labs, Logan, UT) containing 20 µg/ml essential amino acids (Sigma-Aldrich), 10 µg/ml nonessential amino acids (Thermo Fisher Scientific), 4 mg/ml fatty acid free bovine serum albumin (Sigma-Aldrich), and 25 µg/ml gentamicin sulfate (Thermo Fisher Scientific) (Fields, 2011 #2995), and cultured at 38.5°C in 5% O_2, 5% CO_2, 90% N_2.

Endoderm Outgrowth Cultures

At D 8 post-fertilization, individual blastocysts were transferred to 12-well plates (3.8 cm^2; Corning, Tewksbury, MA) coated with Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA). Embryos were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5.5 mM glucose, 20% [v/v] fetal bovine serum (FBS), antibiotic/antimycotic mix (5 IU Penicillin G and 50 µg/ml Streptomycin sulfate; Thermo Fisher Scientific) and 10 ng/ml recombinant bovine FGF2 at 38.5°C in 5% CO_2 in air. At D 10 post-fertilization, an additional 10 ng/ml FGF2 was added to each well without replacing medium. At, D 12, half of the culture medium was replaced with fresh medium containing 10 ng/ml FGF2. Embryos were visualized under a stereomicroscope during media aspiration to ensure that non-adhered embryos were not removed from the well. At D 15, the presence or absence of an outgrowth was determined. The entire volume of medium was replaced with DMEM containing 5.5 mM glucose, antibiotics, and 10% FBS. Cultures were maintained at 38.5°C in 5% CO_2 in air. Medium was exchanged every 2 to 3 days. Cell morphology was used to initially distinguish outgrowths containing END and TE (see Fig. 1 for examples).

Endoderm Cell Line Production

Upon reaching 80-90% confluency, initial END outgrowths passaged as described previously [54]. Outgrowths were passed using 500 μl trypsin-EDTA (0.25%) and passaged onto CELLBind tissue culture plates (Corning; Corning, NY). Initial studies determined that Matrigel-coated plates were not necessary to maintain END cultures. Medium was changed every 2-3 days thereafter, and cells were passaged as they reached 80-90% confluency (approximately every 7-10 days). Cells were frozen after placing in freezing medium (92% [v/v] FBS, 8% [v/v] DMSO [Sigma-Aldrich]).

Culture with STO Feeder Layer

The STO-SNL murine cell line (ATCC #SCRC-1050) were inactivated with 10 μg/ml Mitomycin C for 3 h and plated at a density of 75,000 cell/cm^2. Passage 1 END cells were thawed and seeded onto the STO feeder cells in DMEM containing 5.5 mM glucose, antibiotics, and 10% FBS. Cells underwent serial passages upon reaching 80-90% confluency as previously described. RNA isolation and qRT-PCR analyses were conducted as described below after 6 to 8 weeks of culture.

Fluorescent Staining

END cells were passaged onto 35 mm glass-bottom dishes with a 20 mm glass-bottom well (In Vitro Scientific, Sunnyvale, CA) treated with PureCol, a purified bovine collagen solution (Advanced Biomatrix, San Diego, CA). When reaching the desired confluency (50-90%), cells were then fixed with 4% [w/v] paraformaldehyde in 0.01 M PBS [pH 7.4] and stained with ActinGreen according to manufacturer's instructions (Thermo Fisher Scientific) and DAPI (1 μg/ml; Thermo Fisher Scientific) according to the manufacturer's instructions. Cells

were visualized using an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epiflourescence illumination system and DS-L3 digital camera (Nikon Instruments Inc.).

Quantitative RT-PCR Analysis

RNA extraction was completed when cells were approximately 80-90% confluent by using the TRIzol reagent (Thermo Fisher Scientific) and the PureLink RNA mini kit (Thermo Fisher Scientific). RNA quality was examined using a Nanodrop Spectrophotometer ND-1000 (Nanodrop; Thermo Fisher Scientific). Only samples containing $A_{260/280}$ reading >1.8 were used in subsequent steps.

Prior to RT-PCR, all samples were incubated with RNAse-free DNAse for 30 minutes at 37°C (Thermo Fisher Scientific). Samples (10 ng RNA/reaction) were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). PCR was performed using the SybrGreen detection system (Thermo Fisher Scientific) in combination with primers for endoderm, trophoblast, and epiblast-specific transcripts (Table 1). Primers were designed using the Basic Local Alignment Search Tool (BLAST) from NCBI, and then synthesized by Life Technologies. *RPS9* was used as the internal reference control. Abundance of *RPS9* transcripts was not influenced by confluency, age or cell type (data not shown). Quantitative PCR was completed using primers shown in Table 2, Power SYBR Green PCR Master Mix (Fisher Scientific), and the Eppendorf Realplex4 Mastercycler (Hamburg, Germany). For this, samples were initially denatured at 95°C for 10 minutes and then 40 cycles of denaturation (95°C, 15 sec), annealing (57°C, 15 sec), and synthesis (68°C, 20 sec) were completed. A melting curve analysis was completed to verify amplification of a single product. Primer efficiency was determined for each primer set (efficiencies ranged from 83 to 100%).

Each PCR sample was run in triplicate, and a fourth sample lacking the reverse transcriptase was included as a negative control.

The relationship between the average comparative threshold cycle (C_T) for each gene compared to that of *RPS9* was used to determine mRNA abundance within each sample. In brief, the average C_T value for each gene was determined, and then applied to the following equation: $(2^{-C_T \text{ gene of interest}})/(2^{-C_T \text{ RPS9}})$.

Statistical Analyses

Changes in the relative transcript abundance were determined by least-squares analysis of variance using the general linear model of the statistical analysis system (SAS Institute, Cary, NC). Pair-wise comparisons were completed to further examine differences (PDIFF analysis in SAS).

Results

Outgrowth Production Efficiency

An initial study determined if previous successes with developing END cultures from bovine blastocyst outgrowths could be improved upon [54]. Previous work indicated that fibroblast growth factor 2 (FGF2) supplementation at the beginning of outgrowth culture increased END outgrowth formation in bovine blastocysts exposed to basement membrane matrix (Matrigel™). This same initial FGF2 treatment (10 ng/ml) was provided when the outgrowth cultures were initiated (D 8 post-IVF), and FGF2 was administered again at D10 and D12 (10 ng/ml on each occasion). Also, the amount of FBS in medium was increased from 10 to 20%.

The formation of END outgrowths was initially determined by visual assessment (Fig. 1). Free-floating blastocysts (Figure 2-1A) attached to Matrigel-coated plates between D 10 and D 12 post-fertilization. TE outgrowths also were common in these cultures (Figure 2-1B). These cells are tightly packed and form colonies that surround the remnants of the ICM. Also observed in most of these outgrowths were END cells, which were more sparsely growing, fibroblast-like cells (Figure 2-1B). Initial outgrowths (passage 0) contained an inner ring of TE cells, with the END cells localized around the outer perimeter of the well (Figure 2-1C and -1D).

At D 19 post-fertilization, both TE and END lineages remained in most outgrowths (Figure 2-1C and -1D; 100x and 200x, respectively). Based on these visual evaluations, END cultures were observed in 80.3 ± 5.6% of the blastocyst cultures by D 15 post-fertilization when adding FGF2 every 2 days during initial outgrowth formation and maintaining cultures in medium containing 20% (Figure 2-2). No additional outgrowths formed after D 15 post-fertilization (data not shown).

A follow-up study determined that END outgrowths developed when using regular, expanded or hatched blastocysts selected at D 7 or 8 post-IVF (data not shown). However, END and TE formation was rarely observed when using blastocysts on or after D 9 post-fertilization.

Endoderm Lineage Verification

A series of lineage-specific transcript markers were examined to verify END status and determine the specific types of END present in the cultures. Outgrowths were harvested for RNA extraction and qRT-PCR either between D 16 and 19 or D 21 and 23 post-fertilization (Table 2-1). Regardless of the time of harvest, all outgrowths contained general transcript markers for END lineages (*GATA4, GATA6*). Each outgrowth also contained transcripts preferentially expressed by PE (*CXCR4, HHEX, THBD*) and VE (*BNIP1, VEGFA*). Also, the TE-specific

marker, *CDX2,* was detected in some samples collected at D 16-19 and D 21-23 (4/6 and 9/9, respectively). Transcripts for the pluripotency marker, *NANOG*, were detected in some of the samples collected at D 16 to 19 (3/6) and in all samples collected at D 21-23.

PrE Cell Line Propagation

Two outgrowth cultures were propagated and passed for further testing. The TE was not observed after passage. Both extended END cultures proliferated for 6 weeks before they experienced cell quiescence. During the active phase of their growth in culture, cells divided every 12 to 18 hours. Contact inhibition was observed, and cells required passage prior to 100% confluency to maintain viable cultures.

Cell morphology was examined in both END cell lines, termed END1 and END2 by epifluorescence microscopy (Figure 2-3). Cells were web-like and asymmetrical in appearance and initially grew in low-density colonies (Figure 2-3A). With greater confluency and higher magnification, cell size variations became evident (Figure 2-3 B).

Also, several other END lines were cryopreserved after 1 or 2 passages (1 to 2 weeks of culture). Upon thawing, each line maintained its END morphology and mitotic potential for another 4 to 5 weeks before ceasing to proliferate.

Lineage Specification of Extended END Cultures

Lineage marker profiles were examined in END1 and END2 cultures to describe any changes in expression profiles that may exist as cells were actively proliferating (P2; ~D30), beginning to undergo mitotic arrest (P5; ~D50) or incurring replicative senescence (P6; D60-80) (Figure 2-4). The general END markers, *GATA4* and *GATA6*, were expressed at all stages of culture (Figure 2-4A). Two VE-specific transcripts were examined (Figure 2-4B). Transcripts for *BNIP1* were detected in both cultures at P2 and P5 but were absent in both cultures at P6.

Transcripts for *VEGFA* were observed in both cell cultures at P2 and P5 but only in one culture at P6. Three PE-specific transcripts were also examined (Figure 2-4 C). Transcripts for *CXCR4* were detected in both cultures at P2 but only in one culture at P5 and P6. Transcripts for *HHEX* were detected in both cultures at P2 and P5 but only in one culture at P6. Transcripts for *THBD* were detected in both cultures in all passages.

Transcripts demarking TE (*CDX2, IFNT*) and EPI (*NANOG*) were also examined in the two extended END cultures (Figure 2-5). All markers were detected at each passage. Also, each showed a step-wise increase in relative abundance as passage number increased. Transcript abundance for *CDX2* was increased ($P<0.05$) from P2 to P5 and from P5 to P6, while the abundance of IFNT was increased ($P<0.05$) only at P6. For *NANOG*, transcript abundance was greater ($P<0.05$) at P5 and P6 than at P2.

A final study was completed to determine if the proliferative lifespan of END cultures could be improved by culture with a STO-feeder layer. Initially, early passage END cells proliferated rapidly when maintained on STO feeders, but they also ceased to proliferate by 60 to 80 days in culture. At the end of this culture, none of the cultures contained transcripts for *GATA4* and *GATA6* (data not shown).

Discussion

The importance of the three main germ layers of the developing embryo has long been recognized, but END development and function during embryogenesis remains vastly understudied in cattle and other ruminants. A few bovine END lines have been developed. The low efficiency of acquiring these lines suggests that these cells were self-immortalized, although their development remains tenuous and dependent on feeder layer support [184, 188-190]. Also, these cell lines are not especially helpful for investigating the interplay of cell lineages in pre-

implantation bovine embryos. Studies involving TE function are readily achievable because techniques exist to generate TE outgrowth cultures from bovine blastocysts with high efficiency [54, 184, 191]. This work set out to improve the proficiency with producing bovine END cultures from blastocyst outgrowths so they may be used for studying early embryo development, and particularly yolk sac development in ruminant species. Development of this structure likely is crucial throughout early pregnancy in ruminants, and especially during the extended pre-and peri-implantation stages in these species.

Recently, a major improvement in END outgrowth efficiency was achieved by supplementing FGF2 to medium during initial blastocyst outgrowth formation (Yang et al, 2011). Mechanistically, this underlines the necessity for FGF2 or FGF4 signaling in END formation in bovine embryos [54, 55]. In both studies, supplementing FGF2 promotes END lineage specification whereas blocking FGF2/4 signaling prevents END formation. The same FGF-dependency for END lineage specification exists in mice and presumably other mammals [192, 193]. This lab's previous study reported END outgrowth rates improved from 1.19% to 23.5% at D 15 when providing 5 ng/ml FGF2 to culture medium at D 8 post-fertilization [54]. Improvement in this efficiency was made in this work by supplementing FGF2 at D 8, 10, and 12 post-fertilization. This probably occurred because extending FGF2 activity throughout the pre-attachment phase supported initial END formation. Medium exchanges also were made, and this also likely aided in END outgrowth formation, as this would remove metabolic wastes from cultures.

The need for FGF2 supplementation occurs only as the END cultures are being initiated. Further FGF2 supplementation is not needed to maintain cell lines [54]. However, Yang et al. (2011) determined that END cultures remain responsive to FGF2. Specifically, FGF2

supplementation increased END proliferation. It remains untested whether continuous FGF2 supplementation influences the longevity of endoderm cultures.

Providing excess amounts of FBS (20%) during the time when END outgrowths were being established likely also contributed to improvements in END formation. In mice, 15 to 20% FBS has been traditionally added to culture media to generate TE and END outgrowths, and to provide for optimal stem cell development and proliferation [192, 194]. Previous work only utilized 5-10% FBS to generate END cultures from bovine blastocysts [54, 184, 185]. The specific reason(s) that FBS facilitates END specification is not clear. No controlled studies have been completed to examine if FBS alone is sufficient to induce END formation in bovine embryos. Nonetheless, it is readily apparent that utilizing FGF2 in medium containing large amounts of FBS maximizes the incidence of END outgrowth formation in bovine embryos. After END cultures were established, a reduction in FBS from 20% to 10% had no apparent impact the ability of END to proliferate (data not shown).

Another likely reason for the high success rates for END outgrowth formation was the use of Matrigel as an artificial basement membrane matrix. Matrigel contains several extracellular matrices factors (e.g. collagen, fibronectin, gelatin) and small amounts of several growth factors, including FGFs, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), and transforming growth factor beta (TGFβ). Any of these factors may facilitate or actively encourage END lineage development [195]. It is interesting to note that the bovine END cells did not require further exposure to Matrigel after their initial establishment. Unpublished observations from this group failed to detect any differences in growth rates between END cell lines maintained on Matrigel versus using negative surface area culture ware (CellBind® surface).

The morphology of the cell lines established from these outgrowths is consistent with their classification as END. They contained a web-like appearance and resembled previously described END cells [184, 196]. Also, these early cultures contained several transcript markers of END, VE, and PE [197]. It remains unclear why these cells contain a multiplicity of END, PE and VE markers. Perhaps a single END cell type exists in these cultures, and this cell contains all of these markers. This may indicate its plasticity to develop into several END lineages. Alternatively, perhaps multiple END cell populations exist in these cultures.

The multitude of END lineage markers inspired continued investigations into the longevity of these cells in culture and the type of END lineages that could emerge from these cultures. The END cells grew in colonies when in a continuous culture environment. The two END cultures studied further in this work could be propagated for 6 weeks before their growth rate slowed and eventually stopped. Such cell senescence is common for primary cell cultures, although this outcome may also have a developmental basis. The yolk sac has a definitive life span, and perhaps these cells are programmed by some means to have a limited life span. It was encouraging to observe that the bovine END cells can be frozen away, and then thawed and grown for several weeks. Although the precise timing of senescence after freeze/thawing was not examined, these frozen/thawed cells also ceased to grow after a total of 6 to 8 weeks in culture. This storage of early passage END lines provides a way to utilize these cell lines for various activities, and especially when there is an interest to compare END cultures from batches of embryos provided different treatments.

Various cell sizes were observed in these cultures. No efforts were made to describe END marker specificity for individual cells because the difference in sizes may simply have reflected stages in mitosis or positioning of cells relative to one another. Rather, potential changes in cell type specificity in END cultures were examined by propagating two END cultures until

quiescence. There were indications that END subtype specification occurs over time in culture, but no clear indication of specific cell types was evident. Both cultures continued to express *GATA4* and *GATA6*, general END markers. The relative abundance of these transcripts increased over time. It is not clear why this occurred. There also were increases in transcript markers of PE and VE as time in culture increased. Some selectivity in END cell type may exist with extended END culture, but the loss in specific transcripts does not indicate that cells were preferentially forming into PE or VE. Rather, the presence of all of these markers suggests that the initial END outgrowths have multipotent potential.

An attempt to improve the longevity of END lines was completed by co-culturing END cultures after their first passage onto a STO feeder layer. No substantial extensions in END culture life span were evident. Thus, previous work utilizing STO feeders likely facilitated the establishment of bovine END cultures and the culture of several dozens of outgrowths were needed to identify the few cultures that grew indefinitely [184, 185]. The new, co-culture-free approach developed herein is certainly able to facilitate the establishment of END cultures, but sufficient numbers of extended END cultures were not completed to determine if presumptive self-immortalizing END lines would emerge using these culture techniques.

Not surprisingly, the initial outgrowths contained TE-specific transcripts. TE outgrowths are common in cultures such as this, but their poor attachment to non-Matrigel surfaces, slower growth rate, and poor viability after Trypsin-EDTA treatment likely minimized TE contamination after a few cell passages [56]. The EPI-dependent transcript, *NANOG* also was detected in the initial outgrowths and early passage END cultures. Proliferation of EPI cells is not yet possible in cattle, and it remains uncertain if EPI cells actually presided in these initial cultures or if *NANOG* expression is not limited to EPI cells in bovine outgrowths.

It was surprising, however, that the TE-specific transcripts, *CDX2* and *IFNT*, and *NANOG* were detected in both extended END cultures. Moreover, the relative abundance of these transcripts increased as passage number increased. Microscopic evaluations of these extended cultures failed to identify TE or EPI-like cells. Both cell types form definitive colonies, and those colonies should have been detectable, if they existed. However, we cannot discount the possibility that these cell types remained in these cultures and that they thrived in later END culture passages. Alternatively, it may also be possible that non-committed embryonic cells remained in these cultures. Therefore, at best these cultures can be considered END-enriched cultures, especially as they are passaged for several weeks.

To conclude, this work established a procedure for generating bovine embryonic outgrowths enriched with END cell types. Several END subtypes may exist in these cultures. These cultures undergo quiescence after 6 weeks. They also contain VE and PE markers but do not appear to fully differentiate into either VE or PE. These findings are anticipated to provide a new tool for studying END and yolk sac development in ruminants. Such work is necessary to better understand how END functions to promote the continuation of embryonic and extraembryonic development during early pregnancy.

Table 2-1. The presence (+) or absence (-) of lineage-specific transcripts in bovine embryo outgrowths at day 16 to 19 or day 21 to 23 post-IVF.

Gene of interest	Cell Lineage	Day 16/19*	Day 21/23*
GATA4	END	+	+
GATA6	END	+	+
CXCR4	PE	+	+
THBD	PE	+	+
HHEX	PE	+	+
BNIP1	VE	+	+
VEGFA	VE	+	+
CDX2	TE	+/-	+
IFNT	TE	+	+
NANOG	ICM	+/-	+

*n = 6 samples for D 16/19 and 7 for D 21/23; + indicates detection in all samples, - indicates no detection, and +/- indicates detection in some of the samples.

Table 2-2. List of primers used for qRT-PCR

Gene	Primer Sequence (5′ to 3′)*
GATA4	F: ATGAAGCTCCATGGCGTCCC R: CGCTGCTGGAGCTGCTGGAA
GATA6	F: ATACTTCCCCCACCACACAA R: AGCCCGTCTTGACCTGAGTA
CXCR4	F: ACTTGAGTAGCCGGTAGCCC R: CGTTGCCCACTATGCCAGTC
THBD	F: CACTGCGACACTGGCTATGA R: GCAGATGGTCGGGTAGTGAG
HHEX	F: AGAAATACCTCTCCCCGCCC R: CAAGTCTTGCCTCTGGTCGC
BNIP1	F: TCAGACCTCATGGAGGAAGGC R: AGCTTCCGTCCCAACTGGAT
VEGFA	F: GTCTACCAGCGCAGCTTCTG R: TGCTGGCTTTGGTGAGGTT
CDX2	F: GGCAGCCAAGTGAAAACCAG R: GCTTTCCTCCGGATGGTGAT
IFNT	F: GCCCGAATGAACAGACTCTC R: CCATCTCCTGAGGAAGACCA
NANOG	F: GACACCCTCGACACGGACAC R: CTTGACCGGGACCGTCTCTT
RPS9	F: GAGCTGGGTTTGTCGCAAAA R: GGTCGAGGCGGGACTTCT

*All primers were developed using the NCBI Basic Local Alignment Search Tool (BLAST).
F = Forward primer, R = Reverse primer.

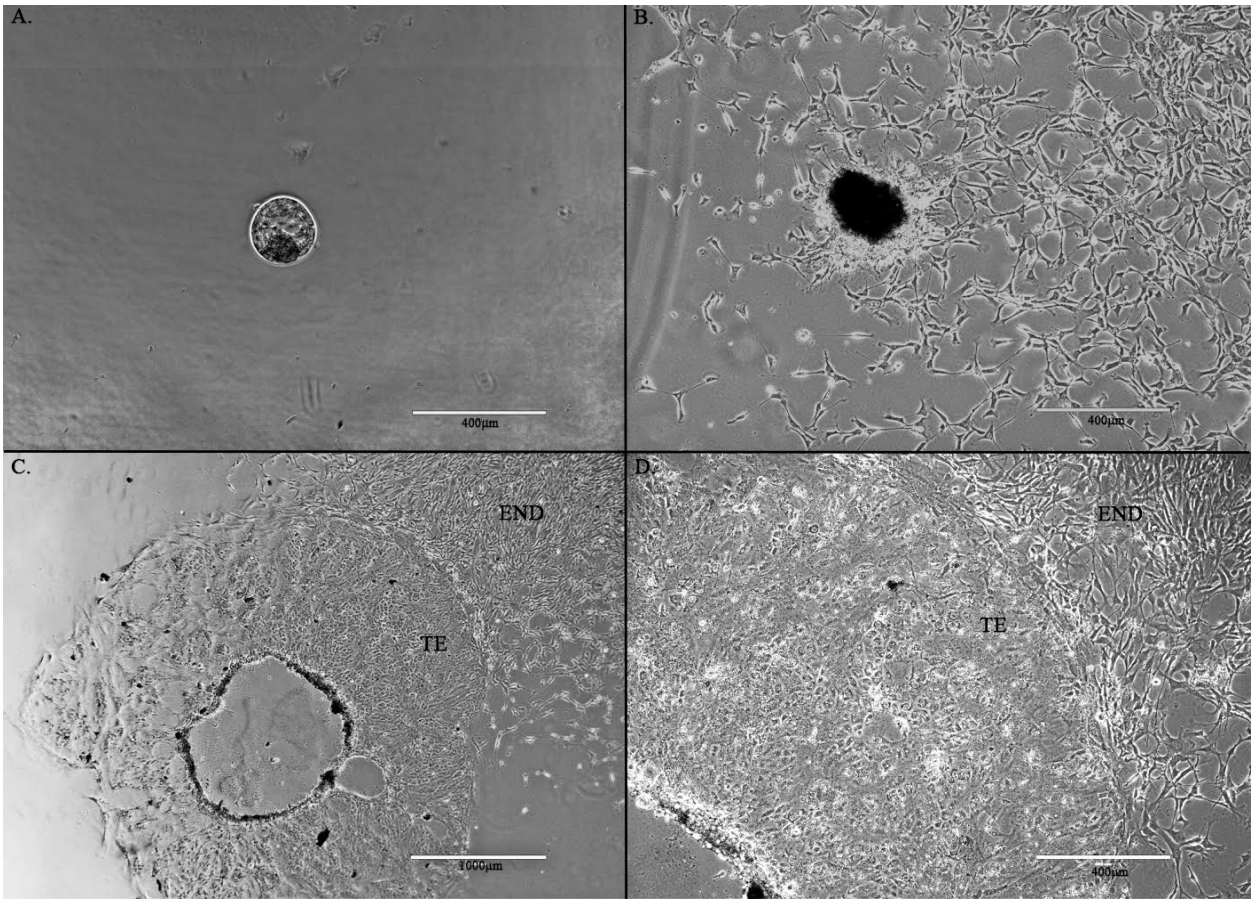


Figure 2-1. Initial outgrowth formation from bovine blastocysts. Bovine blastocysts at D 8 post-fertilization were transferred to 12-well plates (3.8 cm^2) coated with Matrigel™ Basement Membrane Matrixin DMEM containing 5.5 mM glucose, 20% FBS, antibiotic/antimycotic mix, and 10 ng/ml recombinant bovine FGF2. *Panel A*: D8 blastocyst. *Panel B*: D15 embryo with trophectoderm (TE) and endoderm (END) outgrowths. *Panel C:* D19 TE and END outgrowths at 100x magnification. *Panel D:* D19 TE and END outgrowths at 200x magnification.

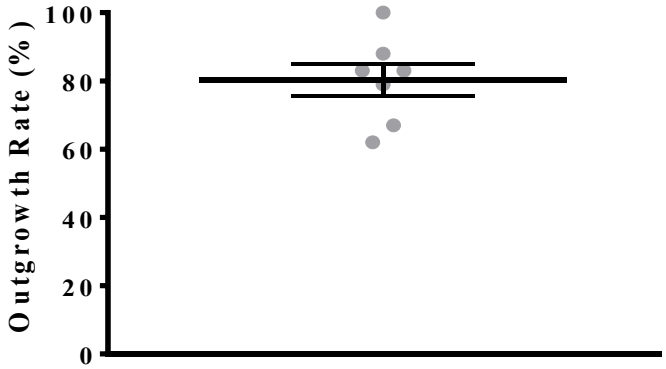


Figure 2-2. The incidence of endoderm outgrowth formation in blastocysts. Single blastocysts were transferred to 12-well plates (3.8 cm^2) coated with Matrigel™ Basement Membrane Matrix. Embryos were cultured in DMEM containing 5.5 mM glucose, 20% FBS, antibiotic/antimycotic mix, and 10 ng/ml recombinant bovine FGF2. Outgrowths were examined at D15 post-fertilization (N=7 replicate studies). The bar indicates the mean incidence of endoderm (END) outgrowth formation. Individual dots represent the incidences of END formation for each replicate study.

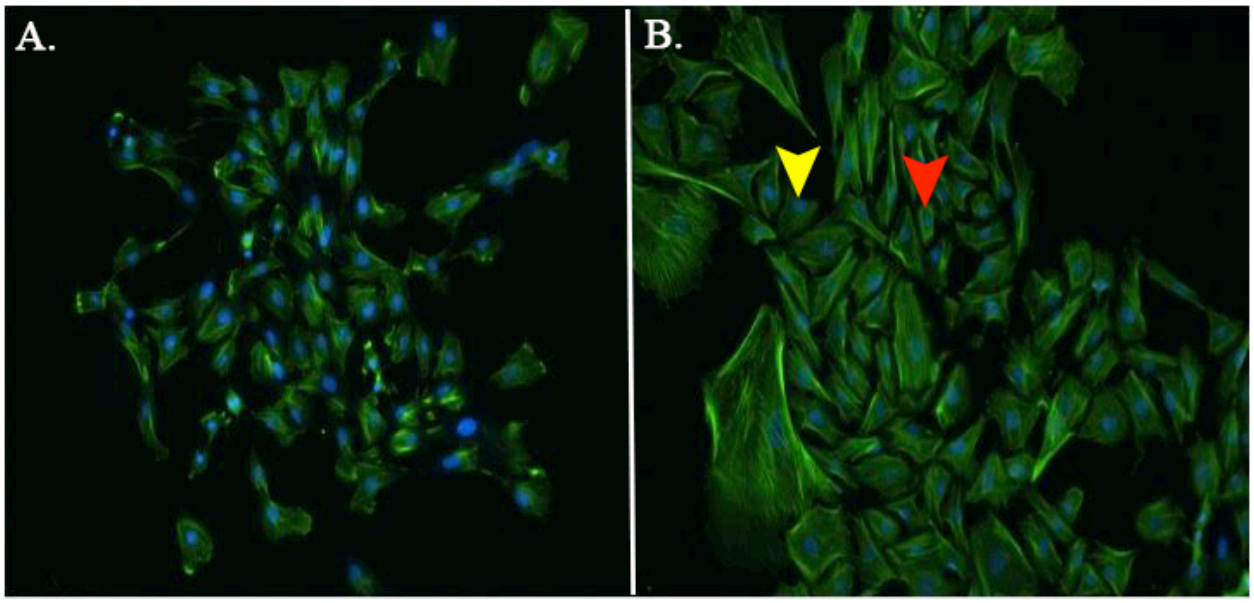


Figure 2-3. ActinGreen and DNA staining of endoderm cultures. Endoderm cells were plated on glass-bottom culture dishes, fixed with 4% paraformaldehyde, and stained with ActinGreen and DAPI. *Panel A*: Fluorescence observed at 100x magnification. *Panel B*: Fluorescence at 200x magnification. Arrows indicate two different cell sizes within the colony.

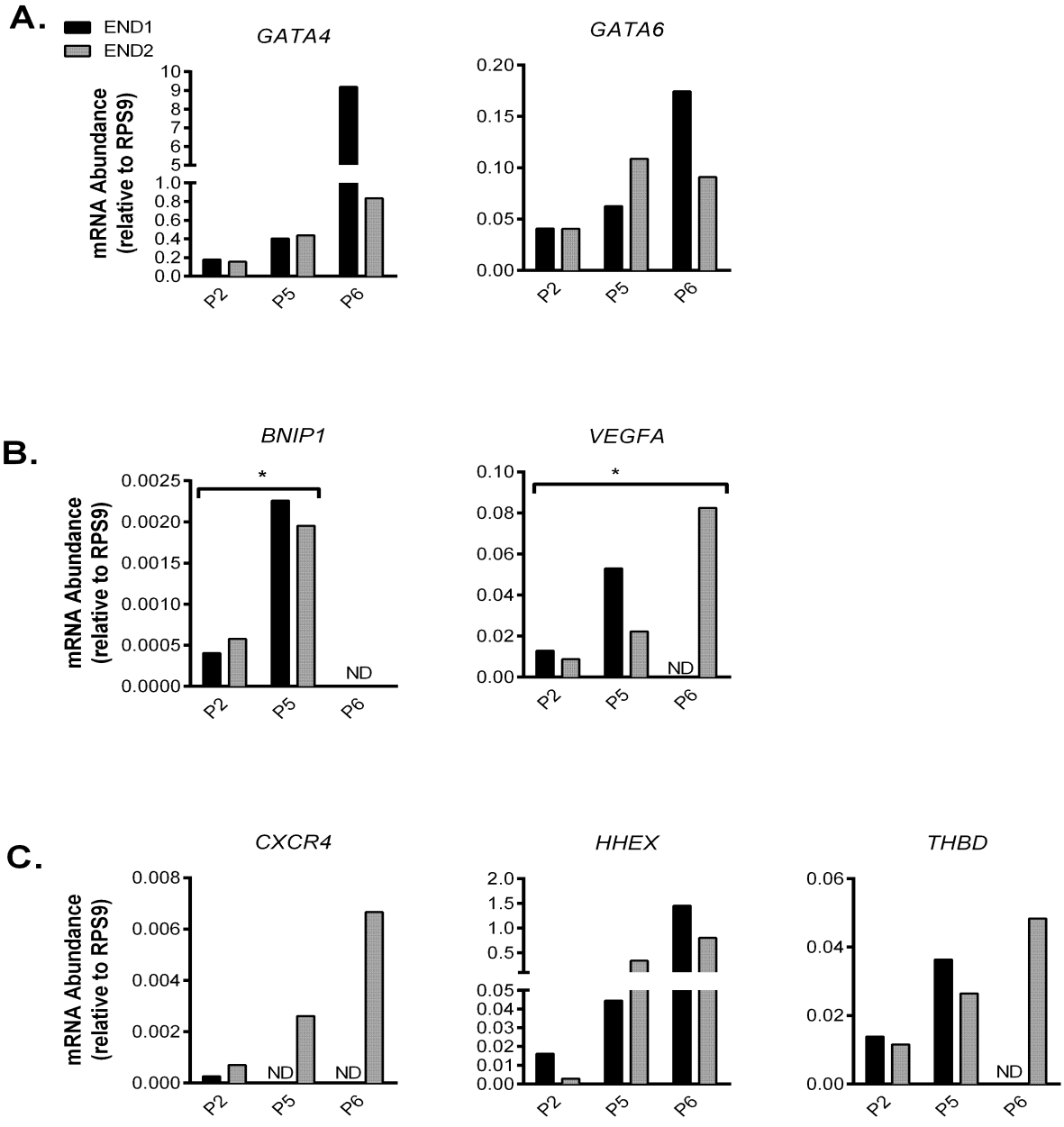


Figure 2-4. Relative abundance of endoderm-specific transcripts in bovine embryo outgrowths. Endoderm (END) outgrowths were continuously cultured in DMEM supplemented with 10% FBS, and antibiotic/antimycotic mix. RNA was collected at passages (P) 2, 5, and 6 (n=3 to 4/passage/culture). P2 is representative of actively dividing cells, P5 represents cells beginning to become quiescent, and P6 represent cells that are likely fully quiescence. Samples were separated between the END1 and END2 cultures. *Panel A*: END-specific transcripts, *Panel B*: visceral END (VE)-specific transcripts, *Panel C*: parietal END (PE)-specific transcripts.

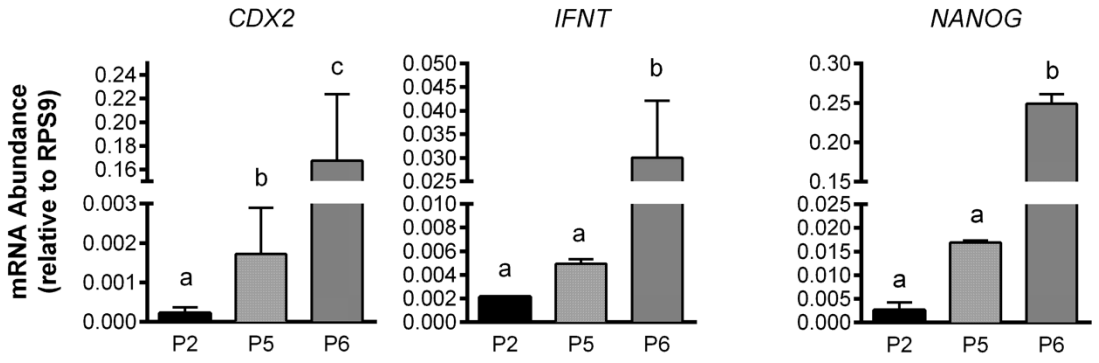


Figure 2-5. Relative transcript abundance of TE and ICM markers during endoderm outgrowth culture. Endoderm (END) outgrowths were continuously cultured in DMEM supplemented with 10% FBS, and antibiotic/antimycotic mix. RNA was collected at passages (P) 2, 5, and 6 (n=3 to 4/passage/culture). P2 is representative of actively dividing cells, P5 represents cells beginning to become quiescent, and P6 represent cells that are likely fully quiescence. TE-specific (*IFNT, CDX2*) and ICM-specific (*NANOG*) are presented. Differing superscripts denote significant differences in transcript expression between passages ($P < 0.05$).

Chapter 3

Exposure to Maternal Obesity Alters Gene Expression in the Preimplantation Ovine Conceptus

Introduction

Obesity is a prominent cause of several adverse health conditions including heart disease, stroke, type 2 diabetes, as well as some cancers in humans and other mammals. These obesity-related conditions make it one of the leading causes of preventable death among adults. Lifestyle choices and poor diet are recognized as the main factors leading to obesity, however, more recent evidence suggests intrauterine exposure to an obesogenic environment is a contributing factor predisposing offspring to obesity-related disorders. Approximately one-third of child-bearing age women (20 to 39 years of age) are overweight, and another one-third are obese in the United States [143]. Postnatal eating and dietary habits of offspring increase the likelihood of childhood and adult obesity in offspring. Also, several obesity-related disorders can manifest in these offspring in the absence of the obese phenotype. This undoubtedly occurs because of embryonic and fetal exposure to maternal obesity in utero.

These and other postnatal outcomes resulting from a poor intrauterine environment is referred to as the Developmental Origins of Adult Health and Disease (DOAHD) [198]. DOAHD highlights the connection between the intrauterine environment and subsequent health and development in later life. Nutritional insults increase the incidence of cardiovascular diseases, metabolic disorders, and reproductive problems [199]. This may be caused by changes in the development of organs in the embryonic and fetal periods of gestation. Intrauterine stresses also modify the epigenetic profile of the fetus and placenta, which then alters gene expression in cell, tissue and organ development well after offspring are exposed to the initial stress [200].

The initial concept of DOAHD applied to human offspring exposed to under nutrition in utero, however, it has since grown to also encompass the state of over nutrition during early development. Animal models have been used extensively to study this phenomenon. Reports in rodents reveal that increased maternal adiposity results in insulin resistance, hyperlipidemia, and increased body weight in offspring [201]. Exposure to maternal obesity is linked to altered skeletal muscle function [202] and reduced muscle mass in 3- and 6-month old male and female offspring [154]. The relationship between nutrition in utero and muscle growth is important in animal agriculture, as skeletal muscle development is directly related to meat quality in various species including the sheep [203-205]. Furthermore, ewes that received fetal exposure to obese ewes were hyperglycemic, hyperinsulinemic, and showed significant increases in pancreatic weight as adults at mid-gestation [160]. Similar to the mouse model, the obese ewe is known to produce lambs exhibiting altered growth, adiposity, and glucose tolerance in adulthood [159]. While the effects of maternal obesity are known to have lasting effects in offspring, methods to alleviate these effects are severely lacking.

The placenta is the key regulator of embryonic and fetal growth. It plays a role in maternal-embryonic cross-talk, nutrient and waste transfer, and hormone production. Maternal obesity has a direct effect on placental nutrient transport, placental vasculature, and blood flow [170, 206-208], and interestingly, exposure to maternal obesity alters placental development in a sexually dimorphic manner [209-214]. Similarly, fetal outcomes observed in offspring exposed to maternal obesity are sexually-dependent, including glucose intolerance, adiposity, blood pressure, and insulin sensitivity [215-217]. The mechanism and timing of the sex-dependent changes in placentation and fetal outcomes are not understood, thus genes involved in placentation were of particular interest in assessing the effects of maternal obesity on the developing embryo.

We were especially interested in understanding how maternal stresses such as obesity impacts pre- and peri-implantation embryogenesis. This is a time significant embryonic and extraembryonic tissue development and cellular restructuring in the embryo and placenta [218]. Critical events occurring during this time include demethylation and remethlyation of embryonic DNA, embryonic cell lineage specification, and embryonic-maternal cross-talk that controls pregnancy recognition [219]. We propose that exposure to environmental stressors and the resulting disruptions in the genes associated with developmental processes will adversely affect early placentation events and thereby adversely affect embryo competency. The following work examined the validity of this premise by examining the effects of obesity status on reproductive performance and conceptus gene expression profiles of ewes at day 14 of pregnancy.

Methods

Animal Use

All animal work was completed in accordance and with the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Dietary treatments were imposed ~4 months prior to the start of the study to establish the obese and lean phenotypes. Dorset ewes, 1-3 years in age, were assigned randomly to lean or obese groups. The obese state was induced by feeding 1 kg corn/day and providing *ad libitum* exposure to high quality pasture in the summer and orchard grass hay in the fall and winter months. Ewes that achieved a body condition score (BCS) >4 (scale of 1-5) were chosen for further study. Lean ewes were kept on a maintenance diet composed of previously grazed pasture in the summer months and poor-quality hay in the fall and winter months. Ewes with a BCS of 3 where chosen from this group. Once an obese and lean ewe model was established, animals underwent an estrous synchronization protocol in fall and winter months (September to

February) that began with controlled internal drug release (CIDR) device (Pfizer, New York, NY) insertion and Cystorelin (Merial, Lyon, France) injection (50 μg; IM) followed 7 days later with CIDR removal and Lutalyse (Zoetis, Parsippany, NJ) injection (15 mg; IM) [220]. Ewes were then bred to genetically-related Dorset rams (three-quarter siblings).

Blood Analysis

Blood samples were collected from the jugular vein at day (D) 0, 6, and 14 of gestation and maintained on ice until plasma was isolated by centrifugation (1,500 g x 15 min). Plasma was stored at -20°C. Ewes were kept off-feed for 12 hours prior to D14 blood collections. Plasma NEFA concentrations were determined using the NEFA-HR(2) Microtiter procedure according to manufacturer instructions (Wako Diagnostics, Mountain View, CA). Plasma P4 concentrations were determined using the IMMULITE 2000 XPi Immunoassay system (Siemens Medical Solutions Diagnostics, Tarrytown, NY state). Plasma glucose concentrations were assessed using Glucose Colorimetric Assay Kit (Ann Arbor, MI).

Conceptus Collection

Ewes were sacrificed on D 14 of gestation (day 0 = day of breeding). Body weight was recorded at the time of sacrifice. The uterus was excised by mid-ventral dissection. Each uterine horn was flushed with 30mL Dulbecco's PBS [pH 7.2] (Gibco, Gaithersburg, MD) to recover conceptuses. Individual conceptuses were teased apart and each length was recorded. Also, the number of corpora lutea (CL) was recorded and used to determine the percentage pregnant per ovulation. Individual conceptuses were snap-frozen in liquid nitrogen, and stored at -80°C.

IFNT Analysis

IFNT protein content was determined in uterine flushes by completion of an ISRE-Luc bioassay describe previously by this laboratory [221]. In brief, Madin-Darby bovine kidney cells (MDBK; ATCC#CCL-22) that were transduced with an ISRE-Luc reporter were plated into 96-well polystyrene plates with opaque walls and optically clear bottoms (Corning Inc., Corning, NY) at a density of 5-10 x 10^5 cells/well in Dulbecco's modified eagle medium (DMEM, 25 mM glucose; Life Technologies, Grand Island, NY) containing 10% (v/v) fetal bovine serum (FBS), and antibiotics (50 IU Penicillin G and 50 μg/ml Streptomycin sulfate). After 4 h incubation at 37°C in 5% CO_2, medium was replaced with 50 μl of medium and either the sample or standard. Recombinant human IFNA was used as the assay standard (3.87 x 10^8 IU/mg; EMD Biosciences, Billerica, MA). A 1:3 serial dilution of IFNA was completed to generate the standard curve. Samples were prepared by mixing DMEM containing 10% FBS and antibiotic with the flush solution (no more than one-half the final volume of medium added to each well). Cells were incubated at 37°C overnight (16-24 h). Luciferase activity was determined by adding 50 μl of One-Glo Luciferase Assay Substrate (Promega Corp., Madison, WI) to each well. After 10 min of agitation, the plate was read using an Infinite M200 PRO Plate Reader (TECAN Systems Inc., San Jose, CA).

RNA and DNA Extraction

Conceptus RNA and DNA were isolated using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany). Prior to PCR analysis, samples underwent an on-column DNase1 digestion (Life Technologies, Carlsbad, CA). Samples were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quality of RNA was examined using the Experion RNA StdSens Analysis Kit (BioRad, Hercules, CA).

Conceptus Sexing

Conceptus sex was determined using a previously described PCR-based approach [222] using GoTaq Green Master Mix (Promega, city state) and an Eppendorf Realplex4 Mastercycler (Hamburg, Germany) programmed for an initial 5 min, 95°C denaturation step followed by 40 cycles of 95C, 56°C. and 72°C and ending with a 5 minute polishing step at 72°C. Samples were then digested with the Sac1 enzyme for 3h at 37°C and loaded onto a 1% (w/v) agarose gel and electrophoresed. DNA was detected using SYBR Safe DNA gel stain (ThermoFisher, Waltham, MA). Male conceptuses were identified by the presence of 3 bands, while females appeared as a double band.

RNA-Sequencing Analysis

RNA samples (n=4 samples/sex/treatment; 16 total samples) were sequenced by Cofactor Genomics (St. Louis, MO). Sequencing was performed with an Illumina-based sequencing platform using single end 75 base reads. Sequencing analysis was performed using Genomics Workbench 10.1.1 (CLC bio). Sequences were mapped to the *Ovis aries* (NCBI; Oar_4.0)*, Bos taurus* (Ensembl;UMB3.1) and *Capra hircus* (NCBI; ASM170441v1) genomes. Expression values were expressed in reads per kilobase of transcript per million (RPKM). Differential gene expression across treatment groups was determined using the Differential Expression analysis within CLC Genomics Workbench. Results were filtered (FDR ≤ 0.05 and ≥ 2-fold change, ≥ 0.2 RPKM), and a list of DEGs was generated. GO-terms associated with the DEGs were determined using the functional classification analysis in the PANTHER Classification System (version 12.0). KEGG Mapper (v3.1) was used for DEG pathway analysis. Placenta-associated genes were identified through a literature search as “placenta” is not currently a GO term.

Statistical Analysis

Ewe body weight, metabolic parameters and reproductive parameters were were analyzed using the general linear model of the statistical analysis system (SAS Institute, Cary, NC). Conceptus sex ratio was analyzed using PROC FREQ of SAS. A repeated measures analysis and within day ANOVA were used to analyze plasma NEFA, glucose and progesterone data (SAS Institute, Cary, NC).

Results

An Increased Plane of Nutrition Affects Body Parameters of Ewes

Providing a corn-based diet altered body conformation of ewes (Table 3-1). Obese ewes had a greater average body weight at the time of collection compared to lean ewes ($P < 0.0001$). Similarly, obese ewes had greater BCS ($P < 0.0001$) and higher back fat measurements ($P=0.002$) than their lean counterparts. Obesity did not affect plasma NEFA concentrations, however, NEFA concentrations were reduced at D14 in both groups ($P = 0.03$) (Table 3-1). Circulating glucose concentrations were unaffected by obesity status.

Obesity Does Not Alter Various Pregnancy Parameters

Ewes were sacrificed at D14 post-breeding, and data were collected to assess the effects of obesity on various pregnancy parameters (Table 3-2). Pregnancy rate, ovulation rate (CL number), and conceptuses/CL (pregnancies/ovulation) were not affected by obesity status. Also, conceptus length, conceptus sex ratio and IFNT production were not affected by maternal obesity status or conceptus sex at D14. Maternal obesity status also had no effect on circulating P4 concentrations at D0, 6 and 14 post-estrus.

Maternal Obesity Affects Conceptus Gene Expression

RNA-sequencing was completed on a subset of samples (n=4 of each sex for obese and lean groups) to assess the effects of maternal obesity exposure on gene transcription in the preimplantation ovine conceptus. There was a concern with the completeness of annotation in the ovine genome assembly, so an initial set of annotations were completed against the ovine, bovine, and caprine genomes. Percentages of reads mapped to each genome were similar among species, with ovine, bovine, and caprine averaging 94.2%, 92.1%, and 94.4%, respectively. These results provided the confidence needed to utilize the ovine genome annotation as the main analysis. The ovine genome identified 32,220,571 reads per sample, 42,390 transcripts, and 28,381 genes.

There were 21 differentially-expressed genes (DEGs) in conceptuses collected from lean versus obese ewes. Of these, 10 DEGs were down-regulated and 11 were up-regulated in conceptuses derived from obese ewes (Figure 3-1; Table 3-3). Analysis with the PANTHER GO-Slim Biological Process system identified cellular process (GO: 0009987), metabolic process (GO: 0008152), and cellular component organization (GO: 0071840) as the three largest GO categories represented in the DEGs (11, 6, 3 genes respectively). KEGG pathway analysis identified DEGs involved in the PI3K-AKT signaling pathway (2 DEGs), with specific involvement cell proliferation, angiogenesis and DNA repair. Also, of the 21 identified DEGs, 4 have a known-role in placenta development and function, and 5 are associated with obesity and insulin resistance as reported in the literature.

Conceptus Sex Dictates Gene Expression

A main effect of conceptus sex on transcript profiles was also observed in this work. A total of 137 DEGs (109 annotated, 28 unannotated) were detected between male and female

conceptuses (Table 4). Of these, 25 DEGs were down-regulated and 112 were up-regulated in male vs female conceptuses (Figure 3-1). Gene ontology terms associated with the DEGs include primary metabolic processes, regulation of biological processes, cell death and transport (23, 18, 4, and 8 DEGs, respectively). KEGG analysis identified 10 DEGs involved in metabolic processes, including glycan biosynthesis and metabolism, carbohydrate metabolism, amino acid metabolism, and the metabolism of cofactors and vitamins, specifically nicotinate and nicotinamide. KEGG analysis also identified protein digestion and absorption (4 genes), and arginine and proline metabolism (3 genes) to be affected by conceptus sex. Lastly, 33 of these DEGs have a reported involvement in placental development and function.

Conceptus Gene Expression Is Impacted In A Sex-By-Treatment Manner

There also were 330 DEGs observed when comparing the four conceptus groups. The largest number of DEGs were present in the lean male versus lean female comparison, with 167 DEGs. The remaining comparisons identified 23 DEGs in obese female-lean female, 38 in obese male-lean female, 67 in lean male-obese female, 27 in obese male-obese female, and 35 in obese male-lean male. Interestingly, of the 330 DEGs, 86 were involved in placenta development and function. These DEGs, including several instances where DEGs contained multiple gene variants, are organized on a heat map to describe differential expression trends between the four groups (Figure 3-2, Supplemental Table 3-1). Based on this figure, DEGs segregated initially based on conceptus sex and thereafter based on obesity status.

Discussion

Human obesity rates continue to climb in the United States. Though predominantly attributed to lifestyle choices, recent findings suggest exposure to maternal obesity *in utero* can illicit similar metabolic and physiological outcomes in offspring regardless of their postnatal diet

[223]. Studies utilizing the mouse model have identified changes in development following obesity exposure during the earliest stages of development [224, 225]; however, an understanding of the timing of these events is currently lacking in sheep. Work until now has focused on characterizing fetal and postnatal outcomes of maternal obesity [159, 160, 226, 227]. The obese ewe produces offspring that exhibit altered growth, adiposity, and glucose tolerance in adulthood [159]. However, the specific times during development when obesity can impact embryonic and fetal programming remained unexplored. This work sought to establish whether programming events resulting from obesity could be detected early in pregnancy, and specifically during the peri-implantation period. This allowed us to examine changes in gene expression that would occur solely from alterations in oocyte maturation, fertilization, and embryonic and conceptus development. The extended period of pre-implantation conceptus development that occurs in the sheep and other ruminants permitted us to collect large conceptus samples that were at least largely and potentially totally devoid of endometrium, given that firm trophoblast-endometrial contact does not begin until on or after day 16 in the sheep [72, 81]. Collecting at this time also provided us with the opportunity to examine conceptuses when they were comprised primarily of extraembryonic membranes, specifically trophectoderm and endoderm. This permitted a detailed description of how obesity status impacts early placental development and allowed us to identify the existence of early developmental programming in the sheep. Ewes in the obese group were significantly heavier at time of conceptus collection and had a higher average BCS than the lean group. These results are not surprising because animals in the obese group were maintained on a higher plane of nutrition, resulting in fat deposition.

One interesting facet of this sheep work was that many of the metabolic and endocrine parameters normally associated with obesity in humans and rodents were not evident in this work. Notably, NEFA concentrations were not affected by treatment in this work. This opposes

the findings of previous studies that report an increased plasma NEFA concentration accompanying the obese phenotype of sheep [228, 229]. The discrepancy may be due to the pregnant state of animals in this study, whereas ewes in the previous studies were not pregnant. Pregnant rats also failed to exhibit changes in NEFA concentrations on high fat diets [230]. Pregnancy is characterized by increased fat deposition during early gestation, and a decrease in fat stores during late pregnancy [231, 232]. The increase in lipogenesis during early gestation would decrease NEFA levels, and this may mask the expected rise in NEFA concentrations observed in obese animals. The lipogenic state of early gestation may also explain the observed decrease in NEFA concentration as pregnancy progressed from D0 to D14. The steady decline in NEFA concentration may the transition to a more lipogenic state at D14. Likewise, glucose concentrations were unaffected by obesity status in this study. This is not surprising given that ruminants utilize volatile fatty acids for a constant-state level of glucose production, whereas monogastrics actively absorb glucose. This means an obese state was achieved in this work without inducing a hyperglycemic or diabetic state.

Obesity status also had no effect on pregnancy parameters in this work. Ovulation rate, pregnancy rate, pregnancies per ovulation, conceptus length, P4 production, and IFNT production were unaffected by obesity status. Obesity can negatively impact the establishment of pregnancy in cow and human models [233, 234]. Alternatively, work in the sheep reported no effect of donor ewe adiposity on ovulation rate, fertilization rate, pregnancy rate, conceptus growth, or birth weight. [227, 235]. These results coupled with the findings of this study indicate that the adverse effects of maternal obesity may not present themselves until later development. It is also important to note that this work was terminated prior to implantation, and it remains possible that pregnancy losses may occur after this time.

Perhaps the most exciting aspect of this study was that obesity-dependent changes in peri-implantation conceptus gene expression was observed in this study despite the absence of alterations in maternal metabolic parameters, macroscopic conceptus assessments and circulating progesterone concentrations. A main effect of obesity exposure was present within the conceptus samples, with 21 DEGs identified, and representative GO terms including cellular and metabolic processes and cellular component organization. These findings are supported by studies in the rodent model, which describe reduced blastocyst rates, retarded embryonic development, and altered regulation of crucial metabolic genes following exposure to maternal obesity [236, 237]. Furthermore, the term metabolic processes can be separated into secondary terms; lipid metabolic processes and protein metabolic processes. Results indicate these mechanisms are altered in obese-derived conceptuses when compared to controls, and may be early signs of the metabolic programming responsible for the increased adiposity observed in offspring of obese ewes [161].

Additional evidence of early programming was observed in 5 DEGs (*MPHOSPH9, BRCA1, ASP, ALCAM, GP2*) associated with obesity and insulin resistance [238-242]. These results fit the paradigm of DOHAD in other mammals. Previous studies in the rodent and human models have highlight the increased incidence of offspring obesity and insulin resistance following exposure to maternal obesity, however these studies focus on postnatal outcomes [154, 155, 243]. Conversely, altered birth weight in lambs exposed to maternal obesity has not been reported, though altered postnatal growth is evident [161, 162]. The current findings suggest the ovine conceptus is programmed for these metabolic disorders prior to implantation, during the earliest stages of development; however, more work is needed to establish the link between gestational stressors and the resulting phenotype in later life.

Conceptuses exposed to maternal obesity also showed differential expression of genes associated with response to oxidative stress. Oxidative stress occurs naturally in the uterus, and oxidation is an essential part of embryogenesis [244]. Oxidative stress may also impair development with decreases in embryonic competency and cell survival in stressed versus non-stressed embryos [245, 246]. These findings compliment the DEGs associated with DNA repair identified in this work. The differential expression of genes involved in the response to oxidative stress may indicate abnormal oxygen environment in utero, and thus resulting in changes in gene expression in those conceptuses exposed to maternal obesity.

Conceptus gene expression was also affected by conceptus sex. This is consistent with previous findings of sexual dimorphism in gene expression throughout development in the murine and bovine models [247, 248]. The mechanisms behind the developmental programming of male and female embryos is not known, though it is possible that male and female embryos respond differently to uterine histotroph during early embryogenesis. This idea is reinforced by studies reporting sexually dimorphic gene expression as early as the morula and blastocyst stages in cattle [248, 249]. Additionally, sex-dependent alterations in postnatal phenotypes are observed when dietary manipulations are implemented during gestation [250]. Sexual dimorphism in response to the maternal obesity may result from potential benefits of one sex over the other depending on the nutrient availability and metabolic status of the mother. DEGs grouped into GO terms including primary metabolic processes, regulation of biological processes, cell death, and transport, and each term is representative of vital mechanisms to embryo development. These data support the idea that females skew the sex ratio of their offspring depending on her nutritional condition and their ability to invest in the offspring's development [251]. The findings provide evidence that the ovine embryo is sensitive to the maternal environment and will respond

with alterations in gene expression in a sex-dependent manner. However, it remains unclear if these changes are beneficial or harmful to future development.

Sex by treatment interactions were observed in conceptus gene expression. Exposure to maternal obesity affected male and female conceptuses differently. A comparison of obese male vs lean male conceptuses identified 35 DEGs, while 23 obese vs lean female DEGs existed, and only one DEG (*ATM*) was shared between the two data sets. However, the GO terms with the highest DEG representation were similar in both male and female conceptuses. These were cellular processes (20 and 11 DEGs, respectively) and metabolic processes (17 and 8 DEGs, respectively). Furthermore, sex by treatment interactions were apparent in in obese male-lean female (43 DEGs), and lean male-obese female (69 DEGs). The data indicate that similar processes are affected in male and female conceptuses, though the extent of the modifications may differ. Work in the area of developmental programming shows that male and female offspring respond differently in the presence of various environmental stressors in utero [210, 252, 253], and a similar phenomenon appears to be present in this model.

Samples were collected at D14 of gestation, during the elongation phase of conceptus development and just prior to implantation into the uterus [81]. This phase of development is marked by an exponential increase in length of the trophectoderm, with the conceptus growing from 1mm on D11 to around 19cm on D15 [254, 255]. At the time of collection, the trophectoderm is the predominant tissue of the conceptus. The trophectoderm is responsible for uterine implantation and will eventually give rise to the placenta. Thus, it is not surprising that a subset of DEGs were associated with placental development and function. Between 19 and 26% of the obesity, sex, and obesity by sex DEGs were related to the placenta. An official GO term is not available for placental development and function, so this DEG category was developed individual assessments of manuscripts pertaining to the placenta of humans, rodents and/or

domestic animals that contained the DEGs. These terms were identified by conducting a literature search for reports identifying the gene in trophectoderm and/or placental tissue.

Specific roles of DEGs included trophoblast adhesion and implantation, placental vasculature and angiogenesis, and response to hypoxia and preeclampsia. These outcomes have been identified in previous reports, though later in gestation than the present data [170, 256, 257]. Interestingly, the biological roles of the placenta-associated DEGs are related. Abnormal TE adhesion and implantation are recognized precursors to preeclampsia in humans, as preeclampsia is characterized by shallow TE invasion [258, 259]. Likewise, pro- and anti-angiogenic factors are misregulated in preeclampsia, resulting in hypertention, the clinical hallmark of preeclampsia [260]. While samples in this study were collected immediately prior to uterine implantation, it appears that the mechanisms responsible for implantation and placentation are already perturbed at D14 of gestation. Furthermore, these maladaptive placental precursors may explain the altered growth trajectory observed in adult animals born to obese ewes [161].

Further analysis of placenta-related DEGs identified 4 pregnancy-associated glycoproteins (PAGs). PAGs are produced in the ruminant TE cells and secreted in the maternal plasma, where they are used as early pregnancy markers in sheep and cows [128, 261]. PAG concentrations appear to be early indicators of pregnancy abnormalities, as increased concentrations were detected in cows that eventually aborted during the first trimester of gestation [262, 263]. PAG concentrations quickly decline following pregnancy loss, closely accompanying embryonic death [264, 265]. The altered PAG expression within samples may be further indications that conceptus development, and placentation thereafter, is altered within the sample set.

Collectively, results indicate that the conceptus genome is susceptible to perturbations caused by maternal obesity early in development, even though morphological changes to the conceptus nor alterations in maternal reproductive parameters are detectable. These effects of maternal obesity also are sexually dimorphic. Furthermore, this work identifies genes involved with placental development, and specifically adhesion, implantation, angiogenesis and placental vasculature as major targets of genetic regulation. The altered expression of these transcripts may be some of the earliest indications of implantation failure and subsequent placental insufficiency that are observed in obese females. Further work should focus on identifying the phenotypic changes resulting from the misregulation of these placental genes in later gestation.

Table 3-1. Body parameters of obese and lean ewes

Parameter*	Obese	Lean
Weight at D14 (kg)	100.6±3.7^{A}	64.9±2.4^{B}
BCS	4.4±0.1^{A}	2.7±0.1^{B}
Back fat (cm)	1.5±0.1^{A}	0.4±1.6e-002^{B}
Plasma glucose (mg/dL)	3.47±0.3	2.49±0.7
Plasma NEFA (mEq/L)		
D0[a]	0.219±0.11	0.250±0.06
D6[a]	0.059±0.03	0.087 ±0.02
D14[b]	0.037±0.003	0.037±0.003

*Uppercase superscripts denote significance between groups while lowercase superscripts indicate differences by day of sampling ($P<0.05$).

Table 3-2. Ewe pregnancy parameters

	Obese	Lean
Pregnancy rate (%)	62.5	68.4
CL number	2.07±0.15	1.84±0.16
Conceptuses/CL (%)	96.2±0.05	85.2±0.07
Male:female ratio*	6:9 (40:60)	7:6 (54:46)
P4 concentration (ng/ml)		
D0	0.5±0.3	0.4±0.3
D6	2.6±0.3	2.9±0.3
D14	4.0±0.3	4.7±0.3

*Data presented numerically and as a percentage of total conceptuses within treatment in parentheses.

Table 3- 3. Obese versus lean DEGs identified in conceptus samples and associated gene ontology (GO) terms associated with these DEGs (indicated by +).

Gene ID	Fold change, obese vs lean (Log_2)*	Cellular process	Metabolic process	Cellular component organization	Placenta development	Proliferation, angiogenesis, DNA repair	Obesity and insulin resistance
MPHOSPH9	8.66						+
INTS12	8.46						
CEP57L1	8.41						
BRCA1	7.44	+	+	+	+	+	+
ASP	6.65	+	+				+
PAPD4	6.53	+					
ALCAM	6.31	+			+		+
RAB4B	5.94						
TTK	5.26	+	+				
RPS3A	3.66	+	+				
TUBA3E	1.62	+		+			
PPP2R3A	-4.42	+	+			+	
GSTA4	-4.78				+		
GP2	-5.55				+		+
DIS3L2	-6.0						
FAM213A	-7.12		+				
SLC35B3	-7.66	+					
PAAF1	-8.04						
ERMARD	-8.24						
DYNLL2	-8.61	+					
TPM1	-8.89	+		+			

*relative fold change (FDR≤0.05, ≥2 fold change, ≥0.2 RPKM)

Table 3-4. Biological GO terms, their associated top 5 differentially expressed genes, total number of DEGs within the GO category, and percentage of total DEGs following sex-dependent analysis.

Biological GO term	Greatest differential expression	# DEGs	% total DEGs
Primary metabolic processes	*DENND4C, ENTPD1, GUCY2C, PAG4, PAG9*	23	16.7
Regulation of biological processes	*CASP6, DKK4, FGFR1, GUCY2C, SS18*	18	13.2
Cell death	*ADAM19, BCL2A1, CASP6, FGFR1*	4	2.9
Transport	*PMM2, RAB31, SLC25A12, SNX16, XPO4*	8	5.8
Placental function	*CPA4, PAG4, TPM1, IL2RB, PRP4,*	33	24.1

*FDR≤0.05, ≥2 fold-change, ≥0.2 RPKM

Table 3-5. Gene IDs of placental DEGs represented in Figure 3-2.

	Average Expression Value (RPKM)			
Gene ID	**Lean Male**	**Obese Male**	**Lean Female**	**Obese Female**
ADAM19v1	0.091089693	0.386400203	0.004556245	0.001135851
ADCYAP1v1	5.954725783	2.456712836	0.413338552	0.209231598
ADMv1	0.230306635	0.640934979	0.010312059	0
AGO4v1	3.916847876	1.133407756	0.43324662	0.576853145
ALCAMv1	0.005528804	0.494075257	0	0.129568694
ALDH1A1v1	6.075242946	5.331024623	12.93640569	12.75289526
ANXA2v2	2.179756804	2.37474655	0	1.288395617
APOC3v1	11.34730036	2.412537267	0.446964227	0.385337321
ARID1Av6	0.401116649	0.123790692	0.221155897	0
ATMv1	0.005342197	0	0.138023743	0
ATMv5	0.205518818	0.671757695	0.36062329	0.001677193
BCL2A1v1	2.724363759	1.045016195	0.09222341	0.053280828
BRCA1v1	0	0.456266625	0.302374837	0
BRCA1v4	0	0.154840174	0.001070645	0.27131103
CALCRLv4	0.264732654	0.074947537	0.001297716	0
CASP6v1	0	0	0	1.046404902
CASP9v8	0.024234634	0	0	0.262013567
CD44v4	0.618520156	0.701646872	0	0.071678842
DSCAML1v1	0.335295537	0.21172564	0.002656442	0.003644467
ERV3-1v1	2.126465605	0.139570479	0.134864986	0.002424724
FETUBv1	45.00449797	30.95512845	4.810183291	6.009594858
FGFR1v5	0	0.031240398	0.173567646	0
FGFR1v9	0	0	0.060979401	0.455130077
FMNL2v1	2.748157309	1.030716013	0.403107615	0.257320122
FOXO4v1	46.91907475	15.01642054	5.007932905	11.95505505
GCM1v1	1.180402886	0.414505251	0.007164014	0.004020614
GJB5v1	5.944825838	0.684884403	0.064103405	0.265836056
GJB5v2	15.93354922	3.744891334	0.28126632	3.087633531
GP2v1	0.249029536	0.008949661	0.388852062	0.002321228
GSSv3	0	0.458029678	0	0.004569328
HMGN3v4	31.85629792	18.40104533	5.237714002	10.24388785
HPSEv1	7.092290826	5.359863798	2.119412848	1.411435071
IDO1v1	0.103584334	0.033356912	0.924427911	0
IL2RBv1	20.65175404	13.65705611	0.037205615	0.042429132

IL2RBv3	0.384139897	0.279420755	0	0
IL33v6	0.206318268	0	0.086509052	0
KDM5Cv4	0.070239474	0	0.22557764	0.006476368
KTN1v2	0	0.190198225	0	0
KTN1v7	0.270982709	0	0.001605727	0.043256257
LAMA1v1	0.026594548	0.747013162	1.317630201	1.079206306
LOC101107232v2 (GSTA4)	1.86745267	0.134747018	1.474533636	0
LOC101107831v8 (GSTM5)	0	0	0	0.28097537
LOC101110239v1	0.009586096	0.003453427	0.027961547	0.013292928
LOC101110259v2 (TKDP1)	4.903343186	34.44845561	0.850353074	4.822012354
LOC101112509v1 (PLET1)	104.4708333	60.71468872	0.403014724	1.031817179
LOC101119768v1 (PRP4)	2.156665826	1.523028672	0	0
LOC101122394v1 (PAG2)	2.532849629	1.475966179	0.01357689	0.018546702
LOC443319v1 (PL)	1.646033917	1.600949984	0.024950376	0
LRP2v2	10.04117628	39.27837604	38.01195284	14.11280823
MMEv8	0.568900241	0.042067093	0	0
MPP7v3	0	0.236657538	0.120844362	0
MUC15v1	3.774971241	1.48724935	0.067926882	0.375898996
NAPEPLDv10	0.392890642	0.002074331	0	0
NAPEPLDv5	0.262434099	0.191813386	0	0
NAPEPLDv6	0.204514632	0.00158886	0	0.012671795
NFAT5v11	0.566574986	0.284130519	0.137314079	0
NMBRv1	0	0.003577064	0.004795828	0.205866792
NRKv3	0.446508964	0.365801222	0.016950797	0.008597909
OCRLv3	0	0.438965552	0.268314138	0.001482916
OGDHv4	0	0.474633897	0.13974736	0
P2RX4v2	1.524944277	0.607849817	1.122337984	0
PAG11v1	3.549042916	2.851858441	0.125596376	0.102396152
PAG1v1	0.300724168	0.144859506	0.288554496	0
PAG4v3	2.13887386	2.590419137	0	0
PREPv1	0	0.129292732	0.246177495	0
PROCRv1	0	0	0	0.210884748
PTPN2v6	0.479603321	0.761845969	1.027439297	0.024826445
PTTG1v4	0	0	0	0.385705601
PVRL4v1	0.264136486	0.127484156	0	0

RFX5v4	0.263394995	0.209555118	0.004455129	0.005719664
RIMKLBv2	0.080249398	0	0.208077342	0
SENP1v1	0.001967485	0.227057604	0	0
SGPL1v2	0.160593014	0.56650555	2.760724727	0.963238735
SGPL1v7	0	0.303524979	0.13108011	0
SIAEv5	0.348932818	0.502297906	0	0
SLC16A10v1	2.556436133	9.582060276	13.01765205	11.86875273
SLC17A5v2	1.120050037	0.011730927	0.020496325	0.549893612
SLC26A2v8	0	0.22438509	0	0.001275514
SLC6A14v1	3.453203647	2.284117547	0.031707821	0.003694713
TFPIv1	5.051518422	4.691764115	0.219638289	0.120448444
THY1v1	2.864030361	1.127287211	0.012637508	0.004205469
TLE2v1	1.712373735	0.652791433	0.042764265	0.020270713
TNFRSF1Bv1	3.596483232	1.946924968	0.323203613	0.330706841
UBAP2v2	2.403746137	0.143601472	0.046666875	3.26640881
USH2Av1	0.002011014	0.121921937	0.38897976	0.18096853
VDAC3v2	0	0	0	0.817494243

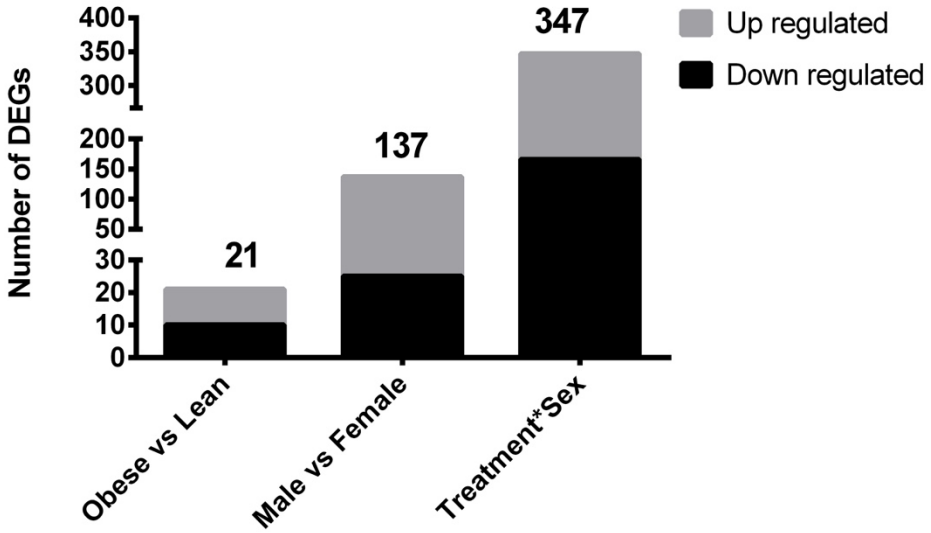


Figure 3-1. The number of number of up- and down- regulated genes across experimental comparisons (FDR≤0.05, ≥2-fold change, ≥0.2 RPKM).

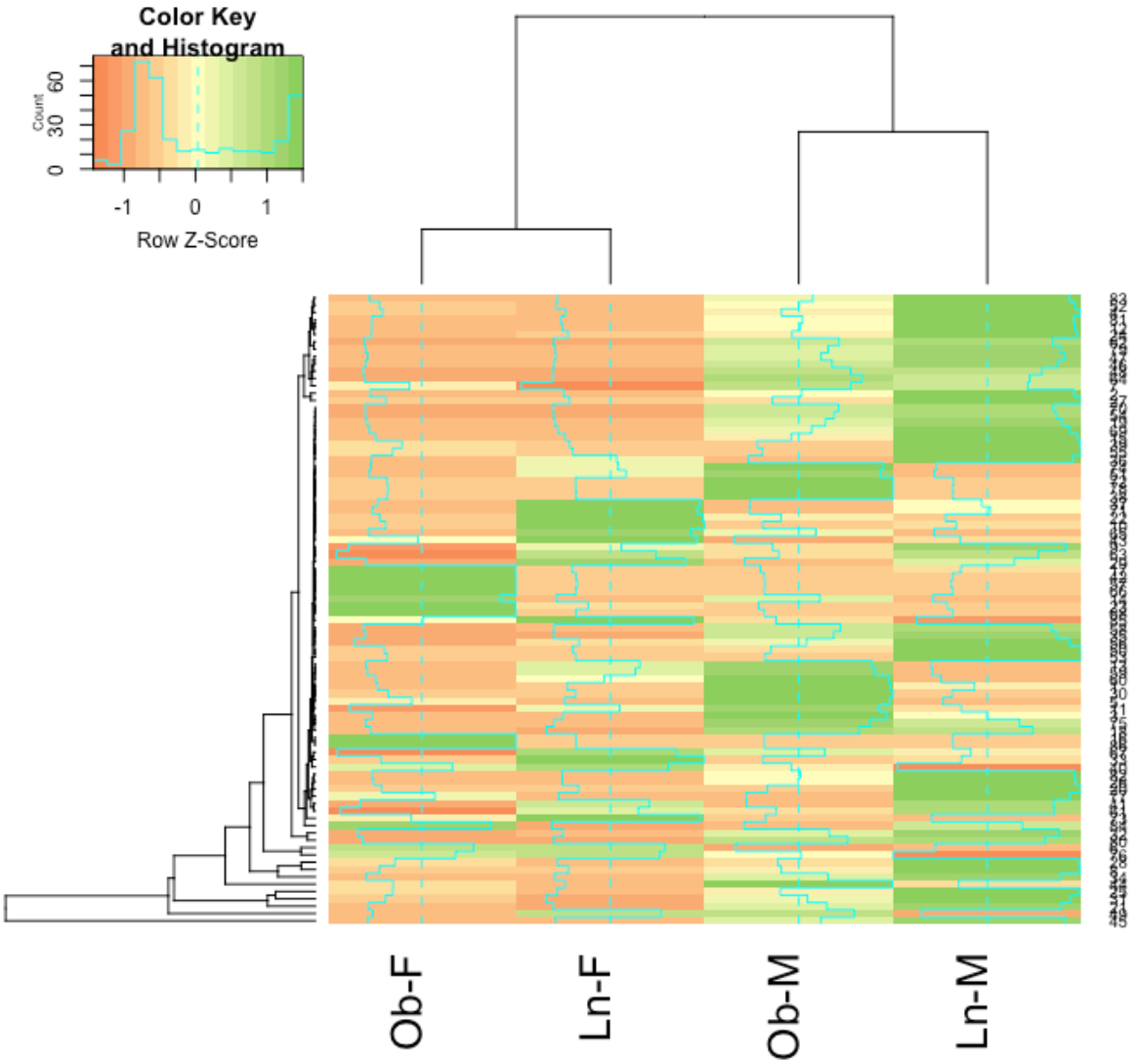


Figure 3-2. Heat map showing clustering pattern of sex*treatment placental DEGs (FDR≤0.05, ≥2-fold change, ≥0.2 RPKM). Gene IDs can be found in Table 3-5.

Chapter 4

Maternal Obesity Alters Transcription Profiles of the Peri-Implantation Ovine Conceptus

Introduction

Obesity in humans is associated with several adverse health outcomes, including heart disease, type 2 diabetes, and some cancers. Poor diet and lifestyle choices are typically blamed for obesity and its related disorders, however, recent research points to the intrauterine exposure to this obese phenotype will predispose fetuses to these adverse health outcomes after birth. The programming of offspring exposed to intrauterine stressors is referred to the Developmental Origins of Adult Health and Disease, or DOHAD [198]. DOHAD highlights the interaction between the maternal and embryonic systems, and the consequential health and development of offspring. Initial studies examining DOHaD focused on the effects of under nutrition on subsequent offspring development. Some of the most notable work includes the Dutch famine birth cohort studies [266]. Studies found that babies exposed to famine during mid-/late gestation were lighter, shorter, thinner, and had smaller heads and placentas, while those exposed during early gestation were heavier and longer at birth. Further examination found that these poor offspring outcomes persisted into adulthood, with people exposed to famine in utero experiencing reduced glucose tolerance [267], a higher body mass index (BMI) [139], and an increased incidence of obstructive airway disease [268]. This was some of the first evidence that early developmental stressors program the embryo in a way that affects future health.

Initial work focused on the effects of under nutrition, however, several animal studies have since examined the effects of maternal obesity on offspring outcomes. Studies utilizing the

rodent model implicate maternal obesity in the emergence of increased body weight, insulin resistance, hyperlipidemia, hypertension, and fatty liver in offspring [201, 216]. Studies have also utilized the sheep as a biomedical model of human fetal development and as a model for ruminants. These studies report decreased circulating insulin and altered muscle development and body composition following exposure to maternal obesity [160, 226, 227]. However, information on the effects of these insults on preimplantation development is limited.

The work presented herein describes the effects of maternal obesity on the peri-implantation ovine conceptus. This time point was selected because it provides a unique glimpse at the early developing placenta. Ruminants undergo extended pre-implantation conceptus development. In the sheep, firm contact with the uterine lining does not occur until day 16 of pregnancy [72]. Immediately preceding this time, the conceptus will grow rapidly and undergo an elongation phase, where exponential development of the trophectoderm (TE) occurs to maximize placental contact with the uterus before implantation. The extraembryonic endoderm, the precursor of the yolk sac, is also rapidly developing at this time. Also, since the conceptus has not firmly attached to the uterus, the entire conceptuses may be harvested without endometrial tissue contaminants.

A portion of this project has been reported elsewhere (see Chapter 3). The work describes transcripts, and notably several placental factors, that are affected by maternal obesity. This report describes follow-up work aimed at describing expression profiles for several transcripts whose protein products are linked to significant features of embryonic and extraembryonic development. The hypothesis of this study is that the effects of maternal obesity can be detected in the pre-implantation conceptus by way of altered expression of transcripts with regulatory roles in embryogenesis.

Methods

Animal Use

All animal work was completed in accordance and with the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC). The animal study design is described in previous work (Chapter 3). Dietary treatments were imposed ~4 months prior to the start of the study to establish the obese and lean phenotypes. Dorset ewes, 1-3 years in age, were assigned randomly to lean or obese groups. The obese state was induced by feeding 1 kg corn/day and providing *ad libitum* exposure to high quality pasture in the summer and orchard grass hay in the fall and winter months. Ewes that achieved a BCS >4 (scale of 1-5) were chosen for further study. Lean ewes were kept on a maintenance diet composed of previously grazed pasture in the summer months and poor-quality hay in the fall and winter months. This yielded an average BCS of 3. Once an obese and lean ewe model was established, animals underwent an estrous synchronization protocol [220] that began with CIDR (Pfizer, New York, NY) insertion and Cystorelin (Merial, Lyon, France) injection (50 μg; IM) followed 7 days later with CIDR removal and Lutalyse (Zoetis, Parsippany, NJ) injection (15 mg; IM). Ewes were then bred to genetically-related Dorset rams (three-quarter siblings).

Conceptus Collection

Ewes were sacrificed on day (D) 14 of gestation (D0 = day of breeding). Body weight was recorded at the time of sacrifice. The uterus was excised by mid-ventral dissection. Each uterine horn was flushed with 30mL Dulbecco's PBS [pH 7.2] (Gibco, Gaithersburg, MD) to recover conceptuses. Individual conceptuses were teased apart and each length was recorded. Also, the number of corpora lutea (CL) was recorded

and used to determine the percentage pregnant per ovulation. Individual conceptuses were snap-frozen in liquid nitrogen and stored at -80°C.

RNA and DNA Isolation

Conceptus DNA and RNA were isolated using the AllPrep Mini Kit (Qiagen). Prior to PCR analysis, samples were incubated with RNAse-free DNAse for 30 minutes at 37°C (Life Technologies, Carlsbad, CA). RNA quality was examined using a Nanodrop Spectrophotometer ND-1000 (Nanodrop; Thermo Fisher) and Bioanalyzer.

Conceptus sex was determined using a previously described PCR-based approach [222] using GoTaq Green Master Mix (Promega, Madison, WI) and an Eppendorf Realplex4 Mastercycler (Hamburg, Germany). Samples underwent an initial 5 min denaturation step at 95°C, followed by 40 cycles of 95C, 56°C. and 72°C, and ending with a 5-min polishing step at 72°C. Samples were then digested with the Sac1 enzyme for 3h at 37°C, and electrophoresed on a 1% (w/v) agarose gel. DNA was detected using SYBR Safe DNA gel stain (ThermoFisher, Waltham, MA). Male conceptuses were identified by the presence of 3 bands, while females appeared as a double band.

Quantitative RT-PCR Analysis

Samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was performed using the SybrGreen detection system (Life Technologies) in combination with primers for transcripts whose protein products are associated with trophectoderm specification (achaete-scute complex 2 [*ASCL2*], caudal type homeobox 2 [*CDX2*], heart and neural crest derivatives-expressed protein 1 [*HAND1*]), endoderm development (apolipoprotein A-1 [*APOA1*], GATA binding protein 4 [*GATA4*]), paracrine factors and receptors (fibroblast growth factor receptor 2 [*FGFR2],* peroxisome proliferator-

activated receptor gamma [*PPARG*], prostaglandin-endoperoxide synthase 2 [*PTGS2*], and interferon-tau [*IFNT*]). and DNA methylation (DNA methyltransferase 1 and 3a/b [*DNMT1, DNMT3a/b*]) (Table 1). Primers were designed either based on published reports or by using the Basic Local Alignment Search Tool (BLAST) from NCBI and synthesized by Life Technologies. *RPS9* was used as the internal reference control. PCR was completed with an Eppendorf Realplex4 Mastercycler (Hamburg, Germany). Samples we initially denatured at 95°C for 10 minutes and then underwent 40 cycles of denaturation (95°C, 15 sec), annealing (57°C, 15 sec), and synthesis (68°C, 20 sec) were completed. A melting curve analysis was completed to verify amplification of a single product. Each PCR sample was run in triplicate, and a fourth sample lacking the reverse transcriptase was included as a negative control. Gene expression data is represented by fold change compared to average lean-derived female conceptus expression.

Results

As described in a previous report (chapter3), at the time of conceptus collection obese ewes were heavier than lean ewes (100.6 ± 3.7 vs. 64.9 ± 2.4 Kg, respectively; $P < 0.001$) and contained a greater BCS (4.4 ± 0.1 vs. 2.7 ± 0.1, respectively; $P < 0.0001$). However, maternal obesity did not affect conceptus length, number of conceptuses, sex ratio of conceptuses, and overall pregnancy rate (pregnancies per corpus luteum).

Quantitative RT-PCR was performed on a set of D14 conceptuses from obese and lean ewes that were used in previously described RNA-sequencing work (Chapter 3; n=16) and from conceptuses that were collected at the same time as these samples but not included in the RNA sequencing analysis (n=11). Also, conceptus sex permitted comparison for how sex influenced maternal obesity effects on gene expression (final n = 6 to 8 conceptuses/sex/obesity status).

Changes in endoderm- and trophectoderm-specifying transcripts (*ASCL2, CDX2,*

HAND1, APOA1, GATA4) were not detected within this sample set (Figure 4-1A). However, maternal obesity exposure and conceptus sex influenced the expression of the FGFR2 hormone receptor produced by TE and endoderm in early pregnancy [269-271] (Figure 4-1B). There was a main effect of sex in *FGFR2* expression, with males having a higher abundance than females (P=0.01). *FGFR2* transcript abundance was lower in lean female conceptuses compared to both lean and obese males (P<0.05). Transcript abundance was also reduced in obese female conceptuses compared to lean male samples (P<0.05). *PPARG and PTGS2* mRNA abundance was greater in male conceptuses than females, regardless of treatment (P=0.0002, and P= 0.03, respectively). There were no treatment effects within sex for either *PPARG* or *PTGS2*.

Transcript abundance for three predominant methylation-associated methyltransferases were also examined in these conceptuses (Figure 4-2). Conceptuses experienced a main effect of sex, as male conceptuses had increased *DNMT1* mRNA abundance compared to females (P = 0.04). A main effect of treatment was also observed for *DNMT1* expression, with obese-derived conceptuses having and increase in transcript abundance compared to lean-derived conceptuses (P = 0.0004). Female conceptuses derived from lean ewes had the lowest *DNMT1* expression, while obese-derived males had the highest (P < 0.05). Transcript abundances for *DNMT3a* and *DNMT3b* were unaffected by obesity, sex or their interactions.

Discussion

The work described herein aimed to determine the impact of maternal obesity on key regulatory transcripts in the pre-implantation ovine conceptus. A majority of work utilizing the sheep model has focused on fetal and postnatal outcomes of maternal obesity [159, 160, 226, 227], leaving the pre-implantation effects of obesity ill-defined. We previously used RNA-sequencing to determine the impacts of maternal obesity on conceptus transcript abundance

(Chapter 3). While analyzing the data, we noticed some of the TE, END, and methyltransferase transcripts met the conventional pairwise P-value cutoff but failed to reach significance using the false discovery rate (FDR) value. This is likely because the FDR is a more conservative indicator of significance, and accounts for the high volume of sequencing data. We also recognized the absence of multiple genes this laboratory is interested in examining. This lead us to investigate if differences in transcript abundances of various genes of interest could be detected via qRT-PCR analysis when a combination of new and previously used conceptus samples were examined.

The conceptus length and other reproductive parameters of this study have been thoroughly described in our previous report (Chapter 3). One interesting outcome of the reproductive parameters is that conceptus length, number of conceptuses and sex ratio was not affected by maternal obesity status, however, 21 differentially expressed genes (DEGs) were identified in conceptuses based on obesity status. The three main gene ontology terms associated with these genes were cellular process, metabolic process, and cellular component organization, suggesting altered development in conceptuses exposed to maternal obesity. Furthermore, transcripts associated with obesity and insulin resistance, and oxidative stress response were also affected by maternal obesity. Together, these finding provide strong evidence that the obese maternal environment alters transcription in the preimplantation ovine conceptus, even in the absence of obvious morphological alterations.

One important novel feature of this work was examining how obesity status affects gene expression in conceptuses of different sexes. It was not surprising that sex influenced how conceptuses responded to maternal obesity status. Sexual dimorphism occurs naturally in the embryo. It can be detected as early as the 8-cell stage in mice and at the morula stage in cattle [247, 249]. Likewise, work in mouse embryonic stem cells reveal distinctive methylation patterning between males and females, resulting in differences in gene expression [247]. These

sex-dependent changes are likely due to differences in response to environmental stressors, as observed in various rodent studies [272-274]. Likewise, sexual dimorphism in response to the embryokine CSF2 has been reported in bovine embryos [275]. This work identifies preimplantation development as period sensitive to the effects of maternal obesity, and suggests that the ovine conceptus also responds to environmental stressors in a sex-dependent manner. Our previous report of conceptus RNA-sequencing found that between 19 and 26% of the DEGs identified in the various obesity, sex, and obesity by sex comparisons were associated with placenta development and function. Interestingly, our work identified 4 transcripts (*FGFR2, PPARG, PTGS2, DNMT1*) with sex-dependent expression, supporting previous findings of developmental disparities in male and female embryos.

We chose to examine FGFR2 and PPARG because each receptor is linked to pregnancy outcomes in several species, including the sheep. There are 4 FGFRs in mammals, and FGFR2 plays an especially important role in early pregnancy in the mouse. Loss of function mutation is embryonic lethal and is characterized with reduced TE proliferation and loss of primitive endoderm development, which prevents yolk sac development [172]. Several FGFs, and notably FGF2, a ligand for FGFR2, are involved with several critical biological activities in bovine TE and embryos, including promotion of blastocyst formation, TE proliferation, TE migration, primitive endoderm formation, and IFNT production [54, 276, 277]. It is not clear if FGFR2 is the sole receptor responsible for these actions in cattle, but the findings in mice implicates FGFR2 as a mediator of at least some of these activities. It is not clear how and why FGFR2 mRNA abundance is influenced by conceptus sex. Further work is needed to evaluate this finding and determine if it is of developmental importance.

The interest in evaluating *PPARG* expression stemmed from recent work identifying PPARG as a TE-expressed receptor that is required for conceptus elongation in sheep [270]. We

also were intrigued by this observation because PPARG is an intracellular lipid-binding receptor that heterodimerizes with retinoic acid receptor X (RXR) to form a complex that binds with various lipids to regulate cellular lipid and glucose uptake [278]. The significance of its sex- and obesity-dependent expression is also not understood.

We examined the expression of a prostaglandin synthase, *PTGS2* (or *COX2*), in D 14 ovine conceptuses. Embryo-derived *PTGS2* has been implicated in conceptus elongation in sheep, and is highly expressed in the preimplantation embryo with expression increasing 30-fold between D 10 and 14 of pregnancy [279, 280]. The termination of PTGS2 occurs around D 17-18, coinciding with uterine attachment. This temporal patterning suggests PTGS2 plays a role in embryonic implantation, an idea supported by work showing enhanced cellular adhesion in intestinal cells programmed to overexpress PTGS2 [281]. The implications of sexually dimorphic *PTGS2* expression is not understood.

Finally, multiple methylation-associated genes were assessed. Unlike the mouse embryo, the sheep does not undergo genome-wide demethylation. Demethylation has not been visualized until the expanded blastocyst stage in the sheep embryo, and it occurs specifically in the TE [282]. This leads to the speculation that demethylation of the TE is required for lineage differentiation. Demethylation and remethlyation events of early development are considered to be due to nuclear uptake or removal of DNMT1 [283, 284]. DNMT1 is responsible for maintaining methylation profiles following DNA replication and is an important factor in epigenetic gene regulation. Work in the mouse showed an overexpression of *Dnmt1* resulted in hypermethylation of the embryonic genome, a loss of imprinting, and ultimately embryonic death [285]. Studies also indicate that a maternal high-fat diet during gestation is associated with altered DNA methylation patterning in fetal and placental tissues [211, 286-288], and the overexpression of *DNMT1* has been identified in several human cancers [289, 290]. This finding

highlights an important facet of *DNMT1* function, as cancers are characterized by unchecked cell growth. Collectively, these findings implicate *DNMT1* is a player in the mechanism behind epigenetic aberrations caused by early obesity exposure. The increase in *DNMT1* expression may be a cause for the altered transcription patterns observed in the previously described genes of this study, and thus alter the epigenome to cause the abnormal postnatal growth observed in lambs exposed to maternal obesity [161]. More work needs to be completed to asses the direct impacts of increased DNMT1 expression on later fetal development, and in particular the potential for lineage-specific effects of *DNMT1*. However, this current data identifies the preimplantation period as a window of methylation susceptibility to the effects of maternal obesity.

To conclude, the findings of this work support the argument that maternal obesity modifies a subset of important mediators of embryonic and extraembryonic development. The data provide needed insight into the mechanisms behind early programming in the sheep, as limited data exists on the effects of maternal obesity during the preimplantation period. Findings also highlight the presence of sexual dimorphism in the conceptus' response to maternal obesity. Future work should focus on the progression of these transcriptional insults into later development, and also the factors controlling sex-dependent changes in the ovine conceptus.

Table 4-1. Selected genes and their corresponding forward and reverse primers used in qRT-PCR analysis.

Gene	Primer sequence (5 to 3)*	Reference
IFNT	F: ATGGCCTTCGTGCTCTCTCT R: CCTGGCATCCAGCATGAGTC	-
CDX2	F: GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	-
HAND1	F: CAAGGACGCACAGGCTGGCGA R: CACTGGTTTAGCTCCAGCGC	-
ASCL2	F: GCTGCTCGACTTCTCCAG R: CGGAACGAGGAACACGG	-
GATA4	F: GGTTCCCAGGCCTCTTGCAATGCGG R: AGTGGCATTGCTGGAGTTACCGCTG	[291]
PTGS2	F: TCCGCCAACTTATAATGTGCAC R: GGCAGTCATCAGGCACAGGA	[292]
APOA1	F: CTCTGAGTTCCACATCGCCA R: TGGCCAGCAGTCTAATCAGC	-
DNMT1	F: AAGTCAAACCAAAGAACC R: TTCTCATCAGAGACTTGTGG	[293]
DNMT3a	F: TGTACGAGGTACGGCAGAAGTG R: GGCTCCCACAAGAGATGCA	[294]
DNMT3b	F: GACGTAGAGGGCAGAGATGC R: ATCACCAAACCACTGGACCC	-
FGFR2	F: CCTGCGGAGACAGGTAACAG R: GCAGCTCATACTCGGAGACC	-
PPARG	F: TAGGTGTGATCTTAACTGT R: CTGATGGCATTATGAGAC	[295]
RPS9	F: CAAGTCCATCCACCATGCCC R: GACGGGATGTTCACCACCTG	-
P1/P2	F: ATAATCACATGGAGAGCCACAAGCT R: GCACTTCTTTGGTATCTGAGAAAGT	[222]

*Forward (F) and reverse (R) primer sequences

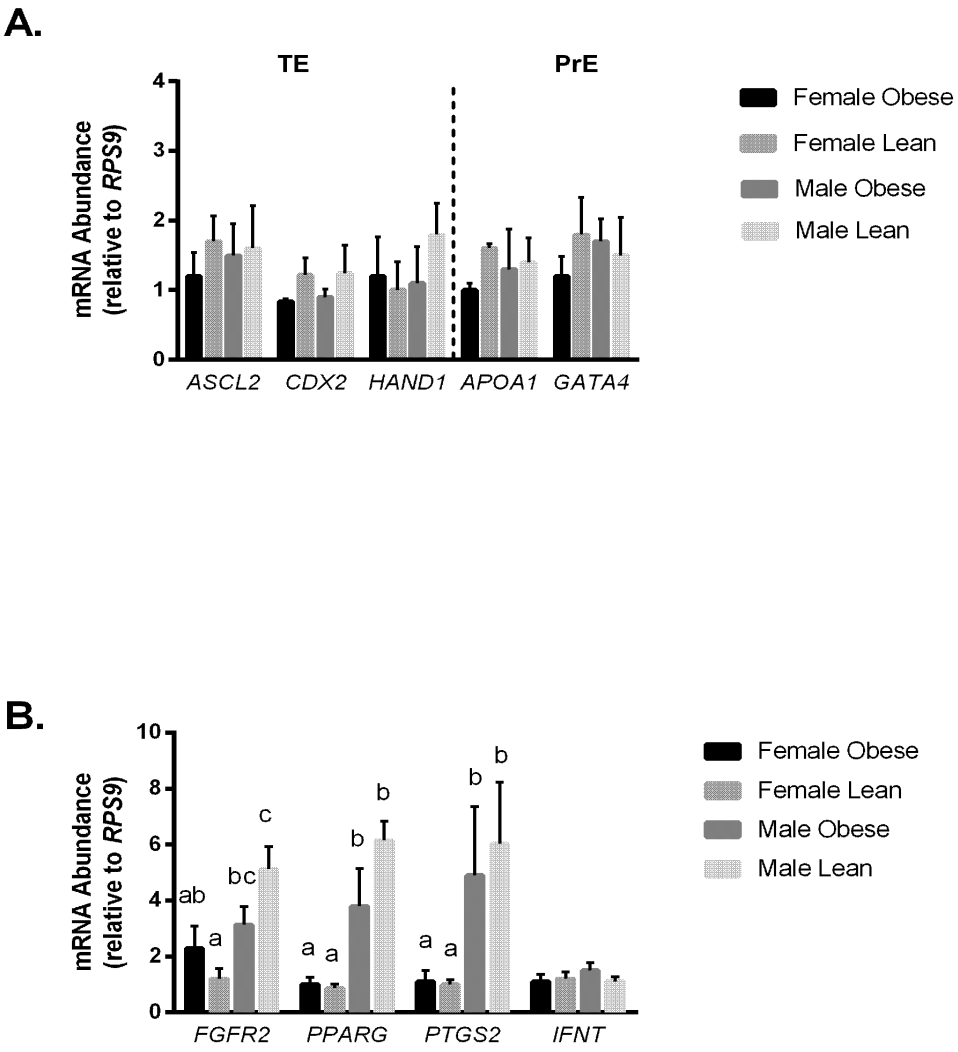


Figure 4-1. Fold change in mRNA abundance of A.) TE- and END-specification transcripts and B.) paracrine factors determined by PCR analysis. Conceptuses were collected from obese and lean ewes on D 14 of gestation. Conceptus sex was determined via PCR. Data are presented at fold change in comparison to the average expression of lean-derived female conceptuses (n=6 to 8 conceptuses/sex/obesity status). qRT-PCR was completed using RPS9 as the internal control. Superscripts denote significance between groups ($P<0.05$).

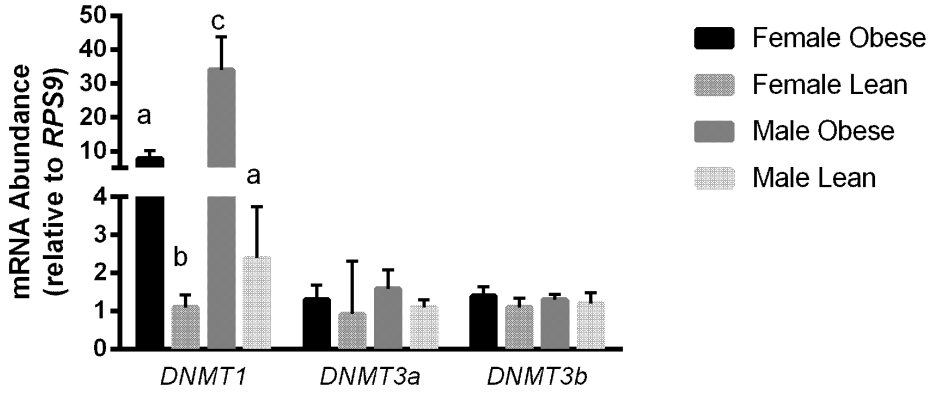


Figure 4-2. Fold change mRNA abundance of methylation associated transcripts as determined by PCR analysis. Conceptuses were collected from obese and lean ewes on D 14 of gestation. Conceptus sex was determined via PCR. Data are presented at fold change in comparison to the average expression of lean-derived female conceptuses (n=6 to 8 conceptuses/sex/obesity status). qRT-PCR was completed using RPS9 as the internal control. Superscripts denote significance between groups ($P<0.05$).

Chapter 5

Maternal Obesity Alters Gene Expression in the Pre-Implantation Ovine Endometrium

Introduction

Obesity and the resulting disorders that accompany obesity are commonly attributed to increased energy intake coupled with reduced energy expenditure; however, evidence now supports the concept of developmental programming as a mediating factor. Developmental programming is referred to as the Developmental Origins of Adult Health and Disease (DOAHD), and it highlights the relationship between maternal health and offspring outcomes [198]. The idea of DOAHD was first developed by the British epidemiologist, David Barker, when he reported that maternal under nutrition at different stages of gestation resulted in differing birth phenotypes and health disparities in adulthood [296]. More recently, the concept of DOAHD has transitioned to include incidences of maternal over nutrition, as over 1/3 of women of child-bearing age are now overweight and another 1/3 are obese [143].

Extensive animal studies have been completed to better understand the DOAHD phenomenon. Work in rodent models showed increased incidence of insulin resistance, hyperlipidemia, and body weight in offspring exposed to maternal obesity during early development [201]. Additionally, poor maternal nutrition was linked to reduced muscle and meat quality in cattle and sheep [203-205]. Obese ewes are also known to produce offspring displaying altered growth and glucose tolerance, and increased adiposity in adulthood [159]. The mechanism behind DOAHD is not completely understood, however, there is evidence from several mammalian showing a direct relationship between maternal nutrition and fetal outcomes.

The endometrium serves as the site of initial maternal-embryonic contact during implantation, and thus describing how maternal obesity influences endometrial function will provide clues about how DOAHD may be manifested during the initial stages of embryonic and placental development. Samples in this study were collected just prior to implantation, a critical window when the endometrium must undergo biochemical and structural changes in order to become receptive to the implanting conceptus. Endometrial receptivity is contingent on proper uterine gland function, the down regulation of anti-adhesion molecules accompanied by the up regulation of pro-adhesion molecules, and the triggering of a local immune system to promote a highly-controlled inflammatory response. Disruptions in these mechanisms commonly result in implantation failure and pregnancy loss [297-300], or abnormal placentation and subsequent placental disorders, such as preeclampsia, which compromise fetal development survival [301]. Though the link between obesity and endometrial function is not entirely understood, animal studies implicate altered receptivity, implantation, and resulting placental insufficiency as probable factors. Work in the over nourished ewe model emphasizes this idea, showing a reduction in placental vascularization, and impaired utero-placental blood flow at mid- and late gestation [302-304]. However, a clear understanding of the direct effects of obesity on ovine endometrial function is still lacking.

Our previous work identified differential gene expression in preimplantation conceptuses of lean and obese ewes. We were then interested in how obesity directly impacts the uterine endometrium during this time, as the preimplantation period comprised of critical cellular and biochemical restructuring, and exposure to stressors, such as obesity, during this time likely impact endometrial function. We hypothesized that maternal obesity alters mechanisms involved in endometrial receptivity, thus impairing conceptus implantation. The following work examined gene expression profiles of endometrial tissue collected from obese and lean ewes just prior to

implantation and established a direct link between obesity status and endometrial function in the ewe.

Methods

Animal Model

All animal work was completed in accordance and with the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC). An obese ewe model was established 4-month period prior to the start of the study. Dorset ewes, 1-3 years in age, were randomly assigned to lean (control) or obese groups. An obese state was induced by feeding 1 kg corn/day and *ad libitum* exposure to high-quality pasture or orchard grass hay until a desired BCS of 4-5 (scale of 1-5) was achieved. Lean ewes were kept on a maintenance diet composed of previously grazed pasture or poor-quality hay. Once an obese and lean ewe model was established, animals underwent a 7-day estrous synchronization protocol. Briefly, ewes received a CIDRs (Pfizer, New York, NY) and an injection of Cystorelin (Merial, Lyon, France). CIDRs were removed 7 days later, and ewes also received and injection of Lutalyse (Zoetis, Parsippany, NJ) (15 mg; IM) [220] at this time. Ewes were then bred to genetically-related Dorset rams (three-quarter siblings).

Endometrial Tissue Collection

On D14 of gestation (D0 = day of breeding), ewes were sacrificed. Ewe body weights were recorded, and the uterus was excised via mid-ventral dissection. Representative caruncular and intercaruncular tissue was collected and snap-frozen in liquid nitrogen. Tissue samples were stored at -80°C until RNA isolation.

RNA Isolation

Endometrial RNA was isolated using the AllPrep Mini Kit (Qiagen) (n=6 samples/treatment). Prior to sequencing, samples were incubated with RNase-free DNase for 30 minutes at 37°C (Life Technologies, Carlsbad, CA). Samples were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quality of RNA was examined using the Experion RNA StdSens Analysis Kit (BioRad, Hercules, CA).

RNA-Sequencing Analysis

Endometrial RNA samples (n=6 samples/treatment) were sequenced by Cofactor Genomics (St. Louis, MO). Sequencing was performed with an Illumina-based sequencing platform, using single end 75 base reads. Reports averaged 49 million reads per sample. Sequencing analysis was performed using Genomics Workbench 10.1.1 (CLC bio). Sequences were mapped to the *Ovis aries* genome (NCBI; Oar_4.0). Expression values were expressed in reads per kilobase of transcript per million (RPKM). Differential gene expression across treatment groups was determined using the Differential Expression analysis within CLC Genomics Workbench. Results were filtered (FDR ≤ 0.05 and ≥ 2, ≥ 5, or ≥ 10-fold change, ≥ 0.2 RPKM), and a list of DEGs was generated. GO-terms associated with the DEGs were determined using the functional classification analysis in the PANTHER Classification System (version 12.0). KEGG Mapper (v3.1) was used for DEG pathway analysis.

GO terms do not currently exist for trophectoderm or placenta, so a literature search was completed to identify DEGs associated with these terms. Search terms included "trophectoderm", "trophoblast", and "placenta". DEGs were identified when at least two sources were available identifying the gene to the search terms.

Results

Pregnancy Parameters Were Unaffected by Obesity Status

As reported previously (Chapter 3), ewes in the obese group were heavier and had a higher BCS (100.6 ± 3.7 kg, BCS = 4.4 ± 0.1) than those in the lean group (64.9 ± 2.4 kg, BCS=2.7 ± 0.1) ($P < 0.0001$). Ewes were sacrificed at D14 post-breeding, and data were collected to assess the effects of obesity on various pregnancy parameters. Pregnancy rate and conceptuses/CL (pregnancies/ovulation) were not affected by obesity in this model. Conceptus length was also unaffected by maternal obesity status and conceptus sex at D14.

Obesity Affects the Transcript Profile of the Ovine Endometrium

RNA-sequencing was completed on endometrial tissue to assess differential gene transcript abundance between lean and obese ewes. Sequencing averaged 49,039,628.25 reads per sample, with 89.69% of reads mapped to the ovine genome. We wanted to identify an appropriate RPKM threshold, so analyses with both ≥0.2 and a more conservative ≥ 5 RPKM threshold were used. At an RPKM threshold of ≥ 0.2, 669 DEGs were identified between obese- and lean-derived endometrial samples. When the threshold was increased to ≥ 5 RPKM, 330 DEGs were identified. We chose to continue analyses at the ≥ 0.2 threshold, as we were concerned that many of the lowly expressed DEGs would be mistakenly overlooked.

Further analysis at fold change ≥ 2 revealed 171 DEGs were down regulated and 498 were up regulated (Figure 5-1) in obese endometrium vs lean. The PANTHER GO-Slim Biological Process system identified cellular process (GO:0009987; n = 303 DEGs), metabolic process (GO:0008152; n = 199 DEGs), and biological regulation (GO:0065007, n= 124) as the largest gene ontology categories represented by the list of DEGs. An overrepresentation test

showed increased enrichment of DEGs representative of immune system process, negative regulation of apoptosis, cell growth, and cell adhesion (FDR < 0.05) (Figure 5-2). The number of DEGs was reduced to 295 when the threshold fold change was set to ≥ 5. Of these DEGs, 85 were down regulated and 210 were up regulated in endometrium from obese ewes compared to their lean counterparts. The largest representative GO terms at this threshold were cellular process (n = 142 DEGs), metabolic processes (n = 109 DEGs), and biological regulation (n = 40 DEGs). The list of DEGs was further reduced to 234 (164 up-regulated, 70 down-regulated) when a threshold fold change ≥ 10 was applied. The most represented GO terms were the same as analyses at the previous fold change thresholds (n = 110, 82, and 37, respectively).

KEGG pathway analysis was used to identify the biological pathways represented by the DEGs. Metabolic pathways (oas01100) contained the highest number of DEGs (n = 34), with particular focus on energy metabolism and amino acid metabolism. Other pathways identified included PI3K-Akt signaling pathway (oas04151; n= 31), and focal adhesion (oas04510; n = 23).

Maternal Obesity Alters Expression of Transcripts Involved in Placentation

A literature search of the initial 669 DEGs that exhibited ≥2-fold change in transcript abundance between lean and obese ewes revealed 125 genes that could be associated with the trophoblast lineage and the placenta. Panther Pathway Analysis revealed DEGs involved in the WNT signaling pathway (P0057), Angiogenesis (P00005) and the Integrin signaling pathway (P00034) (Table 5-1). A subset of these placenta-related DEGs were also present at the ≥ 5-fold (9 DEGs) and ≥ 10-fold (6 DEGs) change thresholds (Supplemental Table 5-1). KEGG Pathway analysis identified pathways in cancer (oas05200; n = 18) as containing the most DEGs. Specific pathways involved sustained angiogenesis, proliferation, and block of differentiation. Interestingly, RNA-sequencing revealed some placenta-associated DEGs were expressed in one

treatment group and completely absent from the other. Endometrium samples from lean ewes contained 5 unique DEGs, while obese-derived endometrium contained 17 (Figure 5-3).

Discussion

Our previous work highlighted the impact of maternal obesity on embryo development by examining changes in gene expression in preimplantation conceptuses. We found that neither pregnancy parameters nor conceptus length were affected by obesity, though RNA-sequencing identified differential gene expression based on conceptus sex, treatment (obese vs lean), and sex by treatment interactions. This lead us to next examine the preimplantation endometrium, as uterine-embryonic cross-talk is vital to successful implantation and this is likely the mechanism by which maternal obesity alters conceptus gene expression [305, 306]. The impact of obesity on endometrial function is well-studied in several mammalian species including rodents [307, 308] and humans [308, 309], however, an understanding of these events in the ovine model is currently lacking.

Our list of DEGs contained an enrichment of genes involved in immune response. Pregnancy requires both a viable embryo and receptive uterus in order to be maintained, and uterine receptivity is strongly influenced by the immune system. During a healthy pregnancy, a local macrophage population alters endometrial cell surface molecules just prior to conceptus attachment, allowing for trophoblast adhesion [310]. Furthermore, immune cells are recruited to the site of implantation, and are responsible for secreting various cytokines and angiogenic factors necessary for implantation [311]. The enrichment of DEGs associated with immune response is concerning, as scenarios of abnormal immune response during the preimplantation period result in implantation failure and pregnancy loss in humans and mice [300, 312]. Our data

suggest obesity impacts the localized immune response of the endometrium during early pregnancy in a way that may alter subsequent conceptus implantation.

The immune system, specifically uterine macrophages, is also responsible for inducing apoptosis at the site of implantation in the gravid uterus. Early work in the mouse identified an increase in the macrophage population, accompanied by an abundant population of apoptotic cells in decidual tissue during implantation [313]. Though sheep undergo epitheliochorial placentation, they too experience an increase in macrophage number in the endometrium during pregnancy [314]. This likely induces apoptosis as in hemochorial species. Our data indicate differential expression of genes associated with apoptosis in the endometrium of lean and obese ewes. We are not able to confirm discrepancies in conceptus adhesion and implantation because samples were collected just prior to implantation. However, these data, paired with the immune alterations reported above, suggest an altered immune response and apoptosis in the endometrium of obese ewes which may affect uterine receptivity.

The idea of altered implantation in obese ewes compared to their lean counterparts is further stressed by a large number of DEGs playing a role in adhesion in general. The list of DEGs included 5 integrins (*ITGA10, ITGA11, ITGA8, ITGAM, ITGB2*). Several integrins have been identified to play a role in conceptus implantation in the sheep [315], and the disruption of integrin signaling in the murine model resulted in implantation failure [316]. DEGs also included 6 collagen genes. Work in cows revealed a decrease in expression of collagen factors in the caruncular tissue of pregnant vs non-pregnant animals at implantation, suggesting a role in initial conceptus adhesion [317]. The high number of DEGs involved in adhesion may prelude atypical conceptus implantation in this model.

KEGG pathway analysis identified genes involved in the PI3K-Akt pathway to be differentially expressed in the endometrium of obese and lean ewes. The importance of the

PI3K-Akt pathway regulation during endometrial decidualization was highlighted in human *in vitro* studies. This work reported a decrease in PI3K-Akt activity and Akt isoforms was reduced during the decidualization process [318]. Similarly, work in the mouse identified this pathway to be involved in embryo implantation, with PI3K inhibition resulting in a reduction of implantation sites [319]. A reduction in the genes involved in the PI3K-Akt signaling pathway indicate altered conceptus implantation in obese ewes compared to lean ewes.

A literature search of DEGs identified a number of genes associated with the placental development. This was unexpected as samples were collected prior to implantation, however, it may indicate that conceptuses began to adhere to the endometrium at the time of tissue collection. An additional explanation is that these genes are derived from the endometrium, not the trophoblast, and act as facilitators of placental development. Several of these placenta-associated DEGs were involved in the WNT signaling pathway. WNT signaling is required for proper placental development, and obstructions in signaling result in placental abnormalities and embryonic lethality in the mouse model [320, 321]. Genes involved in the integrin signaling pathway were also differentially expressed in obese compared to lean endometrial samples. In the human placenta, integrin signaling is involved in modulating both migration and implantation of the trophoblast [322-324]. Finally, a number of placental DEGs were involved in angiogenesis, which plays a direct role in placental efficiency and fetal development. Placentation is marked by extensive angiogenesis in maternal and embryonic tissues, allowing for sufficient maternal-fetal exchange throughout pregnancy, and reductions in placental vasculature are associated with embryonic loss [325, 326]. While pregnancy retention was not affected in this study, these of placental DEGs may explain the abnormal growth trajectory observed in adult animals born to obese ewes [161].

Perhaps one of the most interesting finding in this work was the absence of expression of various placenta-associated genes in either the lean or obese ewe endometrium. Of particular interest was the complete lack of expression of trophoblast-specific pregnancy-associated glycoproteins (PAGs) 4, 6, and 11 in lean endometrial samples. All 3 of these PAGs are products of trophoblast binucleate cells, which are involved in endometrial attachment [101, 125]. This suggests that the conceptuses of the obese ewes may be developing faster and attaching to the endometrium sooner than those of the lean ewes, resulting in potential asynchrony between the conceptus and endometrium. Work in cattle shows an increase in pregnancy loss following the transfer of more advanced embryos into asynchronous recipients [327]. Likewise, the transfer of D 2.5 embryos into gilts at D 1.5 of estrous resulted in smaller implantation sites than those transferred to D 2.5 females [328]. We cannot confirm the more rapid implantation rates in obese ewes in the current study, however, this is a possible mechanism for impaired implantation and later developmental programming observed in obese females.

Collectively, these data suggest maternal obesity affects uterine receptivity, thus altering conceptus implantation and resulting placentation. This work identifies genes involved in immune response, adhesion, and angiogenesis to be differentially expressed in the endometrium of obese ewes compared to lean ewes. Trophoblast- and placenta-associated genes were also differentially expressed in obese and lean ewes. These changes in endometrial gene expression may be the mediator between maternal nutrition and the changes we previously observed in D 14 conceptuses. Future work should focus on the implications of these observations on post-implantation development and placental function.

Table 5-1. Pathways identified in the list of placental- and trophoblast-associated DEGs, the percent of DEGs represented in each category out of total number of DEGs, and gene names (FDR ≤ 0.05).

Pathway (Accession number)	% of DEGs represented	DEG names
Wnt signaling pathway (P00057)	6.4	*BMPR1A, CDH11, CDH3, FSTL1, PRKCE, SFRP2, TGFBR1, WNT4*
Integrin signaling pathway (P00034)	5.6	*RHOB, COL16A1, COL3A1, COL5A1, COL6A3, ITGA8, LAMA2*
Angiogenesis (P00005)	4.8	*RHOB, PDGFRB1, STAT1, PRKCE, FGFRI, ETS1*

Table 5-2. Placenta- and TE-associated DEGs present at various fold change thresholds in preimplantation ovine endometrium (FDR ≤ 0.05).

2-Fold Change	5-Fold Change	10-Fold Change
ABCC9	*C3*	*CD63*
ABCG1	*CD63*	*MRP1*
ABO	*MRP1*	*PAG1*
ACSL4	*PAG1*	*PAG2*
ADAM12	*PAG2*	*PLET1*
ADAM19	*PLET1*	*TKDP1*
ADAMTS12	*SLC7A3*	
ADAMTS4	*TKDP1*	
ADM	*VCAM1*	
AGTR1		
ANO1		
APOE		
AREG		
ATF1		
ATP6AP2		
AXL		
BMPR1A		
C3		
CAPS2		
CASP6		
CCR1		
CCR5		
CD63		
CDH11		
CDH3		
CDK4		
COL16A1		
COL3A1		
COL5A1		
COL6A3		
CRABP2		
CRIM1		
CSF1R		
CTH		

CTSB		
CTSK		
CYP1B1		
CYR61		
DAB2		
DKK3		
DKK4		
DYSF		
EBI3		
ETS1		
FABP3		
FABP5		
FGFR1		
FST		
FSTL1		
GPX3		
GPX7		
HAND1		
HTRA1		
ICAM1		
IGFBP2		
IGFBP5		
IL6		
INHBA		
ITGA8		
LAMA2		
LOXL1		
LOXL2		
MEST		
MICA		
MME		
MMP1		
MRC1		
MRP1		
NEK6		
NID2		
NOS2		
NR3C1		
PAG1		

PAG11		
PAG2		
PAG4		
PAG6		
PDGFRB		
PDPN		
PGF		
PLET1		
POSTN		
PRKCE		
PSEN1		
PTN		
RARB		
RGS5		
RHOB		
ROBO1		
RUNX1		
SDC3		
SECTM1A		
SERPINE2		
SFRP2		
SLC26A4		
SLC28A2		
SLC2A3		
SLC6A14		
SLC7A3		
SLC7A7		
SLCO1A2		
SLCO4A1		
SLPI		
SPARC		
SPI1		
SPINT1		
SPP1		
STAT1		
TCF4		
TGFBI		
TGFBR1		
TGFBR2		

TIMP1		
TIMP3		
TKDP1		
TPM4		
TREM1		
TRPC3		
TRPV6		
UBE2D3		
UCP2		
VASH1		
VCAM1		
VDAC3		
WNT4		

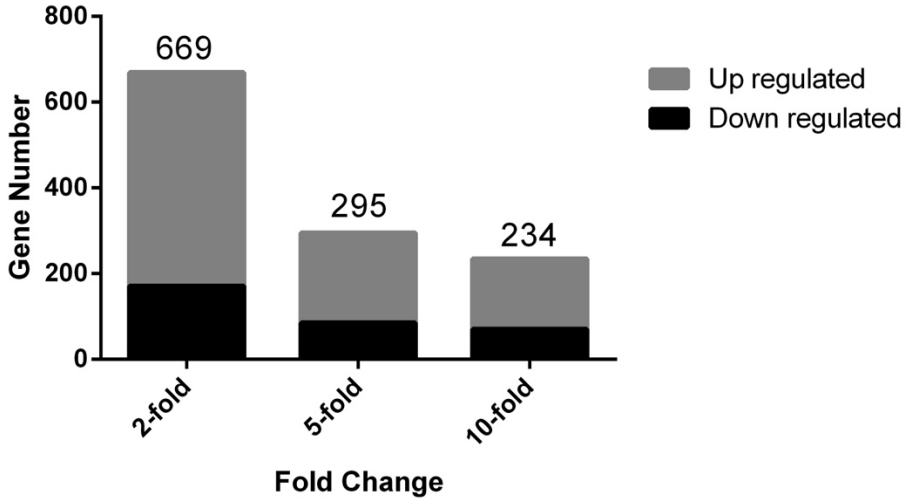


Figure 5-1. The number of up regulated and down regulated genes at the 2-, 5-, and 10-fold change thresholds in obese vs lean ewe endometrial tissue. FDR ≤ 0.05. Total number of genes is written above each bar.

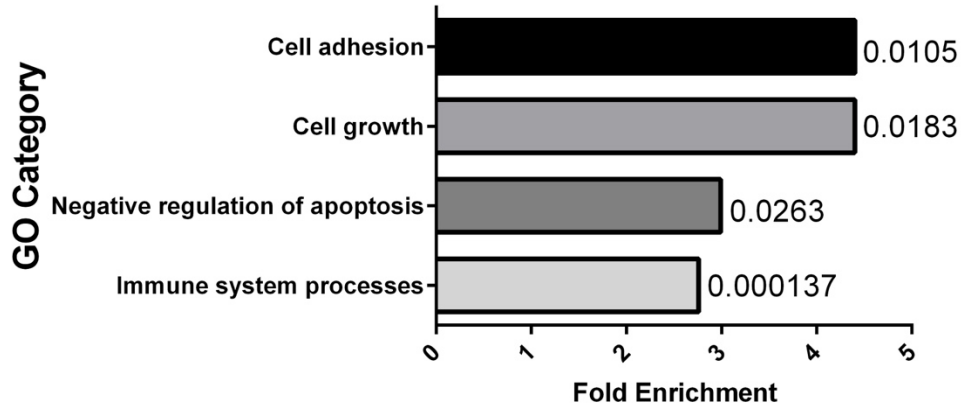


Figure 5-2. Fold enrichment of endometrial DEGs. FDR for each category is marked to the right of each bar. Data were analyzed using PANTHER Classification System.

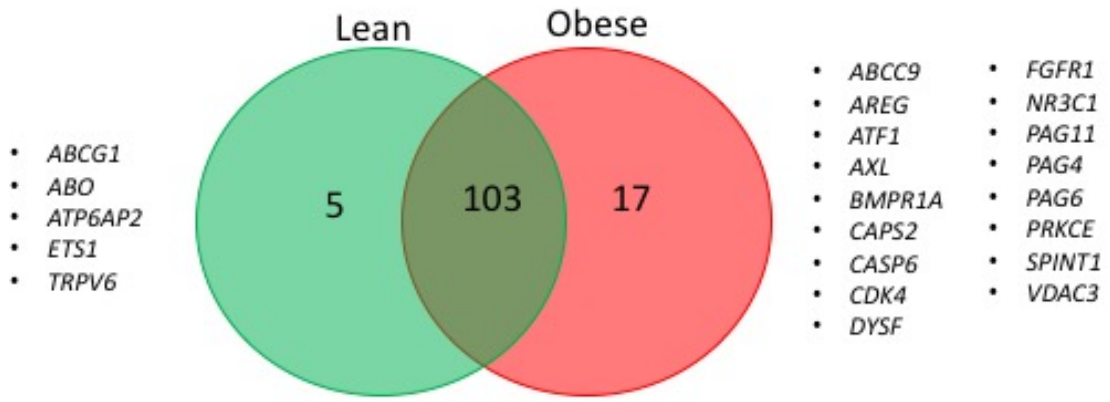


Figure 5-3. Placenta- and trophoblast-associated genes expressed exclusively in lean or obese ewe endometrial tissue.

Conclusions and Implications

Mammalian species experience the highest rates of pregnancy loss during early embryogenesis. The timing of these losses coincides with the development of the initial embryonic lineages. Thus, it is probable that miscues in these specification events are to blame for early embryonic mortality. Based on this work, early embryogenesis appears to be sensitive to environmental stressors. Exposure to adverse intrauterine environments results in maladaptive programming and poor offspring outcomes, including increased rates of developmental disorders and disease susceptibility. Lineage specification events and the impacts of environmental stressors on early embryogenesis remains vastly understudied, particularly in ruminant species. The goal of this work was to improve our understanding of lineage specification events, and to examine the effects of environmental stress on early embryogenesis in ruminants.

The first studies were performed to improve the efficiency of producing primitive endoderm outgrowths *in vitro*. Endoderm cells and the subsequent yolk sac were once believed to be rudimentary in ruminants and other mammals. However, studies now indicate that the yolk sac may play an important role in pregnancy retention, as impaired endoderm development results in embryonic mortality. Even with this knowledge, bovine endoderm remains understudied. Until recently, it has been difficult to produce bovine endoderm in culture, thus limiting a close examination of its development and function. Results from this work showed improvements in outgrowth production, and defined cell behaviors during extended culture periods. These cells will prove to be an invaluable tool in improving our current understanding of early bovine lineage specification.

An exciting facet of this work is the presence of multiple cell types within the outgrowths. Future work should focus on purifying the sample to contain a single endoderm subtype. This will allow for more conclusive assessments of endoderm development. Additionally, the opportunity to isolate multiple endoderm subtypes (PrE, PE, VE) from these outgrowths will allow us to study the progression of endoderm throughout embryogenesis. These endoderm cells may also be used to identify potential interactions with the trophectoderm during early embryogenesis. The two lineages develop in close proximity within the embryo, and potentially interact via chemical or physical signals. This information is not currently available, so these studies will greatly improve our current understanding of bovine embryogenesis.

A second set of studies was performed to examine the effects of maternal obesity on the preimplantation ovine conceptus. Research in this area is predominantly focused on fetal and postnatal effects of maternal obesity. An understanding of the impacts during early embryogenesis is lacking. This work identified potential developmental programming events in the preimplantation conceptus (Figure 6-1 A and B). Sequencing analysis revealed differential expression of genes involved in metabolic processes as well as placentation. This differential expression may be the mechanism behind the developmental programming and poor offspring outcomes observed in later development. Furthermore, this may be some of the earliest evidence of developmental programming in the ovine conceptus.

Follow-up work should be performed to examine any conceptus behavioral changes resulting from differential gene expression. I am especially interested in differences in implantation success and placental function between lean- and obese-derived conceptuses. This work would distinguish effects as sustained outcomes or identify the presence of compensatory mechanisms responsible for preventing complete placentation failure. Future studies may also focus on determining the underlying mechanisms behind the differential gene expression

observed in lean- and obese-derived conceptuses. Of particular interest is the methylation profiles of these conceptuses, as qRT-PCR identified differential transcript abundance of *DNMT1*, a factor involved in methylation patterning. This information may help identify methods to lessen the effects of maternal obesity on conceptus development. Finally, it would be beneficial to identify the lineage specific effects of maternal obesity. This can be done by dissecting the trophectoderm, endoderm, and epiblast from the conceptus, and analyzing each lineage separately. The current study may mask some of the altered gene expression, as genes are naturally differentially expressed among these tissues. This may provide even more insight into conceptus dysfunction. Ultimately, this study identifies the preimplantation period of conceptus development as a time susceptible to the effects of maternal obesity, and sheds light on the effects of maternal obesity on early embryogenesis.

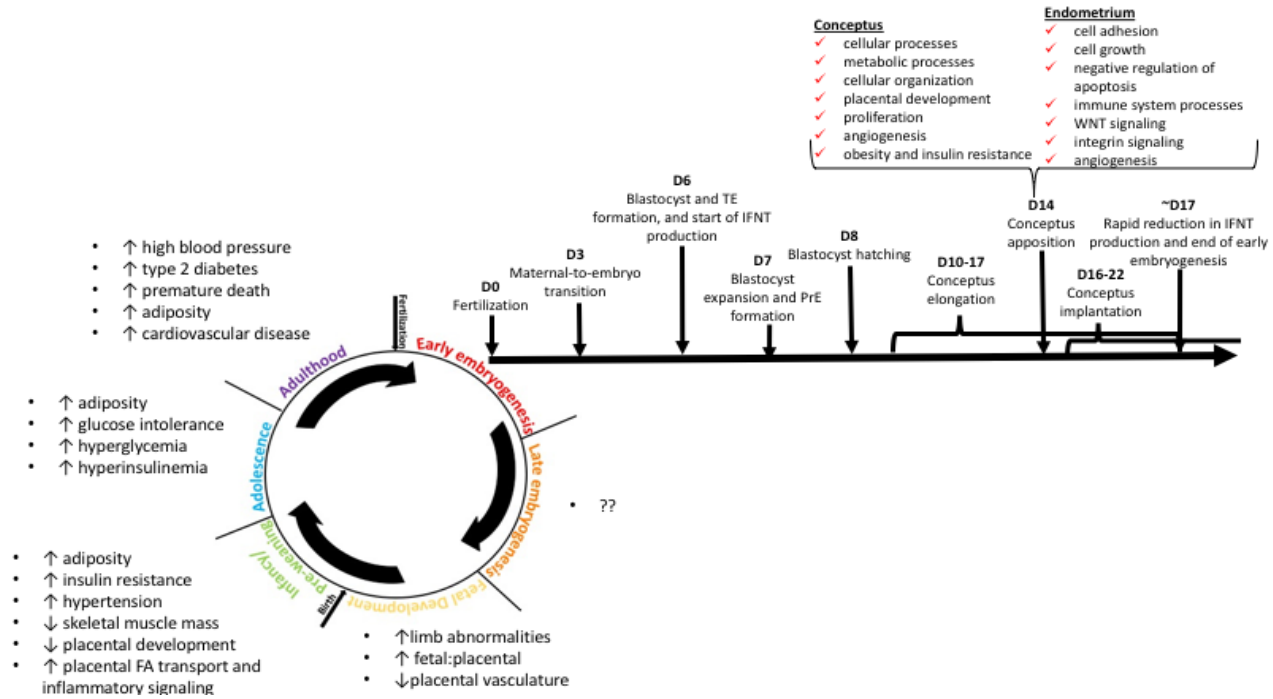
A final study was performed to evaluate the effects of maternal obesity on endometrial gene expression. The endometrium is the initial point of contact between the conceptus and maternal system during the process of implantation, and therefore likely plays a role in programming the developing conceptus. Our results showed altered expression of genes associated with uterine receptivity, including immune response, adhesion, and implantation (Figure 6-1 A and B). These findings complement our previous results of altered expression of genes involved in implantation and placentation in the conceptus. The changes in gene expression implicate the endometrium as a mediator between maternal nutrition and conceptus development.

While endometrial gene expression was affected by maternal obesity, it remains unclear if these changes impact protein production. Future work should include western blot analysis of proteins involved in implantation and placentation. Of particular interest are factors involved in initial adhesion events, including integrins and collagens. An assessment of these proteins would

provide evidence that the events controlling the earliest phase of implantation (trophoblast adhesion to the endometrium) are affected by obesity. I am also interested in examining PAG protein levels, as several were observed to be exclusively expressed in the endometrium of obese females. Expression of these proteins would indicate that BNCs have already migrated to the TE-endometrium interface in obese ewes, an event that implies asynchrony in the implantation cascade. As mentioned previously, future work should assess if uterine receptivity and conceptus implantation is actually impaired in obese ewes. It would be especially useful to determine which facets of uterine receptivity are affected obesity status (i.e. migration of immune cells, increased vasculature, etc). This would allow us to identify the mechanisms involved the abnormal implantation, resulting placentation, and maladaptive programming events.

Collectively, these studies provide new insights into the events controlling early embryogenesis and lineage specification of ruminant species. They also highlight the connection between maternal health, uterine environment, and conceptus development. Continued research in the area of early embryogenesis will elucidate the events involved in pregnancy loss and poor offspring outcomes and will help lessen their prevalence.

A.



B.

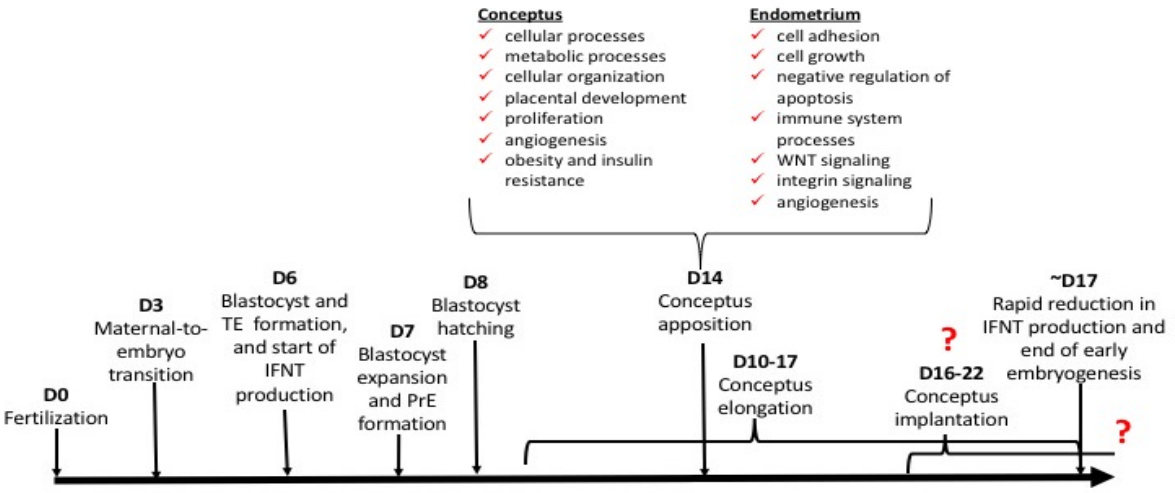


Figure 6-1 AB. Summary figures of the impacts of maternal obesity on offspring development. The figure has been updated to include the affected biological processes in conceptus and endometrial tissue. It remains unclear if these effects persist into late embryogenesis, as indicated by the question marks.

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