FACTORS AFFECTING TROPHOBLAST DIFFERENTIATION, DEVELOPMENT AND FUNCTION IN CATTLE

By

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To Mom, Dad, Chris, and Annie

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FACTORS AFFECTING BOVINE TROPHOBLAST DEVELOPMENT, DIFFERENTIATION AND FUNCTION

By

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In dairy cows, approximately 60% of all pregnancies fail to reach term and generate healthy offspring. A substantial portion of these losses occur during the first three months of gestation. Critical reproductive events must occur during this time period, and insufficiencies in any of these likely contribute to pregnancy failure. Inadequate or retarded placental formation and development are mechanistic causes for some, if not a substantial portion of pregnancy losses in cattle.

The initial events of placental attachment and adhesion with the endometrial epithelium begin by day 20 of gestation in cattle, and the placenta is fully formed between days 40 to 50. One of the first developmental events in the bovine placenta is the formation of binucleated trophoblast cells, referred to as binucleate cells (BNCs). BNCs are the trophoblast cells that attach to the uterine epithelium to form a feto-materal syncytium and produce several hormones important for pregnancy, fetal development and mammary gland development in the mother. Mechanisms controlling BNC formation are not well described. This dissertation research was completed to test the hypothesis that mechanisms controlling trophoblast differentiation, development and

function in cattle are similar to mechanisms utilized to generate invasive trophoblast lineages identified in humans and mice. Three primary objectives completed in this dissertation research include:

1) development of a method for obtaining enriched populations of BNCs from mid-gestation bovine placentae
2) identification of factors controlling BNC formation in cattle
3) evaluation of how bone morphogenetic proteins (BMPs) effect trophoblast development and function.

For the first objective, BNCs were isolated from mid-gestation bovine placentae based on their DNA content using fluorescence activated cell sorting (FACS). Sorting for hyperploidic cells yielded enriched BNC and CSH1 (chorionic somatomammotropin-1)-positive samples versus pre-sorted controls. Transcripts for BNC-specific markers were greater in abundance in BNC-enriched fractions than in MNC samples. FACS-sorted BNCs remained viable after 3.5 days in culture, and greater numbers of BNCs were evident when incubated on Matrigel-coated than non-coated plates. However, regardless of culture matrix, BNCs contained lower amounts of BNC-specific transcripts and were nearly devoid of *CDX2* mRNA after 3.5 d in culture.

The second objective was to examine the expression profile of selected factors associated with formation and activation of BNC-like placental cells identified in primates and rodents and to determine if overexpression of one of these factors (HAND1; heart and neural crest derivatives expressed-1) induced BNC formation in culture. Nearly all of the transcripts of interest (*HAND1, MASH2, ID1, ID2, IMFA, Stra13, GCM1 and E12/E47)* were present in bovine cotyledons obtained from mid-

gestation placentae. One of these, *HAND1*, was characterized by mRNA and protein concentrations being greater in BNC versus MNC. However, HAND1 overexpression did not induce BNC differentiation in a mononucleated trophoblast cell line.

The third objective was to elucidate the function of BMP2 and 4 in bovine trophoblast cells. BMPs play an important role in several reproductive processes and are implicated in controlling placental development in humans and mice. Transcripts for both BMPs were present in day 17 bovine conceptuses, the CT1 bovine trophoblast cell line and endometrium. The BMP antagonist, Noggin, also was detected in day 17 conceptuses but not in CT1 cells or endometrium. All receptors necessary for BMP2 and 4 signaling were found in all tissues examined. Supplementing CT1 with recombinant human BMP2 and 4 did not affect *IFNT* and *CSH1* expression and did not induce BNC formation. Therefore, potential functions for these morphogenic factors in bovine trophoblast cells remain unclear.

In conclusion, FACS is an effective method for isolating enriched populations of BNCs; however culturing BNCs leads to rapid loss of key BNC and trophectoderm specific markers in culture. HAND1 was found in greater abundance in BNCs versus MNCs. However, overexpression of HAND1 did not induce BNC formation in an ovine trophoblast cell line suggesting that HAND1 is not the only causative factor required for BNC formation in ruminants. The signaling pathway controlling BNC differentiation is still unknown. BMP2 and BMP4 mRNA are abundant in day 17 conceptuses; however their actions for regulating trophoblast function is unknown.

CHAPTER 1
INTRODUCTION

Over the past six decades reproductive efficiency in dairy cattle has decreased steadily. This decrease is negatively correlated with increased milk production [1, 2]. Approximately 60% of all pregnancies fail to reach term in dairy cattle [3]. Between 40-50% of pregnancies fail to reach term due to complications during the first three months of gestation [3, 4]. The events leading to pregnancy failure can be evaluated based on the timing of specific developmental events throughout early and mid gestation. Studying the mechanisms controlling specific events during this period of pregnancy loss can enhance our understanding of what may be leading to pregnancy failure. Some of the events this laboratory has focused on include embryo and conceptus development, maternal recognition of pregnancy and placental development [3, 5].

Substantial pregnancy loss occurs during the first 7 days of pregnancy. During this period, the fertilized egg must undergo several cellular divisions. By days 4-5 of gestation, embryos enter the uterus and begin translating from their own genome, an event termed embryonic genome activation [5, 6]. On day 6, the trophoblast lineage is specified and forms an outer layer of cells referred to as the trophectoderm and the blastocyst begins to be formed. Lack of cellular divisions and blastocyst formation can result from cellular miscues, including improper genome activation that includes the expression of lethal genes and deficient expression of housekeeping genes and chromosome abnormalities [7], These problems can be exacerbated by environmental and physiological stressors. One example is the partitioning of energy towards milk synthesis and lactation rather than reproduction causing reduced embryo quality [3, 8].

From 20 to 40% of pregnancies that survive into the beginning of the second week of gestation fail before day 24 of gestation [3, 4, 9]. Several crucial developmental events occur at this time. By day 13-15 of gestation the embryo begins the processes of conceptus elongation, transitioning from a spherical embryo, to a tubular and soon thereafter a filamentous conceptus [10]. During this time the hormone interferon tau (IFNT) is secreted from the mononucleate trophoblast cells (MNCs) [11]. IFNT prevents the pulsatile secretion of prostaglandin $F_{2\alpha}$ to extend luteal function beyond the length of a normal estrous cycle. The production of IFNT radically increases as the conceptus elongates [12, 13]. By day 16 of pregnancy, enough IFNT must be produced by the trophectoderm of the embryo or luteolysis will occur and the pregnancy will be lost [14]. Data indicate that the inability of the embryo to elongate and produce enough IFNT to prevent luteolysis results in pregnancy loss [15, 16].

Pregnancy failures occurring after day 24 of gestation but before day 42 (transition from embryonic to fetal development) are classified as late embryonic losses [4]. These events account for 5-20% of all pregnancy losses [3, 4, 17]. Many of these losses likely result from insufficiencies in placental formation and function. The placental attachment processes begins at approximately day 20 in cattle and is nearly completed by day 42 [13, 18]. BNC formation and migration into the uterine lining is a hallmark feature of early placental formation in ruminants. BNCs are apparent on day 16 of gestation [19]. The mechanisms controlling BNC formation and function are still unknown in dairy cattle. This topic is of central interest in this dissertation given that some studies have found that reductions in BNC numbers may contribute to pregnancy failures in cattle [20, 21].

Pregnancy losses following the sixth week of gestation are usually referred to as fetal losses. These losses are less prevalent than early and late embryonic losses representing between 2-5% of total pregnancy failures. The cause of fetal losses is often undetermined but can result from a pathological condition [3].

Reproductive failures are directly related to economic loss in the dairy industry [3, 4, 8]. It is estimated that each pregnancy failure in lactating cows costs a dairy farm $555. This value is based on repeat breeding and losses associated with lifetime milk production potential [22]. The cost of pregnancy failure is greater in fetal and late embryonic associated losses than early embryonic losses, due to the increased number of days open and costs associated with the loss of a calf. Also high producing dairy cows are estimated to have more costly pregnancy losses than lower producing dairy cows. An extended period of time between the subsequent lactations is more costly for high producing cows than low producing cows [22-24].

Based on these findings, it is clear that insufficiencies in trophoblast development, placental formation and/or placental function during early and mid-gestation can significantly impact pregnancy success in cattle. Understanding the processes controlling trophoblast development, differentiation and function are, therefore, imperative for identifying new ways to limit pregnancy failures in cattle. This dissertation research was completed to test the hypothesis that mechanisms controlling trophoblast differentiation, development and function in cattle is similar to mechanisms utilized to generate invasive trophoblast lineages identified in humans and mice. Three overall objectives were examined in this dissertation research.

1) To develop a method to isolate enriched populations of BNCs from mid-gestation bovine placenta and study the function of these cells in culture.
2) To identify expression differences of suspected trophoblast differentiation factors in MNCs and BNCs and determine if over expression of differentially expressed genes in a trophoblast cell line can induce BNC formation.
3) To evaluate the expression profile and function of bone morphogenetic proteins (BMPs) and their receptors during trophoblast development and differentiation.

CHAPTER 2
LITERATURE REVIEW

The Placenta

The mammalian placenta is a transient endocrine organ that is essential for fetal survival in eutherians [25]. The term 'placenta' was derived from the Latin term for "flat cake", an unleavened loaf of bread found throughout ancient Rome, and undoubtedly reflects the gross morphology of the human placenta [10]. The placenta is essential for fetal growth. It provides gas, nutrient and metabolic waste exchange between the mother and fetus [26-29]. It also produces several growth factors, cytokines, and hormones which help regulate the maternal environment and maintain pregnancy [30, 31].

The first studies of placental function and morphology date back to ancient Egyptian times. The first scientific studies of the placenta were completed by the Greek philosophers, Hippocrates and Aristotle, who hypothesized that the embryo is nourished by maternal blood [32]. In the fifteenth century, Leonardo da Vinci made several illustrations and observations about the placenta and fetal circulation that were based mainly on ruminant placentae, the placental model he had access to [33]. These observations, while anatomically incorrect for the human, did show that the fetal circulation is not continuous with the mother and that the placenta attaches to the uterus [32].

Later, advancing histological technology permitted scientists to complete more substantial investigations about the placenta. In the 1700s brothers William and John Hunter published books about the structure of the uterus and placenta in humans [32, 34]. William Hunter described the structure of the maternal spiral artery using wax

colored injections [35]. John Hunter was the first to describe the anatomical relationship between the fetal and maternal circulation in humans [34, 35]. Another well-known philosopher of the eighteenth century who made significant contributions to the understanding of placental function was Erasmus Darwin. In his published work, "*Zoonomia*", he described the role the placenta plays in delivering oxygen to the fetus [34, 36].

In the nineteenth century, Enrst Heinrich Weber obtained the first microscopic images of the human placenta and was able to describe an epithelial lining separating the maternal supply from fetal blood vessels [35]. In 1889, Ambrosius Hubrecht first coined the term "trophoblast" for cells that differentiate into placenta [35]. The term trophoblast is still used today to describe placental cells.

Placental Formation

Great diversity exists in placentation amongst eutherian, or placental, mammals, but many of the events that control placental formation are similar among most mammals [37]. All eutherian mammals have a chorioallantoic placenta [38, 39]. After fertilization, the embryo goes through a series of cellular divisions, leading to a mass of tightly compacted cells. This stage of embryo development is termed the morula stage [10]. Very early in development, usually before any differentiation event occurs, the translational events controlling development switch from maternal RNAs to the embryonic genome in an event called the Maternal-Zygotic Transition (MZT) [6, 40]. The timing of this event depends on the species; occurring at the late two-cell stage in mouse, between the four to eight cell stage in human, and at the 8 to 16-cell stage in ruminants [41].

The mammalian embryo undergoes its first lineage differentiation event as cells on the outer layer of the embryo differentiate to trophoblast cells [42]. The inner cells, often referred to as inner cell mass (ICM), remain totipotent and eventually develop into various other extraembryonic layers and embryonic germ layers [43]. The trophectoderm is made up of trophoblast cells that form the outer layer of the blastocyst and later the placenta [44, 45]. Another hallmark of blastocyst formation is the presence of a fluid-filled cavity termed the blastocoele cavity. Trophectoderm cells facilitate the formation of this cavity by pumping sodium into the blastocoele. This increase in ionic concentration in the embryo causes water to diffuse into the embryo forming the blastocoele cavity [45, 46].

Following cavity formation, two extraembryonic layers, the primitive endoderm and mesoderm, begin to form. The primitive endoderm differentiates from the ICM and grows to form the yolk sac [43, 47]. In cattle, the primitive endoderm begins to differentiate by day 8 and is completely formed by day 10 [48]. The yolk sac forms in the blastocoele cavity space [10]. The next layer of cells to differentiate is the mesoderm, which forms between the endoderm and trophectoderm. This layer emerges at day 14 in cattle [49, 50]. The trophectoderm and the mesoderm eventually will fuse to form the outer and inner layers of the chorion, respectively [43, 47].

The embryo continues to grow and expand as the now formed chorion folds and surrounds the embryonic disc. These folds eventually meet and fuse together to form the amnion and its fluid-filled amniotic cavity that surrounds the embryo/fetus. Simultaneous with chorion folding the embryonic disc begins to differentiate and eventually form the fetus.

At the same time as the amnion is developing, another extraembryonic membrane begins to form from the fetal hindgut. The posterior region of the hindgut extends and forms a sac-like evagination forming the allantois. The allantois begins to form at day 20 in cattle [50]. This extra embryonic membrane forms a fluid-filled sac that collects liquid fetal waste. The allantois also carries blood vessels that will vascularize the chorion and amnion [10]. The last event to occur is the fusion of the allantois and chorion to become the chorioallantoic placenta [10].

Placental Classification

There is a great diversity in placentation types, and several classification systems exist to account for the various differences in placental types identified. Several schemes for discriminating placentae are used. Some of these include the type of maternal-fetal interdigitation, nutrition, gross morphology, and the number of histological tissue layers separating the maternal and fetal blood supply [25, 51-54]. These classifications have been useful for understanding the evolution of placental diversity among mammals [51, 55], which will be discussed in greater detail later in this section.

Two widely used parameters for classifying placentae are based on gross morphology and histological examination [25, 31]. The gross morphological classification of placentae is based on the distribution of chorionic villi. Chorionic villi develop small finger-like projections that extend away from the chorion and intercalate with the uterine endometrium [31]. Four primary deviations in the distribution pattern of chorionic villi exist. In diffuse placentae, chorionic villi are distributed evenly over the placental surface [25, 31]. Species with this type of placentation include swine [56] and equids [57]. Some other mammals, including cats and dogs, possess a zonary placenta, which is characterized with a ring or band of invasive chorionic villi in the center of the

chorion [58, 59]. The discoid placenta contains a disc of concentrated chorionic villi that form the attachment to the uterine endometrium. The discoid placenta is found in rodents and humans [31, 60]. Ruminant species have a cotyledonary placenta. This placenta is similar to the discoid placenta in regards to having disc-like structures, but numerous chorionic villi structures, termed cotyledons, spread across the chorion and interact with distinct maternal structures, termed caruncles to form placentomes. The placentome is the area of placental attachment. This placenta type is found in ruminant species, including the bovine and ovine [61].

The placental classification system based on histological examination of the number of tissue layers separating the maternal and fetal blood supply was first described in the early 1900's by Grosser [25, 39]. In this classification, the most non-invasive type of placenta is termed epitheliochorial, where six layers of tissue separate the fetal and maternal blood supply (fetal endothelium of the blood vessel, the mesoderm, trophoblast, maternal epithelium, stroma, and endothelium of the maternal blood vessel). These placentae are found in swine and equids [62, 63]. Ruminant species have a synepitheliochorial morphology, where fusion between fetal placental cells and maternal uterine epithelium cells create areas of fusion that form syncytial plaques, or syncytium to various extents depending on the species [61-63].

The third type of placenta in this classification is the endotheliochorial placenta. In this placentation, the maternal epithelium and stroma is eroded away and the chorionic epithelium contacts the maternal endothelium. Species with this type of placentation include the feline and canine [25, 62, 63]. The most invasive type of placenta is called, hemochorial, and is classified as having no maternal tissue layers separating the

maternal blood supply and the fetal chorionic epithelium. This is often referred to as the placenta bathed being in the maternal blood supply. Humans and rodents are both classified as having a hemochorial placenta; however there are differences in the number of fetal tissue layers separating the fetal and maternal blood supply. In rodents, there are three trophoblast cell layers (hemotrichorial), two syncytial layers and a mononucleate cell layer. In humans, there are two trophoblast cell layers (hemodichorial), one syncytial layer and one mononucleate cell layer in the first trimester and by term there is one complete syncytialtrophoblast layer (hemomonochorial) separating the fetal and maternal blood supply and a discontinuous layer of mononucleate trophoblast cells. [62-67]. In the rodent the fetal and maternal interdigitation area is termed the labyrinthine and in humans it is termed villous [68].

Evolution of the Placenta

It is interesting to consider the evolutionary advantages and disadvantages afforded eutherian mammals versus other animals (methatherians, monotremes, and non-mammals [both viviparous and oviparous]). One advantage of viviparity over oviparity (egg laying) is the ability to protect the embryo/fetus as it develops. However, protecting the developing embryo can reduce the fitness and survivability of the mother, and this limitation likely maintained oviparity throughout the evolution of animals [69, 70]. The viviparity-driven conflict hypothesis argues that viviparity allows for genomic conflicts following fertilization. Conflicts over resources arise between the mother and embryo, siblings in the womb, and maternal and paternal genomes. These conflicts have resulted in the diversification in mammalian placentation [70-72]. Methatherian (marsupial) mammals evolved a primitive yolk sac placenta containing a poorly developed maternal-fetal interface [37, 73]. While eutherian mammals developed a

chorioallantoic placenta that allows these mammals to deliver well developed young by having a high degree of fetal-maternal exchange [69, 70, 74]. Overall the evolution to a chorioallantoic placenta has allowed eutherian mammals to produce well developed young with less reproductive wastage than seen in egg-laying species [69, 70, 74, 75].

Understanding the necessity of various placental types throughout mammalian evolution has been the focus of much discussion over the past 100+ years and has been rekindled recently by our ability to re-examine evolutionary relationships at the molecular level [76, 77]. The multiple divergences seen in mammalian placentation are due in part to the continental break-up [78-80]. This continental breakup led to four clades of eutherian mammals: Afrotheria, Xenarthra, Euarchontoglires, and Laurasiatheria [51, 53, 55, 76, 78]. The Australia continental break resulted in a large clustering of marsupials that are often included as an out-grouping in phylogenetic trees describing the evolution of eutherian mammals [37, 39, 51]. Original studies of placental evolution were based on fossil records and indicate that eutherian mammal divergence occurred following the Cretaceous/Tertiary (K/T) boundary (i.e. the end of dinosaurs) [79, 81]. More recent placental evolution analysis based on molecular phylogenetics indicates that eutherian mammals split from the metatherian taxa, or marsupial species, before the K/T boundary [78, 81]. While there is a disagreement between the fossil record and molecular phylogenetics on when eutherian mammals originated, both records show consistency in the order that the placental clades diverged [81, 82].

The superclades Afrotheria and Xenarthra are the closest to the original divergence of eutherian mammals. Interestingly, present-day species within these

groupings contain endotheliochorial or hemochorial placentae, suggesting that one of these placental types likely existed in the original mammals that gave rise to all eutherian mammals [83]. The Afrotheria clade (those arising from ancient Africa) is thought to be the predecessor for the other major clades [53, 77]. The second major clade to diverge likely was Xenarthra (now South America) [84] followed by the Euarchontoglires (now Europe and Asia) [51, 83]. This superclade includes two sister groups; the Glires (rabbits and rodents) and the Euarchonta (primates and tree shrews) [39, 51]. Lastly, the Laurasiatheria superclade diverge from the Euarchontoglires [85]. This fourth clade includes several orders of placental mammals, including perissodyctyls, carnivores, cetaceans, and cetartiodactyla [39].

Examples of epitheliochorial and synepitheliochorial placentae are evident in the Euarchontoglires clade in the lemur and within the expansive cetartidactyla grouping within the Laurasiatheria clade that includes pigs, cattle and whales [51, 53]. The epitheliochorial placenta is a derived adaption in the Euarchontoglires and Laurasiatheria super clades, however the placenta type that this evolved from is unclear [39, 53, 77, 86].

A substantial divergence in placental morphology exists within certartiodactyls. Species with a diffuse epitheliochorial placenta, such equine and porcine, are the more primitive species, and the emergence of a cotyledonary placenta with epitheliochorial or synepitheliochorial characteristics evolved from these species [51, 70]. The evolution of the coteldonary placenta correlates fairly well with the emergence of rumination and the formation of a rumen stomach. Perhaps a reason for this close correlation is that when fermentation occurs glucose availability is limited [51]. Limited glucose availability can

negatively impact the mother causing fatal metabolic diseases. Therefore the mother needs to tightly regulate glucose exchange with the fetus, however glucose is also essential for fetal development [70]. This could explain why placentae in ruminants evolved a more efficient nutrient exchange system than what is found in species with diffuse placentae. The nutrient exchange surface in the cotyledonary placenta is comparable to the endotheliochorial and hemochorial placenta. However, the diffuse equine placenta has a lower placental efficiency when compared to the bovine based on placental surface area and fetal weight gain over time [51, 70]. In conclusion, there are differences between the placentation found in humans, rodents and ruminants however, the epitheliochorial and hemochorial placentae are evolutionary linked and the nutrient exchange rate in these two distinct placentae is similar.

The Ruminant Placenta

As reviewed above, ruminant species have developed a specialized synepitheliochorial placenta also referred to as a cotyledonary placenta. The processes leading to the development and function of the ruminant placenta will be examined here.

Conceptus Elongation and Gastrulation

The process of conceptus elongation refers to the time period when the ruminant embryo changes from a spherical blastocyst to an ovoid then a tubular conceptus during a transition phase before the rapid elongation of the conceptus that leads to a filamentous structure. This rapid change in structure coincides with rapid trophoblast remodeling and proliferation [43, 48]. The timing of these events is species dependent based on the reproductive cycle. For instance, in cattle the embryo begins to transition from spherical to ovoid by day 12 and to tubular by day 14 where the average length is

5-6 mm. By day 16 the bovine conceptus is filamentous and ranges between 10-30cm in length [7, 48, 50].

This elongation process also occurs in non-ruminant ungulates, but some distinctions exist. For example, conceptus elongation in swine occurs because of changes in trophectoderm morphology caused by cytoskeleton re-organization instead of an active proliferation of cells [18, 87-89]. Also, equids do not undergo an elongation process. Instead the equid conceptus migrates freely between both uterine horns until days 16-17 following ovulation when the increase in conceptus diameter prevents this movement through the narrow uterine lumen and it becomes fixed in position [57].

During this same period of elongation, the ICM undergoes gastrulation and neurulation. As discussed previously, primitive endoderm, also referred to as the hypoblast, emerges early during embryonic development and usually is evident by day 8 of gestation in cattle [50]. Over the next few days the outer cells of the ICM become polarized and begin to differentiate into embryonic ectoderm. The hypoblast has formed a complete lining inside of the trophectoderm by day 12 in cattle. At this time Rauber's layer is lost and the epiblast is exposed [48, 50]. The process of gastrulation involves the migration of cells from the epiblast to form the primitive streak, endoderm and mesoderm. The precursor cells of the primitive streak are present in day 14 bovine embryos and accumulate at the caudal end of the epiblast. Also the primitive mesoderm forms between days 14-16 [50, 90]. By day 21 in bovine the primitive streak and definitive endoderm and mesoderm are formed [50].

Placental Attachment

There are several stages of implantation during placental development. First, the blastocyst must hatch from the zona pellucida and orient itself to the uterine epithelium.

Usually the ICM will be positioned on the side opposite of the implantation site [91, 92]. In ruminants, this site is central to the uterine cavity [91]. The embryo undergoes elongation at this time [92]. Following conceptus orientation is the apposition phase when the first cell-to-cell contact is made between the trophoblast and uterine cells [91]. This event occurs at approximately day 19 in cattle at the uterine glands located throughout the intercaruncular spaces on the endometrium [93-95]. Here trophoblast finger-like papillae extend into the openings of the uterine glands anchoring the periattachment conceptus and take up uterine histotroph [91, 93, 94, 96]. Concurrently, the endometrial caruncles develop deep folds that the chorionic villi will interdigitate with to form the placentome [97]. Following apposition, the conceptus begins to firmly adhere to the uterine endometrium. At this time, the trophectoderm and endometrial epithelium interdigitate in both the caruncular and intercarcuclular area. The interdigitation of the fetal villi, or cotyledon, with the caruncle forms the placentome [94, 95, 98].

The initial attachment or apposition of the trophoblast cells to the uterine epithelium is controlled by the loss of anti-adhesive components on the apical surface of the uterine epithelium, such as Mucin 1 (MUC1) [18, 99]. Loss of MUC1 is attributed to the down regulation of progesterone receptors on the uterine epithelium by progesterone [18, 100]. Removal of MUC1 reveals attachment and adhesion molecules. Following this removal, attachment is controlled by low-affinity carbohydrate binding molecules, including galectins and selectins [101-103]. Finally, firm adhesion of the trophoblast to the uterine endometrium is mediated by various integrin heterodimers. These heterodimers interact with extracellular matrix proteins, and

integrin binding molecules such as osteopontin and fibronectin found on the surface of the endometrium [104-108].

Placental Defects

One way to elucidate the essential mechanisms controlling placental development in ruminants is to study improper signaling events that cause abnormal placentation. In this regard, pregnancies produced from somatic cell nuclear transfer (SCNT) are beneficial due to the major placental defects, including placentomegaly, hydrallantois, and poor placental vascularization found in these pregnancies. These defects are due, at least in part to abnormal placentome formation and BNC number resulting in a high percentage of embryonic loss and a greater incidence of large offspring syndrome in SCNT pregnancies [21, 109].

Only 5% of SCNT pregnancies proceed to term in livestock species [110-115]. In cattle, 60-80% of SCNT pregnancies fail between day 30-90 of gestation and a majority of these losses occur between days 30-40 of pregnancy. This period of pregnancy coincides with cotyledon formation in cattle [109, 112]. Histological examination of SCNT placentae during this time period of loss shows smaller, fewer and less vascularized cotyledons when compared to *in-vitro* produced (IVP) embryos or artificial insemination (AI) placentas [21, 116, 117]. A placentomegaly phenomenon exists in bovine and ovine SCNT pregnancies that make it past day 90 of gestation. This phenomenon is characterized by a reduced number of placentomes, but a greater placentomal weight and overall size compared to AI as well as IVP placentae [111, 118, 119].

Along with gross morphological placental differences, the expression pattern of various factors is also different between SCNT and control (IVP and AI) placentae.

These factors include angiogenic factors, such as hypoxia-inducible factor-1 (HIF-1), angiopoieten-1 (ANGPT1), placenta growth factor (PIGF), as well as the vascular endothelial growth factor-A (VEGFa) system [116, 120]. Researchers have also examined SCNT placentas for differences in the insulin-like growth factor axis. Insulin-like growth factor binding proteins (IGFBP) -2 and -3 were increased in SCNT placental tissues in cattle and Insulin-like growth factor 2 (IGF-2) mRNA was reduced in ovine placentae [118, 121]. Glucose transporters, GLUT1, GLUT3, and GLUT8 are also reduced in SCNT ovine placentae coinciding with reduced fetal glucose plasma levels by day 135 of gestation [118]. Major histocompatibility complex I (MHC-I), which is involved in the immunologic rejection of the conceptus, is also reduced in bovine SCNT placentae [122]. Differences in these factors and others may indicate a cause for the SCNT placental abnormalities and also provided insight into potential mechanisms affecting proper placentation.

Another change in SCNT placentas is differences in BNC numbers. Several reports indicate a reduced number of BNCs in SCNT placentas when compared to IVP and AI placentas [21, 117, 123]. Several BNC-specific factors, including chorionic somatmammotropin hormone 1(CSH1), pregnancy associated glycoprotein (PAG) -1 and -9, and prolactin-related protein (PRP) are reduced in SCNT placentas [124, 125]. One report indicates an increase in BNC numbers in SCNT pregnancies [121]. Although there are differing reports of how BNC numbers change in SCNT pregnancies, it is clear that differences in the number of these cells exist. All of the observations made about SCNT pregnancies and placentas highlight essential time periods for proper placental development in ruminant species.

Placental Cell Types

The ruminant placenta contains two morphologically and functionally distinct trophoblast cells, the MNC and BNC [19]. Both of these cell types play essential roles during pregnancy. This section will describe the functional importance of these cell types, review known signaling pathways controlling their function, and discuss how the formation of these cells may be controlled.

Mononucleate Cells

The trophoblast MNCs comprise approximately 80 percent of the trophectoderm throughout gestation in ruminants. They contain features typifying cubodial to columnar epithelial cells located on a basal lamina [126, 127]. The apical surface membrane of these cells forms microvilli that interdigitate with microvilli on the endometrium forming the fetal-maternal contact zone. These contact zones are where maternal-fetal nutrient exchange occurs [10, 19]. Early in pregnancy, the MNCs also produce IFNT responsible for signaling maternal recognition of pregnancy in ruminant species [19, 128, 129].

For a pregnancy to succeed, the embryo must signal its presence to the mother and prevent corpus luteum (CL) regression, a process referred to as maternal recognition of pregnancy [19]. Ruminant species have evolved a unique signaling mechanism early in pregnancy to prevent CL regression or luteolysis. In a non pregnant animal, the CL regresses due to pusatile secretions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) produced by the uterine endometrium [130-135]. Pulsatile secretions of $PGF_{2\alpha}$ are controlled by oxytocin and oxytocin receptor binding on the uterine endometrium. In a pregnant animal, IFNT must be secreted in high enough amounts to prevent oxytocin binding; thus abolishing $PGF_{2\alpha}$ pulsatile secretions and luteolysis [14, 16, 136]. Several

factors influence the production of IFNT and many of these factors are found in the uterine histotroph [15, 137-139].

Trophoblast Cell Lines

Ruminant trophoblast cell lines that resemble MNCs have been established for cattle and other ruminant species (e.g. ovine and caprine) [101, 140, 141]. In cattle, several trophoblast cell lines have been established from *in vivo*, *in vitro*, somatic cell nuclear transfer, and parthenogenetic activation of blastocysts [140, 142-144]. These cell lines secrete IFNT, but are not known to differentiate into BNCs nor do they produce BNC specific markers, such as CSH1 [140, 144]. The *in vitro* blastocyst derived cell line, CT-1, has been used to elucidate the role of several growth factors on IFNT expression [145-147]. Another bovine *in vitro* blastocyst derived trophoblast cell line is the BT-1 cell line, developed in Japan [148]. These cells express IFNT and can differentiate into what is considered a naïve BNC that secrete CSH1 protein but do not produce BNC specific PAGs [148-150].

Sheep trophoblast cell lines have also been established. One of central importance to this dissertation research is the ovine trophoblast cell line, oTr. It was developed from day 15 elongating sheep conceptus [20]. The oTr cell line has been used to elucidate mechanisms controlling trophoblast cell differentiation, migration and attachment [20, 101, 151, 152]. In another study, primary sheep trophoblast cell cultures were produced from the cotyledons of sheep at 120-135 day gestation. These cells were passaged and maintained for 12 to 16 weeks during which time BNC differentiation occurred unlike the oTr cell line which does not form BNCs in culture [153].

There is one report of a caprine trophoblast cell line produced from trophoblast cells isolated from placentae of goats at 100 days of gestation [141]. These cells stain positive for IFNT and CSH1. In this cell line, BNCs also form at a high frequency [141]. Overall the establishment of trophoblast cell lines from various ruminant species has aided researchers in studying the underlying signaling mechanisms controlling trophoblast development and function.

Binucleate Cells

Ruminant BNCs, also termed trophoblast giant cells, differentiate from trophoblast MNCs and have two functions. First, they are the cells that fuse with the maternal epithelium to form the feto-maternal syncytium [61, 154, 155]. Second they are the major endocrine trophoblast cells producing steroid hormones, such as estrogen and progesterone, as well as other hormones including chorionic somatomammotropin-1 (CSH1; also known as placental lactogen) and pregnancy associated glycoproteins (PAGs) [156-158]. These cells are found in both the cotyeldonary and intercotyledonary regions of the ruminant placenta [61, 159]

Placental BNCs are found in all ruminant species examined, including bovine, ovine, cervine, caprine, water buffalo, alpaca, antelope, and giraffe [61, 159-166]. BNCs are also present in the mouse deer, which represents the most primitive ruminant group existing [167-169]. Mouse deer have a placenta that is unique amongst species in the Ruminant suborder because they contain BNCs but do not contain definitive cotyledons [167, 168].

BNCs first appear just before implantation and comprise 15 to 20 percent of all trophoblast cells throughout most of gestation. Their numbers decline approximately a week before parturition [159, 170-172]. The decrease of BNCs in the last week of

gestation is controlled by the concentration of fetal cortisol [172, 173]. Ablation of the prepartum fetal cortisol rise prevented the normal decrease of BNCs while increasing cortisol levels in immature fetuses decreased BNC numbers [173]. In cattle, this decrease in BNCs prior to parturition is associated with placental separation [174].

Beginning at day 16 in cattle, BNCs form from MNCs through either fusion and or acytokinetic mitosis events [175, 176]. Most BNCs contain two nuclei, and all cells are hyperploidic and contain up to 32 DNA copies [155, 165, 175]. Using electron microscopy, it was discovered that BNCs have multiple small mitochondria, rough endoplasmic reticulum cisternae and an extensive golgi body which produces secretory granules [177, 178]. These cytoplasmic secretory granules store synthesized hormones that are delivered to the maternal environment through exocytosis [61, 179].

The migratory nature of BNCs is evident from the time they first appear in the trophoblast. On average, one in seven BNCs can be observed migrating towards the apical surface of the trophoblast epithelium at any one time throughout gestation [159]. BNCs are unique migratory cells because they form a tight junction with neighboring MNCs that allow the trophectoderm to keep the fetal physiological milieu isolated from the mother while allowing the BNCs to deliver hormones to the maternal environment [126, 180]. Following their migration to the uterine epithelium, BNCs fuse with the uterine epithelium. This fusion forms feto-maternal hybrid, or trinucleate, cells which form the placental attachment in ruminant species [61, 181, 182]. The degree of syncytia formed depends on the ruminant species, with ovine and caprine forming a greater syncytia than seen in bovine and cervine [61, 159]. In ovine and caprine the feto-maternal hybrid cells fuse together to form the feto-maternal syncytia containing

numerous nuclei (n=3-20) [161, 171, 183]. In the bovine and cervine this syncytium is formed at the time of placental implantation, however as gestation progresses the syncytium is overgrown by unicellular uterine epithelium cells [184].

One of the major functions of BNCs is to produce and deliver steroid and protein hormones to the maternal environment [61]. BNCs are the primary placental cell producers of steroid hormones [185]. A majority of the placental progesterone in ovine and bovine is produced in BNCs through the conversion of pregnenolone [186-188]. They also are to be the major placental source of prostaglandins and have the ability to convert prostaglandin $F_{2\alpha}$ to prostaglandin E_2 [189]. Oestrogens are also produced by BNCs [185, 190]. Key steroid synthesis enzymes, aromatase and P450 side chain cleavage, are also present in BNCs [191, 192].

One protein hormone expressed by BNCs is placental lactogen (PL) or chorionic somatmammotropin hormone 1 (CSH1) [191, 193, 194]. It is a member of the growth hormone (GH) and prolactin (PRL) family of hormones but is more closely related to PRL [195, 196]. Six isoelectric isoforms of PL have been identified in bovine with a molecular weight of 31-33 kilo Daltons (kDa) [193]. All bovine CSH1 is glycosylated however ovine and caprine CSH1 is not glycosylated resulting in a lower molecular mass of 23 kDa [197]. CSH1 binds and activates the GH/PRL/PL family receptors [193, 198-200].

Various *in vivo* functions have been postulated for CSH1 in both mother and fetus. CSH1 may have a luteotrophic effect in bovine by increasing the size of the corpus luteum and progesterone concentrations [201]. Another proposed role of CSH1 is nutrient intake and partitioning to regulate nutrient supply to the fetus [197, 202, 203].

In conjunction with nutrient intake and partitioning, CSH1 is suggested to regulate fetal growth and development. Research has correlate elevated CSH1 plasma concentrations and increased fetal birth weight [196, 204-207]. Lastly, CSH1 impacts mammary development and lactation. Administration of CSH1 can increase milk yield by acting as a PRL-like compound in cattle [208-211] .

Another member of the GH/PRL gene family produced by BNCs is the group of prolactin-related proteins (PRP) or prolactin-like proteins (PLP) [196, 212]. Nine PRP genes are expressed in bovine placenta but only PRP-I is present at the protein level in bovine [196, 213, 214] Expression has been reported as early as day 17-20 in the conceptus and has been localized to BNCs [215-217]. PRP-1 expression coincides with implantation events in the bovine and therefore this protein may have a potential role in this process. [217]. These proteins are also hypothesized to play a role in regulating ovarian and mammary gland function [30].

BNCs produce several aspartic protease family proteins termed pregnancy associated glycoprotein proteins (PAGs) [158]. These proteins have been characterized in bovine, caprine and ovine ruminant species [218-221]. There are two classes of PAGs: ancient and modern [218, 222, 223]. Both classes of PAGs are present in the cotyledon from implantation to term, although there are different spatial and temporal expression patterns exist for the modern and ancient PAGs [224, 225]. At least some, and likely all of the ancient PAGs are active proteolytic enzymes. These include PAG-2 and -10 and localize to the MNC outer surface [218, 223, 224]. On the other hand, the modern PAGs are unlikely to be active proteases based on their

structure and poor binding to pepstatin [226-228]. The modern PAGs include PAG-1, -6, -7, and -9 are found in BNCs [218, 222, 224].

The function of PAGs during pregnancy is unknown, but PAG measurements in plasma are a useful way to diagnose pregnancy in cattle and other ruminants [229-232]. Based on radioimmuno assay (RIA), a positive pregnancy diagnosis can be made with PAGs beginning between days 28-30 of pregnancy [233]. During early and mid gestation PAG concentrations increase gradually until day 240 at which time they increase exponentially [231, 233]. The concentrations remain elevated until parturition [231]. PAGs remain detectable in bovine for approximately 14 weeks indicating a long half-life for these proteins in cattle [231]. Low concentrations of PAGs (<2.5 ng/ml) in pregnant animals are also predictive of impending fetal losses [234, 235]. Decreased levels of BNC-specific, PAG1, also are seen in high milk production in dairy cattle [8, 236]. Lastly, low levels of PAG1 also are found in placentae from SCNT animals containing fewer BNCs [21, 120].

Other BNC-expressed factors are not as well characterized. For example, lectins such as *Dolichos biflorus* (DBA) are used as a BNC indicator. The function of the carbohydrate moieties it binds with is not known, but it is known that BNCs contain various proteins that are heavily glycosylated (e.g. PAGs) [237-240]. The glycosylation pattern found on BNC secretory granules is conserved across ruminant species and is characterized by containing tri/tetraantennary complex N-glycans and bisecting terminal N-acetylgalactosamine [241]. Another marker used to identify BNCs is the SBU-3 monoclonal antibody. This antibody recognizes a carbohydrate antigen on BNCs [242, 243].

Other factors localized to BNCs have been implicated in regulating BNC migration and fusion. One of these is heparanase (HPA), an extracellular remodeling enzyme that degrades heparin sulfate proteoglycan [244, 245]. HPA plays a similar role in human placental implantation [246, 247]. Another enzyme, termed fertilin β, is a disintegrin and metalloprotease (ADAM) while another protein, termed CD9, is a transmembrane 4 superfamily glycoprotein, are present in a subpopulation of BNCs [248-251]. Both fertilin β and CD9 regulate migration, adhesion and fusion of various cell types, and CD9 specifically has a demonstrated role in human trophoblast invasion [249, 252-254]. The localization of fertilin β and CD9 in BNCs and their established roles in trophoblast cell migration and adhesion also implicates them in ruminant trophoblast migration and adhesion. Integrins also likely participate in regulating BNC migration, adhesion and invasion [255-257]. Various integrins are expressed on placental cells in ovine and bovine [104, 106-108, 258, 259]. Specifically integrin subunits α_6 and β_1 localize in BNCs [259].

BNC Isolation and Culture

Previous efforts to isolate and culture BNCs from mid-gestation placentae have provided insight into various facets of BNC biology including hormone production [189-191]. Several tissue dissociation methods have been utilized to liberate BNCs from the placenta, including mechanical disruption and enzymatic digestion with trypsin, collagenase, or dispase [187, 191, 260-262]. BNCs harvested using collagenase have an increased percentage of viable cells over mechanical disruption and trypsin digestion [260]. Following digestion, BNCs have been typically separated from the whole placenta homogenate using density gradients [186, 187, 191, 260-264]. The purity of BNC samples harvested using these methods ranges from 15% to 80%. The variable

purities and unrepeatability of this BNC isolation method are potentially caused by the narrow range of concentrations (103-1.06 g/ml) used with these density gradients [191, 260, 261, 263].

Following isolation, many of these studies evaluated BNC viability and hormone production in *in vitro* culture systems. BNCs are viable in culture for over 30 days when grown on a collagen substratum [260]. This substrata may be the optimal matrix to culture BNCs on based on cell viability and attachment when compared to plastic [260, 261] The ability of BNCs to produce hormones was also evaluated in culture; BNC produced several steroid hormones including estrogen and progesterone [187, 190, 264]. However the expression of steroid enzymes and CSH1 is decreased following three and seven days of culture [191]. These experiments indicate that while BNCs appear viable throughout culture, they have altered expression of key BNC specific factors.

Trophoblast Cell Differentiation

The signaling events that lead to placental cell differentiation have been best defined in rodents and primates. We hypothesize that the transcriptional regulators of trophoblast development and differentiation are conserved among most mammals, and therefore, the signaling mechanisms controlling ruminant trophoblast cell differentiation likely are similar with those at work in mouse and human trophoblast lineages. The focus of this section is to evaluate the known mechanisms controlling trophoblast cell differentiation in humans, mice and cattle.

Trophectoderm Differentiation

The specification of trophoblast cells from non-committed precursor cells cells during early blastocyst formation involves several transcriptional regulators. Two key

changes in gene expression required for trophoblast cell formation in mice are the loss of octamer-4 (OCT-4; also known as POU class 5 home box 1 [POU5F1]) expression and the gain of caudal type home box transcription factor 2 (CDX2) expression [46]. OCT4 maintains the undifferentiated state of the ICM and is downregulated in the trophectoderm. CDX2 regulates trophectoderm differentiation and is not expressed in the inner cell mass [265-268]. In the mouse, OCT4 and CDX2 are first expressed at the morula stage when OCT4 begins to be restricted to the cells that become the ICM while CDX2 is found more concentrated in the outer cells [269]. Other factors have also emerged in the mouse model of trophoblast cell differentiation, such as TEAD4 (TEA domain family member 4). TEAD4 is necessary for trophoblast development prior to implantation and may drive the expression of CDX2 [266, 269, 270]. The T-box gene Eomesodermin (Eomes) is required for trophoblast and mesoderm development and its expression is stimulated by CDX2 [269, 271].

The pattern of expression of these trophoblast specifying factors is different in cattle. Most notably, OCT4 expression continues in the trophoblast for several days after its formation, and its detection ceases 1-2 days before conceptus elongation [268, 272, 273]. CDX2 is expressed exclusively in the trophectoderm of bovine blastocysts and is implicated in controlling trophectoderm differentiation [42]. Eomes expression has not been found in bovine blastocysts [274]. Expression of TEAD4 remains to be established. These observations indicate that there is a conserved mechanism for involvement of CDX2 in trophectoderm differentiation but other lineage segregation factors differ between mammals.

Trophoblast Lineage Segregation in Mice

There are three distinct trophoblast cell layers in the mouse, the labyrinthine, the spongiotrophoblast, and the trophoblast giant cells. The inner most trophoblast layer to the fetus is the labyrinthine, which is comprised of two layers of syncytium, termed the syncytiotrophoblast. The labyrinthine is highly folded giving it a large surface area essential for maternal-fetal nutrient and gas exchange. The next layer is the spongiotrophoblast layer. This layer whose function is not really known develops from the ectoplacental cone. The trophoblast giant cells are the outer-most cell type of the mouse placenta. These cells mediate trophoblast cell invasion and implantation [31, 275, 276].

Trophoblast Lineage Segregation in Primates

There are two main trophoblast cell layers in primates; the chorionic floating villi and the villous cytotrophoblast. The chorionic floating villi is the inner-most layer and shares analogous function with the labyrinthine layer in mouse. Like the mouse, this layer is where syncytiotrophoblast cells are found. The villous cytotrophoblast are precursors of invasive extravillous cytotrophoblast cells that invade into the maternal spiral artery [31, 275, 277].

Several cell types in rodents and primates share similarities. The trophoblast giant cell in rodents and the extravillous cytotrophoblast cell in primates are homologous based on their hyerploidic and invasive nature [278]. Another placental cell that is also hyperploidic and semi-invasive is the BNC found in ruminants. BNCs and extravillous cytotrophoblast cells are known to have 4-16 DNA copies while the trophoblast giant cells have approximately 100 copies [31, 175, 279]. Many of the same genes that are involved in trophoblast differentiation in rodents are conserved in primates and it is

hypothesized that these same genes may be involved in ruminant trophoblast differentiation [31, 275, 278]. The following sections are aimed at reviewing the known mechanisms controlling trophoblast differentiation and identify conserved pathways that may play a role in ruminant trophoblast differentiation.

Transcriptional Regulation of Trophoblast Differentiation

In both rodents and primates, several members of the basic Helix-Loop-Helix (bHLH) family of transcriptional regulators play essential roles in trophoblast lineage segregation [276, 280]. The bHLHs form homo- and/or hetero- dimers with one another or may associate with E-proteins (e.g. E12/47), depending on the factor. Following dimerization, they bind DNA at specific E-box sites and regulate transcription [281]. The E box (CANNTG) is found in enhancer and promoter regions of numerous genes [281, 282]. The basic region of these proteins is the portion that binds the E box. Specifically, a glutamate residue in the basic region of each dimer subunit makes contact with the cytosine and adenine bases in the E-box. Portions of the loop region and second helix also contact DNA and form van der Waals interactions that help to stabilize the binding of this complex to DNA. [282-284].

There are several classes of bHLH proteins [285]. The class I bHLHs are often referred to as E proteins. Most of these factors are ubiquitously expressed and form dimers with one another or with bHLHs from other classes [286]. Examples of E proteins include Daughterless, HEB, E12, and E47 [282]. The proteins E12 and E47 arise from alternative splicing of the *E2A* gene [287].

The class II bHLHs are expressed in select tissues. Many of these factors can dimerize with one another but their preference usually is to dimerize with E proteins. Members of this second class include the MyoD family of proteins, achaete-scute

complex homolog 2 (ASCL2) and heart and neural crest derivatives expressed 1 (HAND1) [282, 286]. ASCL2 is often referred to as MASH2 or HASH2 (mammalian or human achaete-scute complex-like protein 2, respectively). HAND1 is known by several different names, including Hxt, eHAND, and Thing1 [288]. ASCL2 and HAND1 are antagonists of one another and compete for E protein dimerization, specifically E12 and E47 [275, 289, 290]. Another class of HLH proteins is the ID class (inhibitors of DNA binding; ID1, ID2, ID3 and ID4). These proteins lack the basic DNA binding region and act as dominant negative regulators of E proteins and bHLHs by forming inactive dimers that cannot bind DNA [282, 291, 292].

Several bHLHs appear important for placental development in mice and humans. Of special note is the role that HAND1 may play in regulating trophoblast giant cell formation in mice [275, 276, 293]. HAND1 transcripts are present in placental tissue as early as embryonic day 7.5 [294], and an embryonic lethal phenotype is observed in HAND1 null mice at this time. These mice lack trophoblast giant cells [295, 296].

Use of mouse and rat trophoblast stem cell lines has also provided insight into the role of HAND1 in trophoblast lineage segregation. Trophoblast stem (TS) cell lines have the ability to differentiate into one of four rodent trophoblast cell types, trophoblast giant cells, spongiotrophoblast, syncytiotrophoblast, and glycogen trophoblast [31, 297]. Culturing mouse TS cells with FGF4 maintains a non-differentiated, proliferative population of cells, but its removal results in trophoblast differentiation into trophoblast giant cells and syncytiotrophoblast cells [267, 298-300]. HAND1 over-expression is sufficient to override the FGF4 signal and induce trophoblast giant cell formation [301]. Trophoblast stem cells generated from HAND1 null mutant mice have a reduced rate of

trophoblast giant cell differentiation when FGF-4 is removed. Also, those trophoblast giant cells that did differentiate exhibited a decreased invasion rate when compared to trophoblast giant cells produced from normal trophoblast stem cells [302]. In the rat trophoblast cell line, Rcho-1, over expression of HAND1 induces trophoblast giant cell formation [294, 303].

In rodent species, regulation of HAND1 expression plays a key role in trophoblast lineage specification. Several factors positively and negatively regulate HAND1 in trophoblast differentiation. One such factor is Sox15 (Sry-related HMG box 15). Sox15 is found predominately in trophoblast giant cells, and its overexpression in Rcho-1 cells increased HAND1 expression and induced trophoblast giant cell formation [304].

The expression of Hand1 is tightly regulated by intracellular molecular mechanisms. HICp40 (human inhibitor of myogenic factor [I-mfa] domain containing protein) binds and sequesters HAND1 to the nucleolus [305-308]. HAND1 is activated when Plk4 (Polo-like kinase 4) phosphorylates HAND1 and releases it from HICp40 and the nucleolus [305-308]. Release into the nucleoplasm allows HAND1 to bind DNA. Following release, cells are observed exiting the cell cycle and undergoing trophoblast giant cell differentiation [305, 306].

The regulation of HAND1 activity is a highly coordinated process in rodent placentae, and several HAND1 antagonists play key roles in regulating this factor and placenta development in mice. The HAND1 antagonist, ASCL2, also has a role in trophoblast cell differentiation. ASCL2 null mutants are embryonic lethal between 9.5 and 10.5 due to placental defects. In these mutants the spongiotrophoblast layer of the placenta is absent and a greater number of trophoblast giant cells are present [309].

ASCL2 also promotes spongiotrophoblast differentiation and proliferation *in vitro* [310]. Trophoblast giant cell precursor cells are found in the spongiotrophoblast layer and their growth is promoted by ASCL2 [275, 311]. Over-expression of ASCL2 also inhibits trophoblast giant cell formation. Additionally, expression is down-regulated as trophoblast giant cell differentiation occurs [290, 294, 301, 312].

Other bHLH factors also have a role in trophoblast cell differentiation in mice. I-mfa (inhibitor of myogenic factor) blocks nuclear importation and DNA binding of several bHLHs [313], and I-mfa null mice have a placental defect that is embryonic lethal on day 10.5 of gestation marked by reduced numbers of trophoblast giant cells [312]. This factor inhibits ASCL2, thus promoting trophoblast giant cell differentiation in culture [299, 312]. The bHLH factor, Stra13 (stimulated by retinoic acid 13) also regulates mouse and human trophoblast differentiation [314-316]. Overexpression in mouse TS cells induces trophoblast giant cell formation similar to that observed for HAND1 [301]. The dominant negative bHLH factors, specifically ID1 and ID2, also play crucial roles in trophoblast differentiation [275, 317]. These factors are down-regulated during normal trophoblast differentiation in rodents [294]. Also ID1 overexpression in Rcho-1 cells inhibits trophoblast giant cell differentiation [294].

Many of the same bHLH factors likely also have a role in human trophoblast development. HAND1 is present in various human cytotrophoblastic cell lines, including Jar, Jeg-3 and BeWo [318-320]. The HAND1 antagonist, ASCL2, is localized to human cytotrophoblast progenitor cells [314]. Stra13 regulates human trophoblast differentiation and localizes to the human trophoblast epithelium [314, 315, 320]. Also, ID2 expression is increased in preeclampsia patients. ID2 over expression in human

cytotrophoblast cells reduces cell invasion [321]. These factors are down regulated during normal human trophoblast differentiation [294, 321].

In cattle, the expression of HAND1 and ASCL2 has been examined in normal and SCNT pregnancies. HAND1 is present in the elongating bovine conceptus and in-situ hybridization indicates that HAND1 localizes to BNCs [272, 294]. HAND1 mRNA is also decreased in SCNT placentae with decreased BNC numbers [21]. ASCL2 may be involved negatively in BNC differentiation. It is present in day 8 blastocysts, day 17 conceptus, and day 40 and 60 cotyledonary tissue and was most abundant in the day 17 filamentous conceptus [322]. Expression of ASCL2 expression is increased in SCNT placentae samples that contain decreased BNC numbers [21].

To summarize these findings, several bHLH factors are required for normal trophoblast differentiation in rodents, and apparently these same factors play active roles in controlling placental development in humans. The little information available in ruminants suggests that some of these same factors may control BNC formation and function, although controlled studies have not been completed to support such conjecture.

Endogenous Retroviruses

Endogenous retroviruses have been implicated in trophoblast formation due to their presence in human and animal placenta tissues [74, 323-329]. In multiple species, endogenous retroviruses play a role in placental cell fusion. In humans and mice, the formation of the syncytiotrophoblast cell layer occurs through trophoblast cell fusion. In mice, syncytiotrophoblast formation is regulated by the endogenous retrovirus syncytin-1 [330, 331]. Syncytin-1 is a member of the HERV-W retroviral family. It is an envelope protein [330, 332, 333] that binds to a sodium-dependent neutral amino acid transporter

[334-337]. The over expression of syncytin-1 induces cell fusion and syncytium formation [338].

Expression of synctin-1 is controlled by the transcription factor GCM1 (glial cells missing homolog 1) [276, 330, 339]. There are two binding sites for GCM1 located in the upstream 5'-flanking region of the synctin-1 gene that control its expression [339]. Over-expression of GCM1 induces cell fusion in several trophoblast cell lines and this cell fusion event is linked to increased expression of synctin-1 [301, 340]. GCM1 is localized to syncytiotrophoblast cells in rodents [341-343]. Mutant GCM1 mice are embryonic lethal by day 10 of gestation due to the absence of the placental labyrinth which is formed by syncytiotrophoblast cells [344].

The role of syncytin-1 and GCM1 is similar in human syncytiotrophoblast formation [330, 345]. GCM1 induces syncytin-1 expression and trophoblast cell fusion in the human trophoblast cell line, BeWo [340]. These factors localize to the syncytiotrophoblast of human placenta [338, 342]. The expression of GCM1 is decreased during preeclampsia in direct proportion to a decrease in syncytin-1 also seen during this placental disorder [332, 346]. The decrease in GCM1 expression is due to degradation stimulated by preeclampsia associated hypoxia [345, 347]. The degradation of GCM1 is caused by the suppression of the phosphatidylinositol 3-kinase-Akt signaling that activates GSK-3β (glycogen synthase kinase 3 beta) [345]. Activated GSK-3β phosphrylates GCM1 signaling the recruitment of F-box protein, FBW2 (F-box and WD repeat domain containing 2). FBW2 protein marks GCM1 for ubiquitination and degradation [345, 348]. This information indicates that improper GCM1 levels can affect

the expression the endogenous retrovirus syncytin-1 and trophoblast development resulting in placental insufficiencies.

Endogenous retroviruses also play a role in placental differentiation and development in sheep [20, 176]. In particular, a distinct retroviral envelope protein called endogenous Jaagsiekte sheep retroviruses (enJSRVs) appears vital for this activity. Several enJSRVs are abundant in the uterine epithelial lining and the trophoblast cells of sheep throughout gestation [176, 349-351]. Uterine expression of enJSRVs is progesterone dependent [349]. In the trophectoderm, enJSRVs expression is temporal and coincides with conceptus elongation and BNC formation [351]. The cellular receptor for enJSRVs, hyaluronoglucosaminidase 2 (HYAL2), is detectable beginning at day 16 of gestation and is found exclusively in BNCs and syncytial plaques in the placentomes [351]. Inhibition of enJSRVs in the uterus with morpholinos at day 8 of pregnancy in sheep hampered conceptus elongation and prevented BNC differentiation [20].

Two hypotheses for BNC formation in sheep exist. The first hypothesis is that BNCs develop through endoreduplication followed by enJSRV-mediated fusion between BNCs and uterine epithelium to create trinucleated or multinucleated synchtial cells. The second hypothesis is that the enJSRVs induce both BNC and syncytial formation through fusion with HAND1 likely inducing endoreduplication events either immediately before or after this event [5, 333]. While the importance of endogenous retroviruses has been established in sheep, the same mechanisms have not been identified in other ruminant species. Cattle, for example, do not contain enJSRVs in their reproductive

tract. Therefore, the role of endogenous retroviruses is not known in bovine trophoblast differentiation.

Bone Morphogenetic Proteins

A large family of paracrine factors that play a major role in trophoblast cell differentiation are the bone morphogenetic proteins (BMPs) [267]. These factors are part of the superfamily of transforming growth factor-β (TGF-β) factors that control various aspects of cell, tissue and organ development and differentiation in numerous species [352, 353]. The BMPs are best known for regulating bone formation [354-356], cardiovascular function [357] and various aspects of reproduction [358]. BMPs signal through specific type I and type II serine/threonine kinase receptor complexes. BMP ligand binds the type II receptor, this binding recruits the type I receptor. The type I receptor is then phosphorylated at specific serine and threonine sites by the type II receptor [359]. Several intracellular signaling molecules are activated by this phosphorylation event; most notably the Smads. In this pathway, the receptor regulated (R)-Smad receptor proteins, specifically Smad 1, 5 and 8, are activated by phosphorylating two C-terminal serine residues [352, 353]. Activation allows R-Smads to bind co-Smad, or Smad-4 and form a Smad complex. This complex then translocates into the nucleus, binds DNA and activates target gene transcription [360]. BMPs can also signal through the mitogen-activated protein kinase (MAPK) pathway [361].

Multiple levels of negative regulation for BMP signaling exist in cells. Several antagonists exist for BMPs, and one of central interest in reproduction is Noggin. Noggin binds BMP ligands and blocks the ligand BMP receptor binding site. Noggin is

up-regulated by BMP 2, 4 and 6. Up-regulation of Noggin is part of a negative feedback loop that prevents overstimulation [358, 362].

Another avenue of regulating BMP activity is the inhibition of Smad signaling. Smad signaling is inhibited in part by the inhibitory (I) Smads 6 and 7. These Smads act as antagonists to the R-Smads competing for association with the activated Type I receptor [353]. I-Smads also inhibit signaling by causing receptor degradation. Smad7 recruits ubiquitin ligases, Smurf1 and Smurf2, to form a complex at the activated receptor and induces degradation through proteasomal and lysosomal pathways [352, 353]. Smurfs can also interact with R-Smads and signal for their degradation via similar pathways [353]. Overall the BMP signaling pathway is tightly regulated to prevent over activation, which can lead to various pathologies, including cancer [363].

The BMPs that are of particular interest when considering the paracrine regulation of placental development, differentiation and function are BMP 2 and 4. These factors are closely related TGF-β family members that utilize similar receptors [362, 364]. BMP2 and BMP4 also use the type I serine/theronine kinase receptors, BMPR1A (ALK3) and BMPR1B (ALK6) and type II serine/theronine kinase receptor, BMPR-II [365, 366]. BMP2 also uses the type II receptor, ACVR1 (ALK2) [367]. BMP4 is the more studied of these factors in regards to extra-embryonic membrane development and trophoblast formation. In mice, BMP4 is involved in vascular development between the embryo and placenta [368, 369]. It is also essential for mesoderm formation as indicated by the lack of this tissue layer in a majority of BMP4 knockout mice. Mesoderm formation occurs by day 6.5 in mouse. BMP4 knock-out mice are embryonic lethal, however embryonic arrest occurs between a range of

developmental days (day 6.5 and 9.5). Embryos that survive past day 6.5 develop mesoderm but arrest during other stages of gastrulation [364]. Hypothetically, those embryos that arrest later in gestation likely were rescued by BMP2. BMP2 expression overlaps that of BMP4 [364]. The hypothesis that BMP2 and BMP4 expression overlaps is also supported by data showing the BMP 2 and 4 type II serine/theronine kinase receptor, BMPR-II, knockout mouse is embryonic lethal at day 6.5 due to lack of mesoderm formation [370].

BMPs have also been implicated in controlling human trophoblast differentiation. Supplementation of BMP4 to human embryonic stem cells induces trophoblast formation in culture [371, 372]. This differentiation event is also caused by BMP2 [371]. BMP4 induces this differentiation event by increasing CDX2 expression through SMAD activation [373].

In conclusion, BMPs mediate placental formation in mice and humans. However, little is known about the expression and role of BMPs in bovine trophoblast development and function.

Summary of Previous Literature

For thousands of years we have known that the placenta is vital for fetal development and survival. Eutherian mammals possess a great diversity of chorioallantoic placentae. Multiple placental classification systems have been developed and have helped to describe the evolution of the various placental types . The ruminant has evolved a specialized synepitheliochorial placenta. Based on the predescribed phylogentic assessment of placental formation, this is a placental type derived from a common ancestor of the ungulates with an invasive type of placenta. There are two placental cell types that comprise the ruminant placenta: MNCs and

BNCs. The MNCs produce the maternal recognition of pregnancy hormone IFNT and function in placental nutrient and gas exchange. The BNCs function in placental attachment and the formation of the fetal-maternal syncytium. They also produce and secrete several hormones, including progesterone and CSH1, into the maternal environment. Studies of BNC function are difficult due to the lack of an *in-vitro* model system. The factors regulating trophoblast differentiation are not well understood in ruminants.

Trophoblast differentiation has been studied extensively in the mouse and human. The trophoblast giant cells in mouse and the extra villous cytotrophoblast cells in humans are analogous to BNCs in ruminants. In mice, bHLH transcription factors play essential roles in the formation of the trophoblast giant cell, most notably HAND1 and ASCL2. These factors also are linked to placental defects associated with reduced BNC numbers in SCNT pregnancies. Another family of factors that cause trophoblast cell differentiation in mouse and humans is the BMPs, specifically 2 and 4. The role of these factors in ruminant trophoblast development is not known.

The following chapters were aimed at testing the hypothesis that mechanisms controlling trophoblast differentiation, development and function in cattle are similar to mechanisms utilized to generate invasive trophoblast lineages identified in humans and mice. The first objective was to develop a method to isolate enriched populations of BNCs from mid-gestation bovine placenta and study the function of these cells in culture. Floursence activated cell sorting (FACS) was used to isolate enriched populations of MNCs and BNCs. These cells were then evaluated for differences in gene expression and BNCs were studied in culture. The second objective was to

identify expression differences of suspected trophoblast differentiation factors in MNCs and BNCs and determine if over expression of differentially expressed genes in a trophoblast cell line can induce BNC formation. The third objective was to evaluate the expression profile and function of bone morphogenetic proteins (BMPs) and their receptors during trophoblast development and differentiation. The expression of BMP 2, 4, and receptors was found in day 17 bovine conceptus and endometrium and the function of BMP 2 and 4 on bovine trophectoderm was tested in the bovine trophectoderm cell line CT-1. Overall these objectives evaluated several potential mechanism controlling trophoblast development, differentiation and function.

CHAPTER 3
THE ENRICHMENT AND CULTURE OF BINUCLEATED TROPHOBLAST FROM MID-GESTATION BOVINE PLACENTA USING FLUORESCENCE-ACTIVATED CELL SORTING

Introduction

Placentae from cattle, sheep, deer and other members of the Ruminantia suborder are unique among mammals. Most ruminants have a cotyledonary placenta comprised of multiple maternal and fetal tissue contacts, termed placentomes, instead of the single, large area of contact seen in mammals with discoid placentae [154, 374] . Ruminant placentomes are comprised of fetal cotyledons and maternal caruncles that become highly interdigitated as gestation progresses. Ruminant placentae are also classified as having a synepitheliochorial contact between fetal and maternal tissues. Placental cells invade into the uterine epithelium and form syncytial plaques that persist to varying degrees throughout gestation depending on the species [154, 374]. The placental cell responsible for this invasion is the binucleate cell (BNC). This cell contains two distinct nuclei. It also is called trophoblast giant cell because of its large size when compared to mononucleated trophoblast cells (MNCs) [70, 375]. In cattle, BNCs are apparent by day 16 of gestation. By day 25 of gestation approximately 15 to 20% of the trophectoderm is comprised of BNCs. BNCs are present throughout gestation and begin reducing in number during the last few days preceding parturition [172, 374].

BNCs serve as the primary endocrine cell in ruminant placentae. They produce a variety of hormones, notably estrogens such as estrone- and estriol-sulfate, progesterone, chorionic somatomammotropin-1 (CSH1; also known as placental lactogen) and various prolactin-related proteins [156, 157, 196]. BNCs also secrete

numerous pregnancy-associated glycoproteins (PAGs). These proteins comprise a large group of active and inactive aspartic proteases that are detected in maternal blood throughout gestation in cattle and other ruminants [227]. Detecting PAGs in serum or plasma is one method used to predict pregnancy status in various ruminants [229, 376].

Nearly all of our current knowledge about BNCs has been achieved by observing them *in situ*. BNCs are terminally differentiated and therefore do not proliferate after their collection. It has proven challenging to induce ruminant trophectoderm to differentiate into BNCs *in vitro*. To date only one bovine cell line, termed the BT1 cell, is able to form BNCs, and these cells likely represent a naïve, immature form of BNCs [240, 261]. Others have harvested BNCs from bovine and ovine placentae to complete short-term *in vitro* studies by using density gradient centrifugation to purify BNCs [187, 191, 260, 263]. Work presented in this report describes the use of fluorescence-activated cell sorting (FACS) to isolate BNCs after enzymatic digestion of mid-gestation bovine cotyledons. The hyperploidic nature of BNCs [175] was used in combination with a membrane-permeable fluorescent DNA dye to permit high-speed sorting of BNCs from diploid cells [377]. The overall aims of this work were to examine the efficiency of BNC-enrichment with FACS and examine the limits of using these cells after their collection.

Materials and Methods

Tissue Collection

Pregnant bovine uteri were collected from a nearby abattoir (Central Beef Industries L.L.C.; Center Hill, FL) and transported to the laboratory on ice. Reproductive tracts were dissected, fetuses were excised and crown-rump length was used to estimate the stage of gestation (mean = 135.3 ±2.67 days of gestation; range =

118 to 159 days). For each placenta (n=20), cotyledons (n=5-6/placenta) were separated from caruncles and dissected away from intercotyledonary tissues. Tissue was diced into 5-6 mm sections and incubated in DMEM with high glucose (4.5 g/l D-glucose) (Invitrogen Corp., Carlsbad, CA) containing 25 units/ml Dispase (BD Biosciences, Bedford, MA), 0.625 mg/ml Pancreatin (Invitrogen Corp.), 10% [v/v] fetal bovine serum (FBS; Invitrogen Corp.), and 10 mM HEPES (Invitrogen Corp.) at 37°C for 1 h under constant rotation. Following digestion, homogenates were filtered through a 200 µm mesh and centrifuged (300 x g; 10 minutes at room temperature). In preliminary studies, cells were resuspended in 3 µM Propidium iodide in buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% [v/v] Nonidet P-40) and incubated at room temperature for 15 minutes. Following incubation cells were centrifuged (300 x g; 10 minutes at room temperature), resuspended in buffer, plated on glass slides, and viewed by epifluorescence microscopy. For FACS studies, cells were resuspended in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen Corp.) containing 5% FBS and 10 µM Vybrant® Dye Cycle™ Green (Invitrogen) and incubated at 37°C for 30 minutes in the dark. Samples were transported at 37°C to the University of Florida Interdisciplinary Center for Biotechnology Research Flow Cytometry laboratory (UF-ICBR; Gainesville, FL).

FACS

Homogenates were sorted using a BD FACSAria™ cell sorting system (BD Biosciences) and FACS Diva software version 6.2.1 (BD Biosciences). A 100 mW laser emitting 488 nM light was used for excitation. Thresholds were set at 20,000 on forward light scatter and 5000 on green fluorescence (530 +/- 15 nM) to eliminate excessive cell debris. A gate was set on a forward light scatter and green fluorescence plot to further

assist in debris removal. A green fluorescence histogram was plotted. The photomultiplier voltage was adjusted to maintain the diploid peak to 50 on a linear scale of 0 to 255. Once the diploid peak was established, cells were collected. MNC-enriched samples were collected by harvesting cells that fluoresced at the diploid peak. BNC-enriched samples were harvested in the fluorescence range 2- to 4-times greater than that of the diploid peak. Cell aggregates were avoided by eliminating sample with fluorescence more than 4-times that of the diploid peak. Approximately two million BNCs and six million MNCs were usually collected in a three hour time period. All cells were collected into a collection medium (DMEM with high glucose containing 10% FBS and 10 mM HEPES). Following FACS, cells were centrifuged (300 x g; 10 minutes at room temp) to remove FACS sheath fluid and resuspended in collection medium.

Immunostaining

Tissue homogenates collected before sorting (non-sorted samples) and post-sorted samples were fixed in 4% [w/v] paraformaldehyde (Polysciences Inc, Warrington, PA, USA) for 15 min at room temperature. Cells were permeabilized and blocked in 0.5% [v/v] Triton-X-100 (Thermo-Fisher Scientific Inc., Fairlawn, NJ) and 1% [w/v/] bovine serum albumin (BSA; Thermo-Fisher Scientific) for 20 min at room temperature. Cells were incubated in rabbit antiserum generated against ovine CSH1 (generously provided by Dr. Russell Anthony; Colorado State University; 1:1000 Dilution). After washing, cells were incubated in Alexa Fluor® 594 goat anti-rabbit IgG (Invitrogen Corp.) for one hour, washed again and, mounted onto glass slides using ProLong® Gold antifade reagent (Invitrogen Corp.) and viewed under phase-contrast and epifluorescence microscopy to determine the proportion of cells that were BNCs (cells containing two nuclei in one plasma membrane) and that were CSH1-positive,

respectively. Four individual slides were examined for each placental preparation, and four independent fields were counted per slide (approximately 125-200 cells/field).

BNC Cell Culture

FACS-enriched BNCs were centrifuged (300 x g; 10 min, room temperature) and reconstituted in DMEM with high glucose containing 10% fetal bovine serum and other supplements (100 µM non-essential amino acids, 2 mM glutamine, 2mM sodium pyruvate, 55 µM β-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphoterin B; each from Invitrogen Corp.). Approximately 50,000 cells were seeded onto Lab-Tek II 8-well chamber slides (0.7cm^2/well) (Thermo-Fisher Scientific). Wells either were treated with Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA) or no coating was added (plastic only) in 400 µl medium. Matrigel coating was completed using a 1:3 dilution of Matrigel™ in DMEM following manufacturer instructions. Two replicate Matrigel-coated and non-coated wells were included in each study (n=4 placentae).

After 3.5 days at 38.5°C in 5% CO_2 in air, medium was removed and cells were fixed in 4% [v/v] paraformaldehyde for 15 minutes. Cells were immunostained for CSH1 reactivity as described previously and stained with 8.1 µM Hoechst 33342 (Invitrogen Corp.). Cells were viewed under phase-contrast and epifluorescence microscopy to determine total cell number and number of BNCs and CSH1-positive cells (5 fields/well; ~50 cells/well). Percentages of CSH1-positive cells and BNCs in FASC preparations before culture served as a control in the analysis.

Quantitative (q) RT-PCR

In some studies, total cellular (tc) RNA was extracted from cells immediately after FACS. TRIzol® (Invitrogen Corp.) was added to MNC- and BNC-enriched populations

after centrifugation to remove residual medium and tcRNA was extracted using the PureLink™ Total RNA Purification System (Invitrogen Corp.). In the culture studies (n=4 placentae), tcRNA was extracted using the PicoPure® RNA Isolation Kit (MDS Analytical Technologies, Sunnyvale, CA).

qRT-PCR was completed in both sample sets as described previously [147] to determine the relative abundance of *CDX2*, *CSH1*, *PAG1*, *CYP19* and *18S* mRNA. Samples were incubated with RNase-free DNase (New England Biolabs, Ipswich, MA) before reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA). Primers (200 nM) specific for *CDX2*, *CSH1*, *PAG1*, *Cyp19* and *18S* (internal control) (Table 1) were used in combination with a SYBR® Green Detector System (Applied Biosystems Inc.) and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to quantify target gene abundance. Following an initial activation/denaturation step (50°C for 2 min; 95°C for 10 min), 40 cycles of a two-step amplification procedure (60°C for 1 min; 95°C for 15 s) were completed. A dissociation curve analysis (60-95°C) was used to verify the amplification of a single product. Primer efficiencies were tested on RNA collected from cotyledonary samples by using the relative standard curve approach [146]. Each sample was completed in triplicate reactions. A fourth reaction lacking reverse transcriptase was included to control for genomic DNA contamination. Non-processed, whole cotyledonary tissue was used as a control.

In most studies, the comparative threshold cycle (C_T) method was used to quantify mRNA abundance [146]. Briefly, the average ΔC_T value for each sample was calculated (gene of interest C_T – C_T for *18S*) and used to calculate the fold changes in relative

abundance of each transcript. For one study (BNC culture study), the abundance of *18S* RNA differed over time relative to that of total RNA concentrations. Since the same amount of starting tcRNA was used for this study, raw C_T values were inverted by solving the equation, 40-C_T, to examine differences in mRNA abundance. The value 40 represents the total number of PCR cycles completed.

Statistical Analysis

All analyses were completed by analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (SAS Institute, Cary, NC). When analyzing qRT-PCR data, the ΔC_T values or raw C_T values were used for analyses [146, 147]. Either ΔC_T values were transformed to fold differences or raw C_T values were inverted (40-C_T) for illustration on graphs. Results are presented as arithmetic means ± SEM.

Results

BNC Enrichment Using FACS

A series of pilot studies were completed to identify an optimal strategy for dissociating cotyledonary tissue. Two enzymatic approaches, trypsin and Dispase/Pancreatin, were examined along with a mechanical disruption technique described previously [191, 260]. After each dissociation procedure, placental cells were stained with propidium iodide (Invitrogen Corp.) to identify BNCs and determine cell viability, respectively. Overall viability of placental cells was markedly greater after cotyledons were digested enzymatically (>95% propidium iodide exclusion rates) than in placental cells obtained by mechanical disruption (~10% propidium iodide exclusion rate). Although digestion with 500 µg/ml trypsin proved effective at maintaining cell viability, it yielded substantially more cell clumps when compared to cotyledons

dissociated with Dispase/Pancreatin (data not shown). Based on these observations, Dispase/Pancreatin was used in the remaining studies.

Placental homogenates were sorted via FACS to determine the relative purity of BNCs in the hyperploidic cell population. A consistent diploid peak was observed in placental homogenates. A broad range in hyperploidic cells was evident in most samples, although defined hyperploidic peak(s) could not be detected in most samples (fig. 3-1A & B). To determine whether these hyperploidic cells were indeed BNCs, FACS was completed on placental homogenates (n=5 samples) and cells fluorescing with 2- to 4-times the intensity of those within the diploid peak were collected and analyzed. Under phase-contrast microscopy, substantially more (P=0.0001) BNCs presided in the hyperploidic sample than were evident before sorting began (fig. 3-2). Immunofluorescence was completed to verify that the binucleated cells identified in these sorted samples also produced CSH1, a BNC-specific product. A greater (P=0.0002) proportion of CSH1-positive cells existed in the FACS-sorted preparation than in the cotyledonary samples before sorting (fig. 3-2). The purity of these BNC enriched populations ranged from 65-70% (fig. 3-2).

Attempts to improve BNC purity via FACS beyond what was achieved were not successful. Selecting cells greater than 4-times the intensity of those found in the diploid peak yielded fewer BNCs. In fact, most of these cells represented clumps of MNCs. Utilizing more narrow ranges of green fluorescence intensity (e.g. 2-3, 3-4 times that of the diploid peak) yielded similar BNC purities when compared with that of selecting cells that were 2- to 4-times the intensity of cells found at the diploid peak (data not shown). It also was difficult to collect ample BNC numbers when using

smaller gates. Broadening the sorting gate to 2- to 4-times the intensity of the diploid peak usually permitted collection of two million cells over three hours, although this sometimes varied substantially. Occasionally it took substantially less time to sort these cells (e.g. two million in one and one-half hours) and on other occasions cell sorted proceeded so slowly that sorting had to be terminated before ample cells were sorted. In most instances (85% of the time), however, FACS proceeded with repeatable timing and outcomes.

To determine if MNC-enriched samples also could be collected with FACS, placental homogenates (n=5) were subjected to FACS and cells with fluorescence intensities at the diploid peak and at 2- to 4-times greater than the peak were separated and analyzed (fig. 3-3). Cells with DNA content similar to that of the diploid peak (presumptive MNCs) contained fewer ($P<0.01$) BNCs than the pre-sorted preparations whereas the hyperploidic cells (BNC-enriched samples) contained substantially more ($P<0.01$) BNCs (fig 3-3A). Differences in the relative abundance of selected transcripts also were evident in the MNC- and BNC-enriched samples. Relative abundances of *CSH1* and *PAG1*, two BNC-specific transcripts [224, 378], were greater ($P<0.05$) in BNC-enriched samples than in the MNC samples (fig. 3-3B). Concentrations of CDX2 mRNA, a transcription factor involved with trophectoderm lineage specification and placental gene expression [42], were decreased ($P=0.03$) in BNCs versus MNCs.

BNC Culture

FACS-sorted BNCs were incubated on selective substrata to determine if they would maintain their morphology and gene expression profile following culture. BNCs could be visualized after 3.5 days in culture when grown on either Matrigel™ or no coating (fig. 3-4A). Matrigel coating was better able to maintain BNC numbers during

culture. Total numbers of cells, number of BNCs, and number of CSH1-positive cells in each field were greater ($P<0.01$) after 3.5 days on Matrigel™ than on plastic only (fig. 4B). The percentage of total bound cells that were BNCs were greater ($P<0.01$) on the Matrigel-coated than non-coated wells (75.7±2.5% vs. 58.7±3.9%, respectively). Also, a greater ($P<0.01$) proportion of the BNCs on Matrigel-coated wells stained positive for CSH1 than those on non-coated wells (91.9±2.2% vs. 51.0±7.3%, respectively).

Although the BNCs remained reactive to CSH1 antisera after 3.5 days in culture, marked changes in BNC-specific gene expression were observed after culture. Lower ($P<0.05$) mRNA abundances were evident for *CDX2*, *CSH1*, *PAG1* and *CYP19* after 3.5 day of culture than before culture (fig. 3-5). *CDX2* mRNA levels were barely detectable after culture. The presence of Matrigel did not affect gene expression profiles compared with non-coated wells. Abundance of *18S* RNA also was decreased ($P<0.002$) after culture, and because of this, C_T values were not normalized to this factor.

Discussion

Work described here shows that FACS can be used for selecting BNCs from bovine cotyledonary homogenates. Yields of 1-2 million BNCs could be isolated within 3 h at 70-85% purity when using Vybrant® Dye Cycle™ Green nuclear stain as the indicator. Several placental dissociation methods were tested in preliminary work, and using a Dispase/Pancreatin dissociation approach described for dissociating ovine endometrium [379] proved most effective at dissociating placental tissue into single cells without negatively affecting viability. Different gating strategies also were examined to improve the purity of BNC preparations. Reducing the size of the range in dye intensity

for gating cells did not improve the proportions of BNCs isolated and the speed at which BNCs could be collected was notably reduced.

Devising strategies to further improve the purity of BNCs through FACS could not be conceived by using the fluorescent DNA dye. The most prominent cell contaminant in BNC-enriched samples was non-digested clusters of diploid cells. These clusters likely represented MNCs or maternal endometrial cells. It is nearly impossible to completely separate maternal caruncles and fetal cotyledons in mid-gestation bovine placentae because of the extensive interdigitation of fetal and maternal tissues. Further gains in selecting BNCs over other cells in placental homogenates likely will require the use something other than DNA staining as the FACS indicator. Of particular interest would be to identify a cell-surface marker that can be used to discriminate BNCs from other cells. Such markers have not been identified to the best of our knowledge.

FACS also could be used to purify diploidic cells. In general, these gated samples consistently contained only 5-8% BNCs. These diploid cells were termed MNCs (*i.e.* diploid trophoblast) in the results, although these samples likely also contained some endometrial cells. Current studies were not aimed at purifying placental MNCs away from endometrial cells and other diploid cells. The proportion of maternal cells in diploid samples was not determined, but RNA extracted from these samples contained ample amounts of *CDX2* mRNA, a trophectoderm marker [380, 381], indicating that a fair portion, and likely a majority, of the diploid cells collected were placental MNCs.

Good quality tcRNA also could be extracted from FACS-sorted MNCs and BNCs. One trophectoderm gene (*CDX2*) and two BNC-specific genes (*CSH1* and *PAG1*) were

examined to further describe the efficiency of cell sorting. Not surprising, *CSH1* and *PAG1* mRNA were abundant in BNC-enriched samples. Placental lactogen, the protein product of CSH1, is produced predominately by BNCs in ruminants [194, 196]. PAG1, or PSPB (pregnancy-specific protein B), is produced in BNCs and represents one of the predominant, if not the most abundant PAGs in the maternal bloodstream throughout most of pregnancy in cattle [224, 227, 229, 376]. *CDX2* encodes a transcription factor that regulates placental gene expression and trophectoderm lineage specification [380, 381]. Its expression was different between the MNC and BNC samples, with a reduction in *CDX2* mRNA concentrations existed in the BNC samples.

It also was possible to culture BNCs isolated by FACS. Previous reports of BNC culture exists [191, 260, 261]. Coating plastic plates with collagen proved useful in maximizing BNC attachment in two studies [191, 261]. However, another report found that BNCs can be cultured for extended periods of time without a collagen substratum [260]. Our general observations were that most BNCs attached within 48 h in culture in Matrigel-coated plates whereas from 60 to 72 h was required for BNCs to attach to well with no matrix (data not shown). Collagen-only matrixes were not tested here. After 3.5 days in culture, BNCs appeared normal morphologically regardless of whether they were cultured on Matrigel or plastic-only. However, markedly more BNCs were attached to Matrigel-coated wells than cultures lacking this matrix. Moreover, more of the BNCs attached to Matrigel contained measurable amounts of immunoreactive CSH1 protein over those BNCs found on plastic (see Fig. 4). Based on these observations, Matrigel was a more suitable matrix for BNC culture than using no matrix. The primary component of Matrigel is laminin, a central component of basement membranes [382].

It also contains trace amounts of several growth factors, including transforming growth factor-β, basic fibroblast growth factor (FGF2) and insulin-like growth factor 1. Further research is needed to determine if these or potentially other molecules promotes BNC attachment and survival in vitro.

Marked changes in gene expression profiles were noted after the 3.5 day culture. Reductions in abundance for each transcript investigated were evident. A previous report observed decreases in the expression of *CYP19*, the gene encoding aromatase, following BNC culture [191]. Other genes encoding steroidogenic enzymes also were modified in that report. For example, *CYP17* mRNA abundance increased with progressive culture whereas *3BHSD* expression increased during the first 3 days of culture and decreased thereafter. The present work also observed marked decreases in *CSH1* and *PAG1* mRNA concentrations and nearly the complete absence of *CDX2* mRNA after 3.5 days in culture. Reductions in *CSH1* mRNA concentrations also were evident in another BNC culture study [191]. Based on these observations, BNCs appeared to be losing their ability to express genes normally associated with their activity *in vivo*.

In summary, this work describes a new method for selecting BNCs from bovine placental homogenates. BNC purities ranged from 70 to 85% when using FACS. These sorted cells maintain their viability for several days after collection. Sufficient RNA could be extracted from these cells. Also, the BNC-enriched samples could be cultured for several days. Providing Matrigel improved the speed and efficiency of BNC attachment and may have delayed the rate at which these cells lost their ability to produce CSH1. However, neither culture method proved successful at preventing

BNCs from losing their ability to express several BNC-specific genes. The nearly complete loss in *CDX2* expression suggests that these cells are losing key trophectoderm-specifying molecules. These observations indicate that BNC cultures likely do not adequately represent trophectoderm and certainly do not appear to represent BNCs after a short time in culture.

Table 3-1. Trophectoderm marker primers used for qRT-PCR.

Gene of Interest	GeneID#	Primer	Sequence (5'-3')
CDX2	618679	Forward	GCCACCATGTACGTGAGCTA
		Reverse	GACTACGGCGGATACCATGT
CSH2	281097	Forward	CATCCTGGGATTTCTCTCCA
		Reverse	AAAACCAACCTGGCAACTG
PAG1	281964	Forward	TGTACACATGGACCGCATCT
		Reverse	ACAACTACCCAGTGCCAGG
CYP19A1	281740	Forward	TCTCGAAAGCTGTTCGACCT
		Reverse	GACTTGGGCTATGTGGACGT
18S[a]	493779	Forward	GCCTGAGAAACGGCTACCAC
		Reverse	CACCAGACTTGCCCTCCAAT

[a] [23]

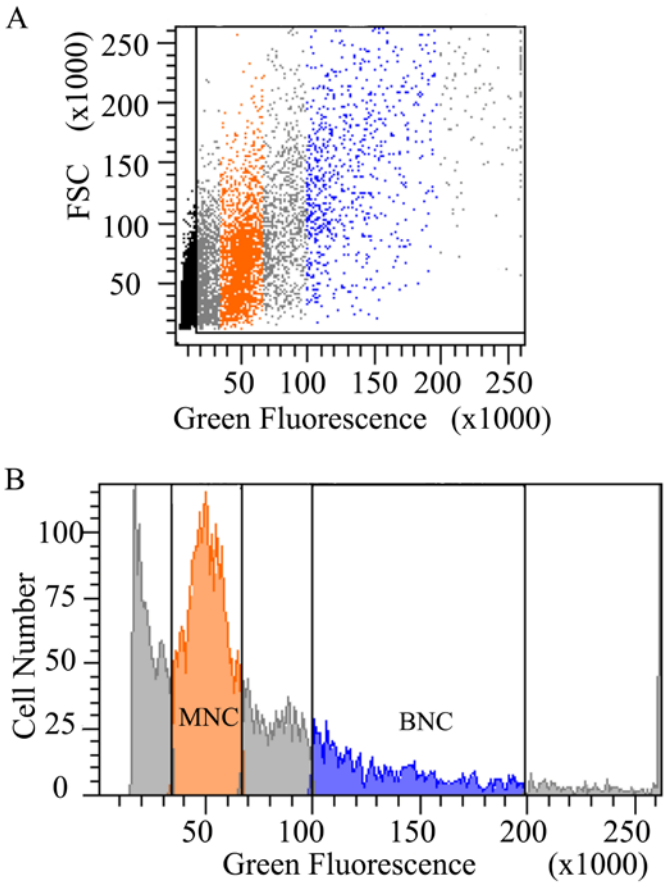

Figure 3-1. Representative FACS plots of a bovine placenta homogenate. Placenta homogenates were incubated in Vybrant® DyeCycle™ Green dye and subjected to FACS. Panel A) Scatter plot of cell densities (forward scatter; Y-axis) and DNA intensity (x-axis) for one sample. The boxed region contains cells with >20,000 forward scatter eliminating small debris particles from the collection sample. B) Distribution of DNA Fluorescence activity (x-Axis) in placental homogenates. The quadrants used to sort for MNC- and BNC-enriched populations are indicated.

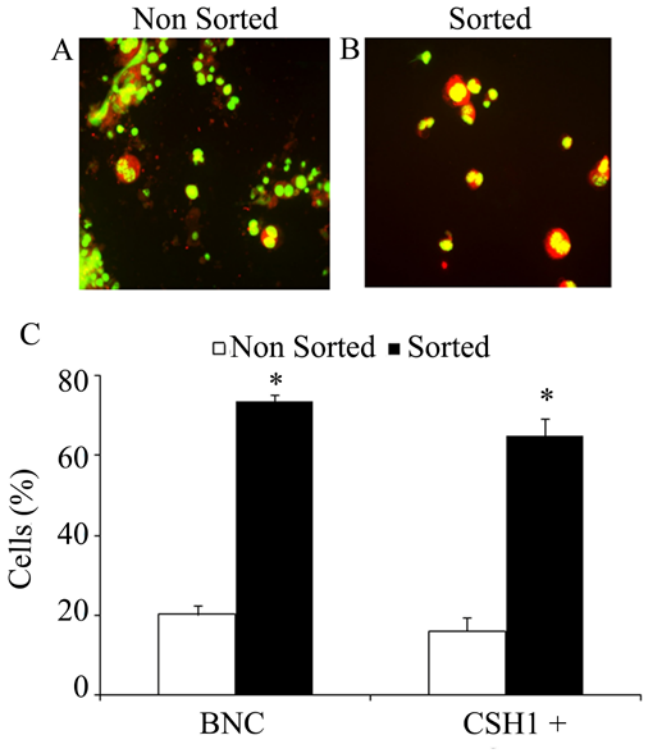

Figure 3-2. Enrichment of BNCs after FACS. Panels A & B) The incidence of CSH1-positive cell staining before (A) and after (B) FACS. CSH1-reactive cells are indicated in red and DNA staining is represented in green (Vybrant® dye staining). Panel C) The proportion of CSH1-positive and BNCs in samples before and after FACS for cells with DNA contents 2-4 times greater than that of the diploid peak identified in samples (n=5 placentae). The asterisk indicates differences in percentages of BNC (P=0.0001) and CSH1-positive cells (P=0.0002) before and after FACS.

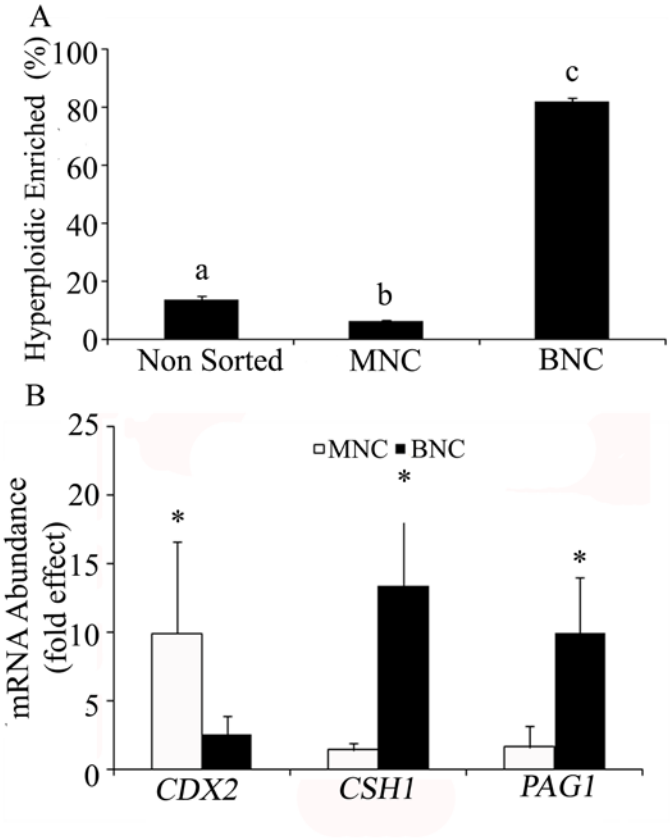

Figure 3-3. Percentage MNC and BNC populations after FACS and gene expression profiles for each. FACS was completed on bovine placental homogenates (n=5-8 placentae) and MNC and BNC fractions were collected. Panel A) The percentage of BNCs and MNCs represented in each sorted group. The presence of 2-nuclei was used to distinguish BNCs from MNCs. Different subscripts represent differences between cell populations ($P<0.05$). Panel B) Expression profiles for candidate genes in MNC- and BNC-enriched samples. qRT-PCR was completed to determine the relative abundances of *CDX2*, *CSH1*, and *PAG1* mRNA. Abundance of *18s* RNA was used to normalize values. The asterisks represent differences ($P<0.05$) in mRNA abundance between MNC and BNC samples.

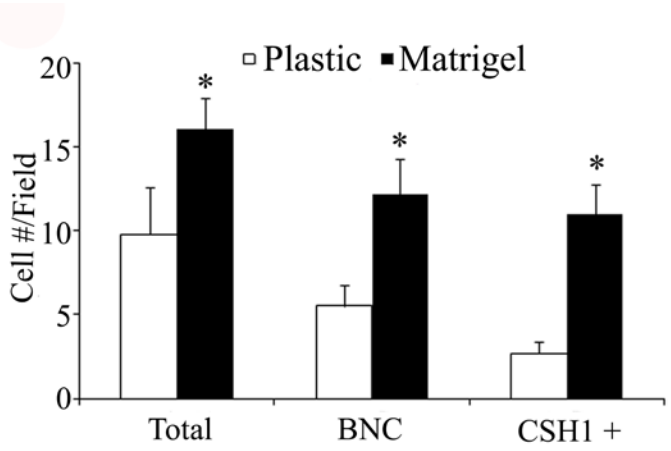

Figure 3-4. Outcomes of culturing BNC-enriched populations for 3.5 days. FACS-sorted BNCs were cultured in wells coated in Matrigel™ or no matrix. After 3.5 days at 38.5°C in 5% CO_2 in air, attached cells were examined for the presence of CSH1 via immunostaining. Hoechst-staining of DNA also was completed (n=4 placental homogenates). A) Representative bright-field image of cultured cells (400-fold magnification). B) Number of attached cells after 3.5 days in culture. Asterisks indicates differences between plastic and Matrigel-coated surfaces (P<0.05).

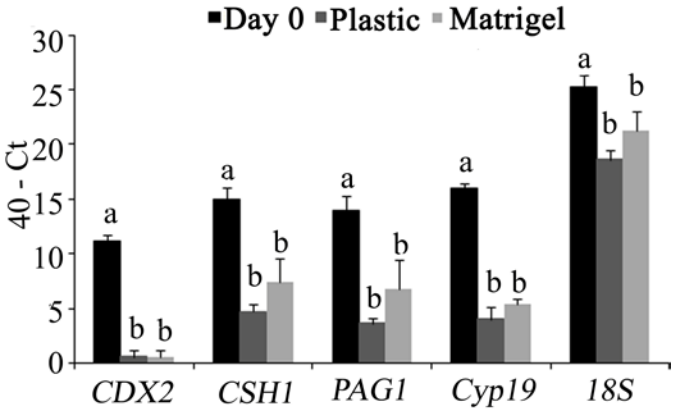

Figure 3-5. Gene expression profiles for BNCs after 3.5 day culture. TcRNA was extracted from BNCs on either Matrigel™ or no coating after 3.5 days in culture (n=4 replicate studies). TcRNA extracted from samples before culture also were included in the analysis. qRT-PCR was completed. *18S* mRNA concentrations differed between samples collected before and after culture, so raw C_T values were analyzed and are graphs after inversion (40-C_T). The same tcRNA concentration was used in qRT-PCR for each sample. Different subscripts represent differences between cell populations (P<0.005).

CHAPTER 4
EXPRESSION OF SEVERAL PUTATIVE TROPHOBLAST DIFFERENTIATION FACTORS IN BOVINE MONONUCLEATE AND BINUCLEATE CELLS

Introduction

Ruminant trophoblast binucleate cells (BNCs) share several common features with trophoblast giant cells in rodents and extravillous cytotrophoblast cells in humans [61, 280]. One class of transcriptional regulators that play a predominant role in the formation of trophoblast giant cells and extravillous cytotrophoblast cells are selected basic helix loop helix (bHLH) factors [31, 275, 320].

Numerous bHLH factors regulate trophoblast differentiation in the mouse and appear to function in similar ways in the human placenta. The role of HAND1 in trophoblast giant cell formation has been studied extensively. In the mouse, HAND1 knockouts are placental lethal due to a lack of trophoblast giant cell formation [295, 296]. HAND1 induces trophoblast giant cell formation in mouse and rat trophoblast stem cells [294, 301]. In the human, HAND1 impacts trophoblast cell differentiation [318, 320]. In cattle, HAND1 localizes to BNCs [272]. A large portion of bovine somatic cell nuclear transfer (SCNT) pregnancies fail to reach term, and a reduction of HAND1 mRNA expression and decreased BNC numbers is evident in these placentae [21].

The natural antagonist of HAND1, ASCL2, negatively impacts trophoblast giant cell formation [275, 294, 309]. ASCL2 and HAND1 compete for the same E-protein binding partners and E-box binding sites. Over-expression of ASCL2 in rodent trophoblast stem cells inhibits the formation of trophoblast giant cells [301]. In bovine SCNT pregnancies with reduced BNC numbers, ASCL2 expression is increased [21, 120].

Other bHLHs of interest include inhibitor of MyoD family-form a (I-mfa) and stimulated by retinoic acid 13 (Stra13). Both factors induce trophoblast giant cell formation when over expressed in rodent trophoblast cell lines [301, 312, 316]. The dominant negative regulators, ID1 and ID2, also play a role in placental differentiation [320]. ID2 specifically is reduced in human placental disease preeclampsia indicating for this factor in trophoblast differentiation and migration [321].

BNCs also share similarities with syncytiotrophoblast cells. Both of these cell types fuse to form syncytium. The HLH factor, glial cell missing homologue 1 (GCM1), plays a role in placental cell fusion in human and mouse [301, 340, 383]. GCM1 induces syncytiotrophoblast formation by regulating the endogenous retrovirus, syncytin-1 [276]. This factor is also down regulated in women with preeclampsia [346]. Over expression of GCM1 in trophoblast stem cells induces cell fusion and increases syncytin-1 expression [340].

Since BNCs are potentially analogous to trophoblast giant cells and extravillous cytotrophoblast cells, we hypothesize that many of the same differentiation factors regulating their formation also may control BNC formation. The objectives of this study were to characterize the expression of these factors in MNC and BNC isolated samples and determine if over expression of differentially expressed factors induce bovine BNC formation *in vitro*.

Methods

Tissue Collection

Tissue and placenta homogenates were collected as described in the previous chapter. Fetuses crown-rump length estimated the stage of gestation to be mean = 135.8 ± 3.6 days; range = 118-159 days. For each placenta (n=12 in all), cotyledons

(n=5-6/placenta) were separated from caruncles and dissected away from intercotyledonary tissues. Whole cotyledonary tissue was collected and snap frozen in liquid nitrogen for future use.

End Point RT-PCR

The PureLink Micro-to-Midi Total RNA Purification System with Trizol (Invitrogen Corp.) was used to extract tcRNA from whole cotyledonary tissue (n=4 animals) following manufactures guidelines. tcRNA (10-250 ng) was incubated in RNase-free DNase (New England Biolabs, Ipswich, MA) for 30 minutes at 37°C and heat inactivated for 10 min at 75°C prior to reverse transcription (RT). The SuperScript III First-Strand Sythesis System Kit (Invitrogen Corp.) and random hexemers were used for RT of tcRNA. Non-reverse transcribed Dnase-treated RNA was used as a negative control. Gene-specific primer sets were used to amplify products for possible BNC differentiation factors (see Table 4-1). A primer pair for β-actin (ACTB) was included as a positive control. PCR amplification was performed using ThermalAce DNA Polymerase (Invitrogen Corp.). A total of 35 cycles of denaturation (95°C for 15 sec), annealing (55-59°C for 1 min, depending on primer set) and DNA synthesis (72°C for 1 min) were completed and were followed by a DNA polishing stage (72°C for 10 min). PCR products were electrophoresed (1% (w/v) agarose gel containing ethidium bromide (100ng/ml) and visualized on an ultraviolet light box. PCR products with a single amplicon at the appropriate size were PCR purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen Corp.) and submitted for DNA sequencing using gene specific primer sets at the University of Florida DNA Core Facility.

Quantitative (q), Real-Time RT-PCR

tcRNA was extracted from FACS sorted MNC and BNC samples (n=8 preparations) and incubated with RNase-free Dnase (New England Biolabs) as described above. The High Capacity cDNA Archive Kit (Applied Biosystems Inc.) was used for RT. Following RT, Primers (200 nM) specific for *HAND1, GCM1, Stra13, ID1, ID2* and *18S* (internal control) (Table 4-1) were used in combination with a SYBR® Green Detector System (Applied Biosystems Inc.) and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to quantify target gene abundance. Following an initial activation/denaturation step (50°C for 2 min; 95°C for 10 min), 40 cycles of a two-step amplification procedure (60°C for 1 min; 95°C for 15 s) were completed. A dissociation curve analysis (60-95°C) was used to verify the amplification of a single product. Primer efficiencies ranged from 90 to 107% when tested on RNA collected from cotyledonary samples by using the relative standard curve approach [146]. Each sample was completed in triplicate reactions. A forth reaction lacking reverse transcriptase was included to control for genomic DNA contamination. Non-processed, whole cotyledonary tissue was used as a control.

Cell Culture

The ovine trophectoderm cell line (oTr) was generously provided by Dr. Thomas Spencer (Texas A&M University, College Station, TX) and cultured as previously described [104, 151]. Briefly, cells were cultured in Dulbecco modified Eagle medium with F-12 salts (DMEM-F12; Invitrogen Corp.) with supplements (10% FBS, 700 nM insulin, 0.1 mM nonessential amino acids, 100 U penicillin, 100 µg streptomycin, and 0.25 µg/ml amphotericin B; Invitrogen Corp.). Cultures were maintained at 5% CO_2 in air at 37°C.

Hand1 Over-Expression

The oTr cells were seeded into 12-well plates. At 50% confluency, cells were transfected using Lipofectamine 2000 (Invitrogen Corp.) following the manufacturer's instructions. Expression plasmids for HAND1 (pCMV-Express-1; Thermo-Fisher Scientific) and β-Gal (pCMV-Sport; negative control; Invitrogen Corp.) were co-transfected with GFP (pCMV-PCS2) reporter plasmid. Transfection efficiency averaged 10% (see Fig 4-4G). Cells were examined at 3 and 6 days post transfection for changes in cell morphology. Western blotting and immunohistochemistry were also preformed for HAND1 and CSH1.

The activity of over expressed HAND1 was assessed using a dual luciferase reporter assay. Hand1 plasmid was co-transfected with the E-box promoter plasmid, 4Rtk-luc (generously provided by Dr. Sally Johnson) and a *Renilla* report control (pRL-TK) plasmid. Cells were cultured for 36 h following transfection and then submitted to the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following manufactures guidelines.

Western Blotting

Samples were washed twice in cold PBS and incubated in RIPA buffer (Thermo Scientific) containing Halt protease inhibitor and Halt phosphatase inhibitor cocktails (Thermo Scientific) and incubated on ice for 15 minutes with constant agitation. Samples were sonicated and centrifuged (14,000 g for 15 minutes) and supernatants were harvested and stored at -20°C.

Protein concentrations were determined using the BCA Protein Assay (Pierce/Thermo Scientific, Rockford, IL). Samples (20 µg) were boiled on ice for 5 minutes with 10% non-reducing lane marker solution (Thermo Scientific) containing

20% β-mercaptoethanol (Fisher Scientific, Pittsburgh, PA) then placed on ice. Samples were loaded into 12.5% poly-acrylamide gels and electrophoresed. Proteins were transferred onto Immobilon-P PDVF membrane (Millipore, Jaffery, NH) using a Hoefer SemiPhor Semi-dry transfer unit (Amersham Biosciences/ GE Healthcare, Piscataway, NJ) at 1 mAMP/cm^2 of membrane and constant voltage (500 V) for 1 h. Membranes were then dried and then blocked in 5% [w/v] non-fat dry milk (NFDM) for 1 h at room temperature. Samples were incubated in a 1:2500 dilution of rabbit-anti HAND1 (Abcam Inc., Cambridge, MA) or 1:5000 dilution of mouse-anti GCM1 (Abcam Inc.) in 3% [w/v] bovine serum albumin (BSA; Fisher Scientific) overnight at 4°C. Membranes were washed in TBST and incubated in a 1:5000 dilution of either anti-rabbit or anti-mouse conjugated HRP secondary antibody (Cell Signaling), depending on primary antibody, in 5% NFDM for 1 h at room temperature. Blots were developed using the Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare). Following developing, membranes were placed in stripping buffer (100 mM β-Mercaptoethanol, 2% [w/v] SDS, 62.5 mM Tris-HCl pH 6.7) for 30 min at 50°C and reused for rabbit-anti alpha tubulin detection (loading control; 1:5000 Cell Signaling, Danvers, MA).

Immunocytochemistry

Transfected oTr cells were fixed in 4% [w/v] paraformaldehyde (Polysciences Inc, Warrington, PA) for 15 min at room temperature following 3 and 6 days of culture. Cells were permeabilized and blocked in 0.5% [v/v] Triton-X-100 (Thermo-Fisher Scientific) and 1% [w/v/] BSA (Thermo-Fisher Scientific) for 20 min at room temperature. Cells were incubated in primary antibody, either a rabbit-anti CSH1 (generously provided by Dr. Russell Anthony; Colorado State University; 1:1000 Dilution) or rabbit-anti HAND1 (1:500 dilution) overnight at 4°C. Cells were then washed in PBS and then incubated in

Alexa Fluor® 594 goat anti-rabbit IgG (Invitrogen Corp.) with 8.1 µM Hoechst 33342 (Invitrogen Corp.) nuclear counterstain. In some studies, F-actin filaments were stained using Texas Red-X phalloidin (Invitrogen Corp.) according to manufacturer's guidelines with Hoechst 33342 nuclear counterstain.

Statistical Analysis

All analyses were completed by analysis of variance using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute, Cary, NC). When analyzing qRT-PCR data, the ΔC_T values were used for analyses [146, 147]. ΔC_T values were transformed to fold differences for illustration on graphs. Results are presented as arithmetic means ± SEM.

Results

Expression Pattern of Potential BNC Differentiation Regulators

The first experiment examined the expression of several transcription factors in bovine trophoblast cells that are linked with placental development and differentiation in humans and mice. When using end-point RT-PCR, transcripts for *HAND1, GCM1, ASCL2, I-mfa, Stra13, ID1* and *ID2* were detected in mid-gestation bovine placentae (figure 4-1). The relative abundance of some of these factors was examined by using qRT-PCR. Specifically, differential expression between MNC and BNC populations were examined after FACS. Expression of *GCM1*, *Stra13*, *ID1*, and *ID2* was not different between MNC and BNC populations (figure 4-2). However, *HAND1* mRNA abundance was greater (P=0.02) in BNC than in MNC preparations (figure 4-2).

The protein expression pattern was also analyzed for HAND1 and GCM1. GCM1 protein was not differentially expressed between MNC and BNC samples (Fig 4-3). Conversely, HAND1 protein was more abundant in BNC samples versus MNC samples.

To date, fully functional BNCs do not differentiate in ruminant trophectoderm cell lines, including the bovine trophectoderm cell line, CT1, and the ovine trophectoderm cell line, oTr. There for the abundance of these factors was measured to missing factors that may regulate BNC differenitation. *Stra13, ID1,* and *ID2* transcripts were present. A limited amount of *HAND1* mRNA was found in either cell line and *GCM1* transcripts were not identified in either cell line (n=5; data not shown).

The Role of HAND1 in BNC Differentiation

HAND1 mRNA and protein was more abundant in BNCs than MNCs, and HAND1 expression was limited in trophoblast cell lines that do not differentiate into BNCs. Hence, a study was completed to determine if HAND1 expression induces BNC formation in oTr cells. The transfection efficiency of both control and HAND1 samples was 6-11% as measured by GFP expression from co-transfected plasmid (Fig 4-4 a & b). The functional capacity of the HAND1 expression system was validated with a luciferase reporter assay with MyoD as a positive control. The experiment showed that over-expression of HAND1 induced ($P<0.05$) luciferase activity over the negative control (Fig 4-5).

The next experiment focused on examining the morphological effects of HAND1 over-expression in oTr cells. HAND1 did not cause oTr cells to differentiate into BNCs when observed by using phase contrast and epiflourescence microscopy of F-actin filaments (Fig 4-4 c & d). Over-expressed HAND1 protein was localized to the nucleus, as expected [305]. Also, HAND1 expressing oTr cells did not produce CSH1 as determined by Immunocytochemistry (data not shown).

Discussion

The first portion of this work describes the expression profile of several suspected BNC differentiation factors. The factors under investigation are involved with differentiation of human and mouse trophoblast cells but their actions on bovine trophoblast cells has not been described. Transcripts for all factors were detected in the bovine placenta.

Interestingly, the only factor that exhibited differential gene expression between MNCs and BNCs was HAND1. Expression of HAND1 was limited in CT1 and oTr cell lines. CT1 and oTr cells do not differentiate into functional BNCs implicating these factors as potential regulators of BNC differentiation [20, 142, 151]. Due to the limited expression of HAND1 in the cell lines and the mRNA abundance results, HAND1 protein expression was analyzed in MNC and BNC samples. HAND1 protein expression was increased in BNCs. This result confirmed the qRT-PCR results and implicates HAND1 as a potential BNC differentiation regulator.

Insufficiencies in HAND1 expression are associated with pregnancy failures in cattle. A substantial portion of SCNT pregnancies are lost during placental formation, or shortly thereafter, and HAND1 mRNA abundance is less in these placentae than IVP and AI placentas [384]. Based on present findings, this decrease likely reflects either that fewer BNCs exist in these pregnancies or that the BNCs present do not produce sufficient amounts of HAND1.

Due to the differential expression of HAND1 in MNC and BNCs and the evidence from SCNT pregnancies, the function of HAND1 in trophoblast differentiation was examined. Over-expression of HAND1 did not alter oTr cell appearance nor induce CSH1 expression [194]. It did appear that HAND1 was being over-expressed in these

cells and was present in the nucleus instead of being sequestered in the nucleolus. This localization is important based on previous reports showing that the nucleolar release of HAND1 promotes its activation and trophoblast giant cell differentiation whereas nulceolar compartmentalization prevents HAND1 activation [305, 306]. The function of over-expressed HAND1 was confirmed using an E box luciferase reporter assay. E boxes are the DNA site for all bHLHs [282]. Although HAND1 did not induce the same response as the positive control, MyoD, it did significantly activate the E box promoter over that of the control. This shows that the over expressed HAND1 is functioning.

Several reasons could explain why HAND1 over-expression in oTr cells did not induce BNC differentiation. First, the transfection efficiency was low in these experiments. However, if HAND1 was the essential factor controlling BNC differentiation the expected result would be that those cells with over-expressed HAND1 would show changes in morphology and/or produce CSH1. An alternative reason for the in sufficiency of HAND1 to cause a morphological change is a problem with the cell line. BNCs begin to appear *in vivo* several days later then when these cell lines were made. Perhaps these cells are not set up for this differentiation event and are missing several key factors, in addition to HAND1. Also, ID1 and ID2, inhibitors of bHLH factors are expressed at significant levels in the oTr cell line. Perhaps these factors prevent HAND1 from functioning at the appropriate level. Another factor that may be playing a role and that was not examined is ASCL2, the competitive inhibitor of HAND1 [275]. A high abundance of this factor may prevent differentiation events. In future endeavors it would be interesting to examine ASCL2 expression in the cell line. Other factors that

also impact HAND1 activity include the proteins HICp40 (human I-mfa domain containing protein) and Plk4 (Polo-like kinase 4). HICp40 sequesters HAND1 to the nucleolus while Plk4 phosphorylates HAND1 releasing it from the nucleolus and activating the protein [305, 306]. These factors should also be examined to ensure their proper expression in oTr cells.

The expression of several other factors was compared between MNC and BNC samples. The expression of *Stra13*, *ID1* and *ID2* was similar in these samples indicates that they may not play an essential role in BNC differentiation. These factors were also examined in the CT1 and oTr trophectoderm cell lines. *Stra13, ID1 and ID2* expression was present in both cell lines. Although ASCL2 expression was found in whole placenta samples and is different in SCNT placentae versus controls, we were unable to accurately examine differences in this transcript abundance between MNCs and BNCs due to difficulties with identifying primers with acceptable primer efficiencies.

GCM1 is a trophoblastic fusigenic factor in several species. In mouse, GCM1 null mice lack syncytiotrophoblast and are embryonic lethal [344]. Decreased GCM1 expression is associated with the human placental disease, preeclampsia [346]. It also is expressed in the equine binucleate chorionic girdle [385]. In the cow, however, we were not able to detect differences in the expression profile for GCM1 between MNCs and BNCs. There was a noted absence of GCM1 from the trophoblast cell lines, suggesting this factor may be needed for trophoblast differentiation or function in ruminants. An interesting finding with GCM1 expression was that it seemed to be in lower abundance when compared to HAND1. In fact, 50 times more starting RNA was needed for the GCM1 mRNA abundance assay than for HAND1. Also the protein

analysis indicated weak GCM1 signals. Perhaps GCM1 is more of a transient factor that is difficult to detect. Also GCM1 may not play a role in BNC differentiation but may have a potential role in signaling BNC fusion to the uterine epithelium.

In conclusion, several potential trophoblast differentiation factors are present in the placenta. HAND1 was differentially expressed in MNCs and BNCs. HAND1 did not induce BNC development in the oTr cell line. Several reasons have been proposed for why overexpression of HAND1 did not cause the expect result. Further experiments are needed to test these hypotheses.

Table 4-1. Primers used for end-point and quantitative RT-PCR

Gene of Interest	Primer	Sequence (5'-3')	Annealing Temp(°C)
HAND1	Forward	ACATCGCCTACCTGATGGAC	57
	Reverse	GCGCCCTTTAATCCTCTTCT	
GCM1	Forward	AGCAGCTGGATAGACGGAAA	57
	Reverse	TCGTCGGAGCTGTAGATGTG	
ASCL2	Forward	ACCCAAGGCTAGTGTGCAAG	57
	Reverse	TAAGCCTTCATACCGCCAGT	
ID1	Forward	TCTGGGATCTGGAGTTGGAG	59
	Reverse	CTGGAAGGACCAGAGAGCAC	
ID2	Forward	CCATTTCACAAGGAGGAGGA	55
	Reverse	TCCCCATGGTGGGAATAGTA	
I-mfa	Forward	CACTAGTGGCGAATGGCTCT	57
	Reverse	TGGACACAGCAGTCTTCCTG	
Stra13	Forward	CTGACCCACAACGTTCTCCT	57
	Reverse	CTTCCCAGTGACCAAATGCT	
E12/E47	Forward	ATAGTGACGGTGCCCACTTC	57
	Reverse	AGGGTGCCCAGAGTAGTAGGAAT	
ACTB[a]	Forward	CTGTCCCTGTATGCCTCTGG	55
	Reverse	AGGAAGGAAGGCTGGAAGAG	
18S[a]	Forward	GCCTGAGAAACGGCTACCAC	59
	Reverse	CACCAGACTTGCCCTCCAAT	

[a][147]

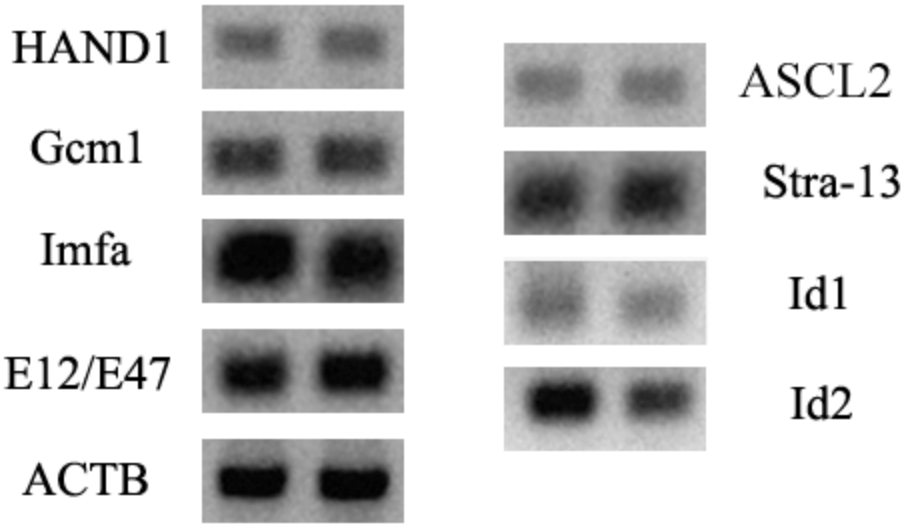

Figure 4-1. Expression pattern of selected trophoblast cell differentiation in the ruminant placenta. End point RT-PCR was performed on whole bovine cotyledonary tissue harvested at mid-gestation (n=4). All factors of interest were present in cotyledonary tissue and products were verified by sequence analysis.

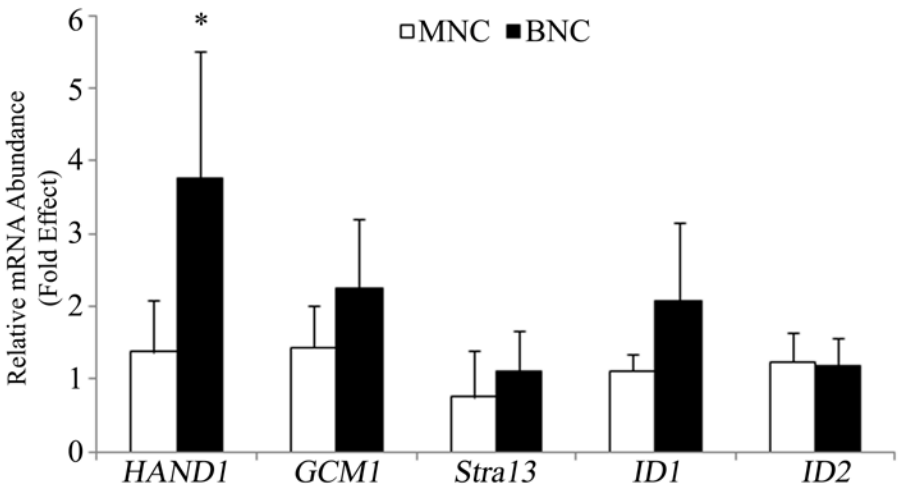

Figure 4-2. Gene expression profile of selected potential BNC differentiation regulators. qRT-PCR was performed on MNC and BNC sorted populations to determine differences in gene expression (n=5-8 replicates). *HAND1* mRNA abundance was greater in BNC versus MNC samples ($p<0.05$) as indicated by the asterisk.

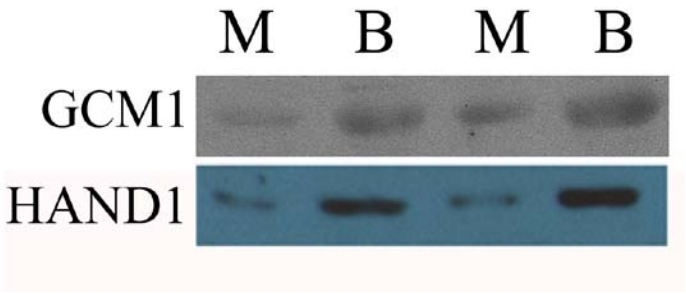

Figure 4-3. Western blot analysis of GCM1 and HAND1 protein expression in MNC (M) and BNC (B) samples. GCM1 protein expression was not different between samples. HAND1 protein expression was higher in BNC samples versus MNC samples. All samples had the same amount of protein loaded as measured by BCA protein concentration assay (n=6 samples).

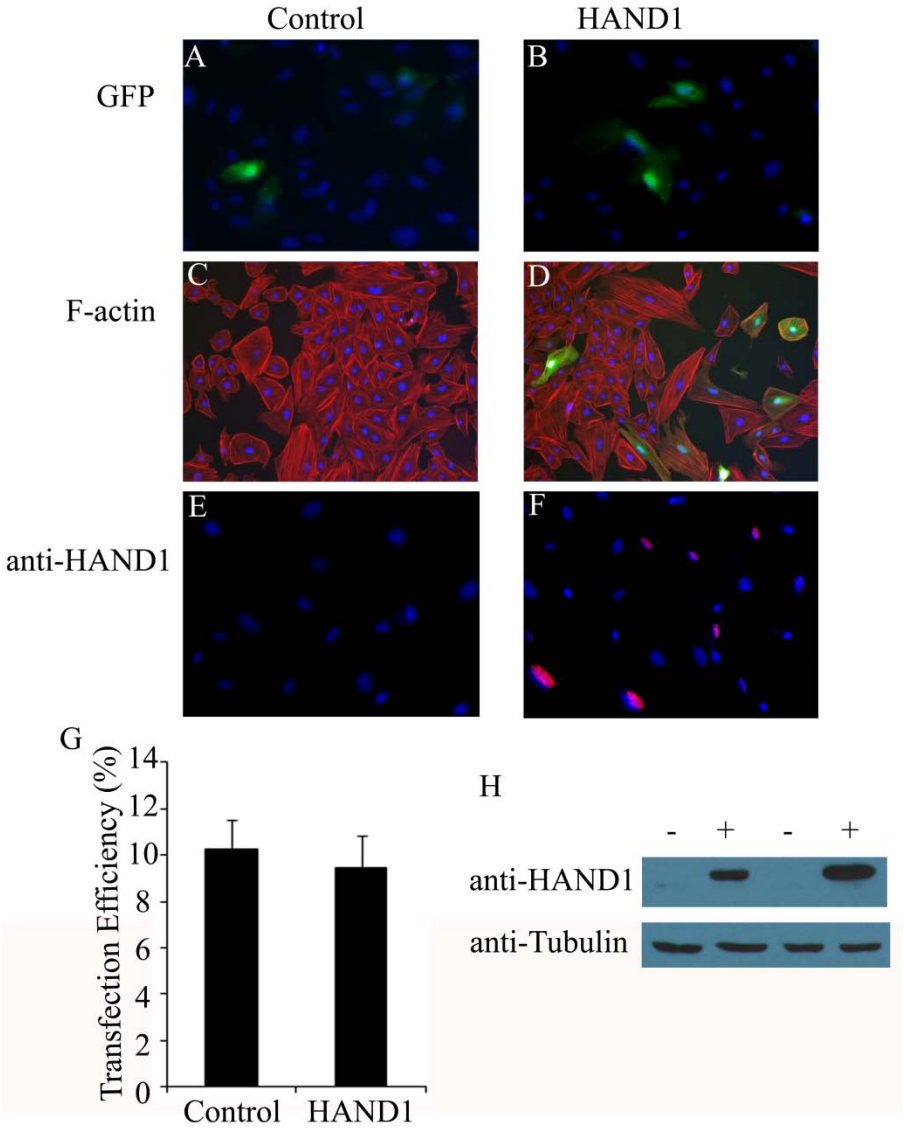

Figure 4-4. Overexpression of HAND1 in oTr cells. Figures A, C, E represent β-Gal transfected control samples and B, D, and F represent HAND1 transfected samples. A&B) Representative figures of samples co-transfected with GFP to measure transfection efficiency. C&D) Samples stained for F-actin to determine if there were changes in cell morphology or cell fusion events. E&F) Localization of over expressed HAND1 to the cell nucleus. G) Graphical representation of transfection efficiency. H) Western blot analysis of HAND1 expression in control (-) and HAND1 transfected (+) samples.

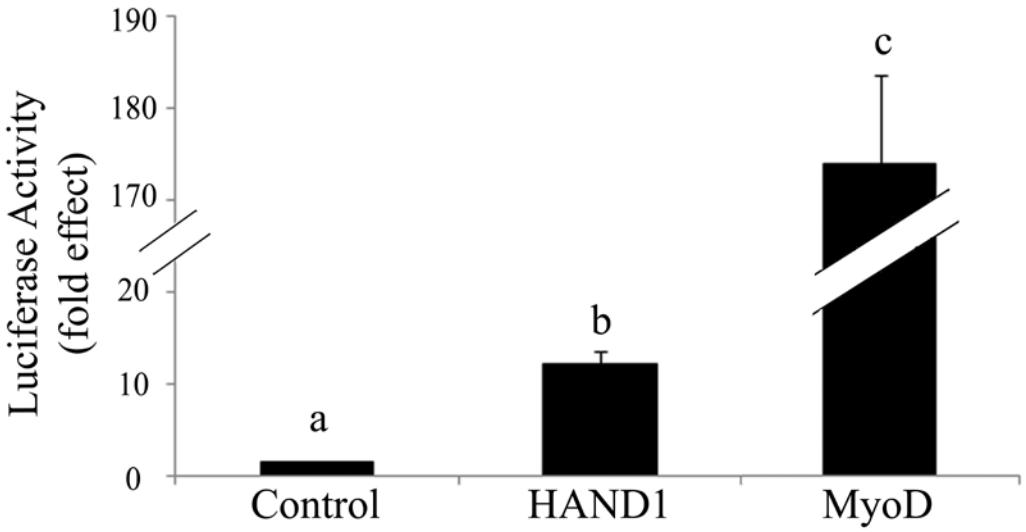

Figure 4-5. HAND1 activity as measured by luciferase activity. B-Gal (negative control), HAND1, and MyoD (positive control) were co-transfected with the E-box promoter (4Rtk-Luc) and luciferase activity was measured following 36 h of culture. Positive control, MyoD, indicates that the reporter assay is functional. Different subscripts represent differences between groups ($P<0.05$)

CHAPTER 5
EXPRESSION AND FUNCTION OF BMP2 AND BMP4 IN THE PERI-ATTACHMENT BOVINE CONCEPTUS

Introduction

The transforming growth factor-β (TGF-β) superfamily is a multifunctional group of paracrine factors [352, 386] that regulate various cell differentiation, and migration events throughout development [363]. Improper regulation of these factors causes multiple disorders, including cancer and auto-immune diseases [352, 363]. TGF-β superfamily members include TGF-βs, activins, nodal, growth and differentiation factors (GDFs), and bone morphogenic proteins (BMPs) [352, 353, 363, 386].

The BMPs mediate various physiological and developmental processes including bone formation [354-356], cardiovascular, nervous and urogenital function [357] and reproduction [358]. BMP4 and 8b play a critical role in primordial germ cell formation [387-390]. Other BMPs, specifically 6 and 15, are localized to the oocyte and are essential during folliculogenesis for controlling follicle turnover selection and dominance [358, 391-394]. In the mouse placenta, BMP4, 7 and 8a are expressed and play a role in trophoblast proliferation and differentiation [358, 395, 396].

Two BMPs of special interest in regards to trophoblast development and function are BMP2 and BMP4, two closely related TGF-β family members [362, 364]. BMP4 is the best studied of these factors. It is especially important for regulating placental vascular development in mice [368, 369]. It also serves a major function in mesoderm formation. BMP4 knockout mice arrest between day 6.5 and 9.5 with majority of embryonic death occurring at day 6.5. Those embryos that arrest at day 6.5 lack mesoderm [364, 368]. Those BMP4 knockouts that do not die until embryonic day 9.5 form mesoderm and are believed to be partially rescued by BMP2 [364]. This

hypothesis is supported by theBMP2 and BMP4 type II receptor knockout, BMPR-II. This null mouse is embryonic lethal at day 6.5 due to lack of mesoderm formation [370].

Signaling for all TGF-β superfamily members occurs through ligand binding to a heterodimeric receptor complex of specific type I and II serine/threonine kinase receptors. The type II receptor phosphorylates the type I receptor initiating the signaling cascade [352, 386]. BMP 2 and 4 interact primarily with the type II serine/theronine kinase receptor, BMPR-II. They also use the type I serine/theronine kinase receptors, BMPR1A (ALK3) and BMPR1B (ALK6) [365, 366]. BMP2 also reacts with the type I receptor, ACVR1 (ALK2) [367]. BMP binding and activation of their receptors impacts several signal transduction pathways including the Smads and the mitogen-activated protein kinase (MAPK) pathway [360, 361].

BMP2 and 4 interaction with their receptors regulate Smad1, 5, and 8 activity [352, 353]. Upon complexing with Smad4, the Smads are translocated into the nucleus, bind DNA at specific sites (Smad binding elements) and regulate transcription of targeted genes [353, 397, 398]. Several levels of negative regulation exist in the BMP signaling pathway and one of special interest is Noggin, which is secreted from cells and serves as a competitive inhibitor of BMP2 and 4 for their receptors [352, 353].

BMP4 can also induce human trophoblast differentiation when added to embryonic stem cells [371, 372]. BMP2 can also induce trophectoderm differentiation in human embryonic stem cells, although greater doses of BMP2 are needed to mimic the effects of BMP4 [371]. Little is known about the expression and role of BMPs in the bovine uterus and pre-attachment conceptus. The goals of this work were to examine the expression pattern of the BMP2/4 ligand, receptor and inhibitor profile in the bovine

uterus and pre-attachment conceptus and explore the functions of these paracrine factors during early pregnancy.

Materials and Methods

Animal Use and Tissue Collection

All animal use was completed with the approval of the Institutional Animal Care and Use Committee at the University of Florida. Healthy, non-lactating Holstein cows (n=12) were housed at the University of Florida Dairy Unit (Hague, Fl, USA) and fed a maintenance diet. Tissues were harvested at day 17 of gestation following slaughter in superovulated cows [147]. Pregnant and non-pregnant endometrial samples were collected from non-superovulated cows subjected to estrus synchronization as described previously [146]. Four biopsies were taken from the endometrium ipsilateral to the functional corpus luteum; with biopsy location ranging from the horn tip to the uterine body. Biopsies were pooled in one tube, snap-frozen and stored at -80°C.

Total cellular RNA was extracted from d 17 conceptuses using the RNAqueous-Midi RNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX). Other tissues and CT1 tcRNA were extracted using the PureLink Micro-to-Midi Total RNA Purification System with Trizol (Invitrogen Corp.). RNA concentration and integrity were evaluated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Bovine Trophectoderm Cell (CT1) Culture

Cells were cultured as previously described [142, 146, 147] in DMEM with high glucose containing 10% fetal bovine serum and other supplements on Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA) at 38.5°C in 5% CO_2 in air. CT1 cells were seeded onto 12-well plates and allowed to attach for 48 h. Upon reaching ~50% confluence, cells were placed in fresh DMEM lacking FBS but

containing other supplements plus a serum substitute (insulin/transferring/selenium; ITS; Invitrogen Corp.). 24 h after serum free culture, cells were placed in treatments containing 0, 0.1, 1, 10, and 100 ng/ml of either recombinant human (rh) BMP2 (R&D Systems, Minneapolis, MN) or rhBMP4 (R&D Systems) (n=2 wells/BMP treatment/replicate experiment). BMP2 and BMP4 were reconstituted in sterile 4 mM HCl according to manufacturer's guidelines. The same amount of carrier was placed into all cultures.

SuperArray

Total cellular RNA from d 17 conceptuses (n=3), pregnant endometrium (n=4) and non-pregnant endometrium (n=4) were analyzed using the Oligo GEArray® System (SABiosciences/Qiagen, Frederick, MD) and the TrueLabeling-AMP 2.0 kit (SABiosciences/Qiagen) according to manufacturer's guidelines. Custom nylon membrane arrays were supplied with 60-mer oligonucleotide probes for specific genes (see Fig. 5-1).

End Point RT-PCR

All samples were processed as described in Chapter 4. Gene-specific primer sets were used to amplify products for BMP2, BMP4, Noggin, BMPRII, ACVR1, BMPR1A, and BMPR1B (see Table 5-1). A primer pair for β-actin (ACTB) was included as a positive PCR control (Table 5-1). PCR amplification was performed using ThermalAce DNA Polymerase (Invitrogen Corp.). 35 cycles of denaturation (95°C for 1 min), annealing (55-59°C for 1 min, depending on primer set) and DNA synthesis (74°C for 1 min) followed by a DNA polishing stage (72°C for 10 min) were completed. PCR products were analyzed, cloned and sequenced as described previously.

Quantitative (q) RT-PCR

After 24 h, 4 d or 8 d, tcRNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System with Trizol following manufacturers guidelines (n=4 replicate experiments). TcRNA was stored at -80°C until further use. For the 4 d and 8 d studies, medium with BMP2 or BMP4 supplements (0, 1, 10 or 100 ng/ml) were changed every three days. DNase treatment and RT was performed as previously described.

The abundance of *IFNT*, *CSH1and 18S* RNA (internal RNA loading control) in BMP2 and BMP4 treated CT1 samples were then determined by TaqMan based qRT-PCR. Primers and probes specific for *IFNT* and *CSH1* were synthesized (Applied Biosystems Inc.; Table 5-2) and labeled with a fluorescent 5' 6-FAM reported dye and 3' TAMRA quencher. The *IFNT* probe was designed to recognize all know bovine *IFNT* isoforms [146, 147]. After an initial activation/denaturation step (50°C for 2 min; 95°C for 10 min), 40 cycles of a two-step amplification procedure (60°C for 1 min; 95°C for 15 s) was completed with TaqMan reagent (Applied Biosystems Inc.) and a 7300 Real-Time PCR System to quantify mRNA abundance. *18S* abundance was quantified using the 18S RNA Control Reagent Kit (Applied Biosystems Inc.) containing a 5'-VIC-labeled probe with a 3'-6-carboxy-tetramethylrhodamine quencher. Each RNA sample was analyzed in triplicate (50 ng tcRNA). A negative control was also run which lacked reverse transcriptase for each sample to verify they were free from genomic DNA contamination. TheΔC_t method was used to contrast abundance of *IFNT* and *CSH1* transcripts relative to the *18S* RNA.

Proliferation Assay

CT1 cells were seeded into 24 well Matrigel™ coated plates and allowed to attach for 48 h. Cells were then placed in serum free medium for 24 h. After serum starvation,

fresh serum-free medium containing 0, 0.1, 1, 10, and 100 ng/ml of rhBMP2 or rhBMP4 was added to cultures (4 wells/treatment). After 48 h, the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) was used to determine relative cell numbers.

Alkaline Phosphatase Staining

CT1 cells were seeded into plates and cultured for 2 days to allow cell attachment. Medium was then changed into serum-free medium as before and, 24 h later, medium was changed into serum-free medium containing 0, 10, 100 ng/ml of rhBMP2 or rhBMP4 (n=2 wells/treatment). Cells were then cultured for 4 or 8 d. Medium and BMP supplements were changed every 3 days. On 4 d or 8 d, cells were fixed in 4% [w/v] paraformaldehyde (Polysciences Inc, Warrington, PA, USA) for 15 min at room temperature. Staining solution (500 µl-1M $MgCl_2$, 310µl-5M NaCl, 1ml-1 M Tris (pH 9.0), 8 ml-dH_2O, 66 µl NBT [Nitro-Blue Tetrazolium Chloride], and 33 µl BCIP [5=Bromo-4Chloro-3'-Indolyphosphate p-Toluidine Salt]) was placed on cells and incubated at room temperature in the dark overnight. Cells were then viewed under phase contrast microscopy for positive alkaline phosphatase activity as indicated by purple staining.

Smad 1, 5, 8 Western Blotting

CT1 cells were seeded onto 6 well Matrigel™ coated plates and allowed to attach for 48 h. Medium was then changed into serum free medium as described above for 24 h. Cells were then placed in 100 ng/ml of rhBMP2 or rhBMP4, for 0, 5, 15, 60, or 120 min. Following treatment, cells were placed in NP-40 buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 2 mM EDTA, 1% [w/v] NP40) for 20 min on ice. Samples were then stored at -20°C until further use. Samples were sonicated and then centrifuged (10,000 g for 10 min at 4°C).

Western blotting was performed as described previously (Chapter 4). Membranes were blocked in 5% NFDM at room temperature for 1 h and then incubated in rabbit-anti phoshorylated Smad1 (Ser 463/465) / Smad5 (Ser 463/465) / Smad8 (Ser 426/428) antibody (1:5000; Cell Signaling, Danvers, MA) over night at 4°C. Membranes were then washed and incubated in secondary antibody. Membranes were then developed as describe in the previous chapter. Following developing, membranes were placed in stripping buffer, blocked in 5% NFDM for 1 h at room temperature, and then incubated with rabbit anti SMAD1/5/8 (1:5000; Santa Cruz Biotechnology inc., Santa Cruz, CA) in 3% BSA over night at 4°C. Following incubation, membranes were washed and then incubated in secondary antibody, washed in TBST, and developed as above.

Statistical Analysis

All analyses were completed by analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (SAS Institute, Cary, NC). When analyzing qRT-PCR data, the ΔC_T values were used for analyses [146, 147]. ΔC_T values were transformed to fold differences for illustration on graphs. Results are presented as arithmetic means ± SEM.

Results

Expression of BMP Ligands and Receptors in Bovine Conceptus and Endometrium

Customized SuperArrays were generated to evaluate the expression of several factors, including FGFs, IGFs, VEGFs, Notch signaling molecules and BMPs in endometrium and conceptuses. These factors are involved in a variety of cell differentiation and signaling events throughout development. We isolated RNA from day 17 conceptuses, pregnant and non-pregnant endometrial which was used to

generate a template for biotinalated cDNAs that were used to probe this customized SuperArray (fig 5-1). Transcripts for BMP2 and BMP4 were present in all samples tested (n=4 arrays/tissue). The relative expression of these transcripts appeared greater than many other growth and differentiation factors expressed by conceptuses and endometrium. Specifically, substantially more BMP2 and 4 mRNA was detected than FGF2 and 10 mRNA, two factors known to be expressed by conceptuses and endometrium during early pregnancy [146, 147]. Other transcripts, notably IGF2, VEGFa and VEGFb, also were detected.

End-point RT-PCR was completed to confirm the observations made when using the SuperArray (fig 5-2). Both *BMP2* and *BMP4* mRNA were detected in bovine endometrium and day 17 conceptuses (fig. 5-2). *BMP2* and *4* mRNA also were detected in CT1 cells (fig 5-2).

The presence of BMP2/4 signaling components was also examined by end-point RT-PCR. Each of the major receptor subtypes utilized by BMP2 and BMP4 binding were found in endometrium, d 17 conceptuses and CT1 cells (fig. 5-3). The type II receptor, *BMPR-II,* was found in all tissues; although the abundance of this transcript appeared to vary between conceptus samples. The type I receptor BMPR1A was found in all tissues examined. *BMPR1B* was found in d 17 conceptus and endometrium but there was limited expression of this transcript in CT1 cells. *ACVR1* was also found to be expressed in all tissues examined.

The expression profile of *Noggin* was examined in these tissues. *Noggin* expression was confirmed in the d 17 conceptus; but was not identified in the endometrium. CT1 cells had limited transcript expression of *Noggin* (fig 5-2).

Biological Activities of BMP2 and BMP4 in Bovine Trophectoderm

Several studies were completed to examine the biological potential for BMP2 and 4 in bovine trophoblast. The CT1 cell line was used for all studies.

The initial study investigated whether BMP2/4 affected *IFNT* expression in CT1 cells. BMP2 and BMP4 supplementation had no effect on *IFNT* mRNA abundance after 24, 96, and 192 h (fig. 5-4). In a separate set of experiments, the ability of BMP2/4 to regulate *CSH1* expression was determined. Mid-gestation placenta RNA was used as a positive control. Following 96 and 192 h of BMP2 and BMP4 treatment, no change in the production of *CSH1* mRNA was detected (data is not shown).

The ability of BMP2 and BMP4 to control trophoblast proliferation was examined in CT1 cells after 48 h exposure to BMP2 or 4. BMP2 did not affect CT1 cell numbers after 48 h (fig. 5-5A) but BMP4 decreased ($P<0.05$) CT1 proliferation when provided at 1, 10, and 100 ng/ml (fig C-5B).

To ensure bone formation and mineralization was not induced in BMP treated CT1 cells, alkaline phosphatase activity was measured [399]. As seen in figure 5-6, alkaline phosphatase staining was evident in all treatments and no increases in staining intensities were observed between the non-treated controls and treated groups.

The ability of BMP2/4 to activate Smad 1/5/8 signaling was examined in CT1 cells. Phosphorylated Smad was detected in non-treated controls and BMP2 supplementation did not increase the presence of p-Smad1/5/8 (fig 5-7). BMP4 supplementation appears to have increased the activation of Smad1/5/8 signaling in the 60 minute sample as compared to the control, 5, and 15 minute supplementation samples. Given that only a single analysis was completed, further verification is needed to confirm that BMP4 increased Smad activation (fig 5-7).

Discussion

The BMPs are utilized to control various reproductive processes across species [358] but little is known about how these factors impact the events of early pregnancy in cattle. These studies were completed to discover some of these functions. Transcripts for BMP2 and BMP4 were readily detectable in both elongated conceptuses and endometrium collected from pregnant cattle, and upon further study it became evident that all the receptor subtypes needed to elicit a BMP2/4 response were present in conceptuses and endometrium.

Noggin regulates BMP expression in various systems and is also up-regulated by BMP2 and BMP4 to prevent overstimulation by BMP2 and/or BMP4 [362]. The BMP2 and 4 antagonist, Noggin, was found in day 17 conceptus but had limited expression in CT1 cells and endometrium. Perhaps the lack of Noggin expression allows BMP2 and BMP4 present in these cells to activate their signaling pathway, while in other tissues where Noggin is present there is more control of this signaling system.

A main focus of this laboratory is to better understand how IFNT expression is controlled during early pregnancy, and a study was completed to determine if BMP2/4 affects IFNT expression. In humans BMP4 induced trophoblast cell differentiation from embryonic stem cells, however the cell line used in these studies is an already differentiated trophectoderm cell line [371]. A major function of BMP4 in mice is development of the mesoderm [368]. However, this function could not be tested in the CT1 cell line. A potentially better model system may be an *in vitro* produced bovine embryo.

BMP4 supplementation decreases CT1 cell number, however this result needs to be further investigated to identify if this is a result of decreased cell proliferation or cell

death. BMP4 supplementation may be inducing cell differentiation, which would decrease cell number, however other experiments conducted here did not observe any signs of differentiation. No biological effect was seen because the signaling system is already activated by endogenous BMP 2 and 4 as indicated by the endogenous activation of Smad 1/5/8. In order to determine if the endogenous expression of BMP2 and BMP4 is the cause of continuous Smad activation, studies are needed to inhibit BMP2 and BMP4 activity. Also to determine the function of the endogenously stimulated Smads, inhibitors can be used to block activation and then examine IFNT expression, proliferation and differentiation events in these cells.

It is quite possible that the primary site of BMP2/4 action is on the uterus. BMPR1B knockout mouse lack uterine glands [358]. Previous studies in ewes show that endometrial glands are essential for conceptus development and pregnancy [15]. The uterine glands secrete a wide range of factors into the uterine lumen that nourish the conceptus. The BMP 2 and 4 ligands produced by both the endometrium and day 17 conceptus may function to promote endometrial gland formation by signaling through this essential receptor.

While the examination of alkaline phosphatase staining did not indicate any mineralization it is also used as a marker for pluripotency in stem cells [400]. The presence of alkaline phosphatase throughout control and treated samples may indicate that CT1 cells, which are derived from an in-vitro produced bovine blastocyst [142], may maintain a small amount of pluripotency. However since a bovine embryonic stem cell line has not been established, it is unknown whether alkaline phosphatase will be a good marker for pluirpotency in these cells.

Overall, BMP2 and 4 and their receptors are present in the bovine endometrium and day 17 conceptus. However, the role the BMP2 and BMP4 play in the pre-attachment bovine conceptus has yet to be identified. Determining the potential role of BMP2 and BMP4 negative regulation has on this system may lead to a better understanding of what function these factors have in trophoblast function.

Table 5-1. Primers used for end point RT-PCR

Gene of Interest	Primer	Sequence (5'-3')	Annealing Temp(°C)
BMP2	Forward	CTTAGACGGTCTGCGGTCTC	59
	Reverse	CGAAGCTCTCCCACCTACTG	
BMP4	Forward	TGAGCCTTTCCAGCAAGTTT	55
	Reverse	TACGATGAAAGCCCTGATCC	
Noggin	Forward	GAACACCCGGACCCTATCTT	57
	Reverse	ATGGGGTACTGGATGGGAAT	
BMPRII	Forward	AGACTGTTGGGACCAGGATG	57
	Reverse	GTCTGGCCCACTGAATTGTT	
ACVR1	Forward	AAATGGGATCGCTGTACGAC	57
	Reverse	CTGTGAGTCTGGCAGATGGA	
BMPR1A	Forward	CAGGTTCCTGGACTCAGCTC	59
	Reverse	CACACCACCTCACGCATATC	
BMPR1B	Forward	AGGTCGCTATGGGGAAGTTT	55
	Reverse	CTCCCAAAGGATGAGTCCAA	
ACTB[a]	Forward	CTGTCCCTGTATGCCTCTGG	55
	Reverse	AGGAAGGAAGGCTGGAAGAG	

[a][147]

Table 5-2. Primer and Probe sets used for real-time qRT-PCR

Gene of Interest	*Primer/ Probe*	*Sequence (5'-3')*[a]
IFNT[b]	Forward	TGCAGGACAGAAAAGACTTTGGT
	Reverse	CCTGATCCTTCTGGAGCTGG
	Probe	TTCCTCAGGAGATGGTGGTAGGGCA
CSH1	Forward	GTGGATTTGTGACCTTGTTCGA
	Reverse	CCTGGCACAAGAGTAGATTTGACA
	Probe	TCCTGCCTGCTCCTGCTGCTGGTA

[a]Each probe was synthesized with a 6-FAM reporter dye and TAMRA quencher
[b][147]

Blank	FGF1	FGF2	FGF3	FGF4	FGF5	FGF6	FGF7
FGF8	FGF9	FGF10	FGF11	FGF12	Fgf13	Fgf14	Fgf16
FGF18	FGF20	FGF21	FGF22	FGF23	Blank	HGF	Blank
IGF1	**IGF2**	Blank	**VEGFa**	**VEGFb**	VEGFc	**BMP2**	**BMP4**
BMP6	Blank	GDF5	GDF8	GDF10	GDF11	TGFb1	TGFb2
DII1	DII3	DII4	**JAG1**	JAG2	Blank	Blank	Blank
Blank	Blank	**PUC18**	Blank	Blank	AS1R2	AS1R1	AS1
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
GAPD	**B2M**	**PPIA**	**PPIA**	**ACTB**	**ACTB**	**DAS2C**	**DAS2C**

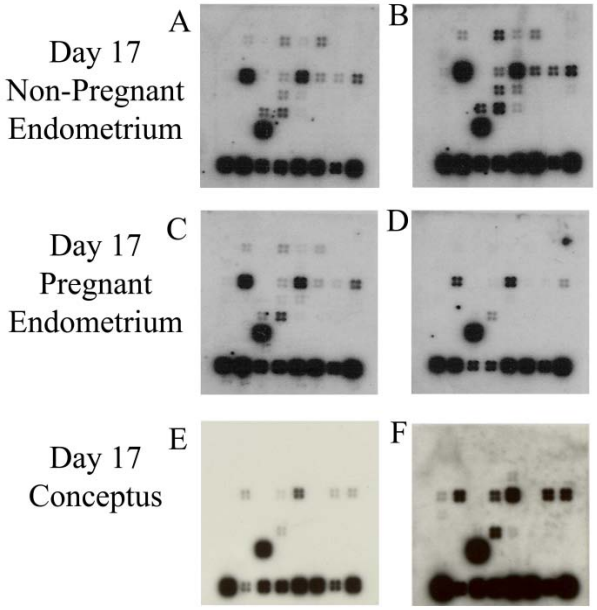

Figure 5-1. SuperArray Gene Expression Analysis. Panels A and B are day 17 non-pregnant endometrium, panels C and D are day 17 pregnant endometrium and panels E and F are day 17 conceptus samples. The grid provided is a list of genes represented and their position on the SuperArray. Genes in bold on the grid are those that appear in all samples.

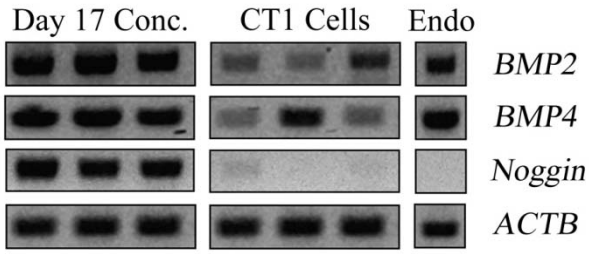

Figure 5-2. End point PCR of BMP ligands in Day 17 bovine conceptus, bovine trophectoderm and bovine endometrium. Three samples of day 17 Conceptus and CT1 cells and one sample of endometrium were run per replicate (n=2 replicates).

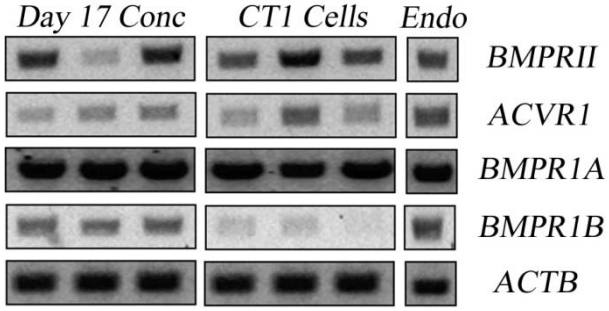

Figure 5-3. End point PCR of BMP receptors in Day 17 bovine conceptus, bovine trophectoderm and bovine endometrium. Three samples of day 17 Conceptus and CT1 cells and one sample of endometrium were run per replicate (n=2 replicates).

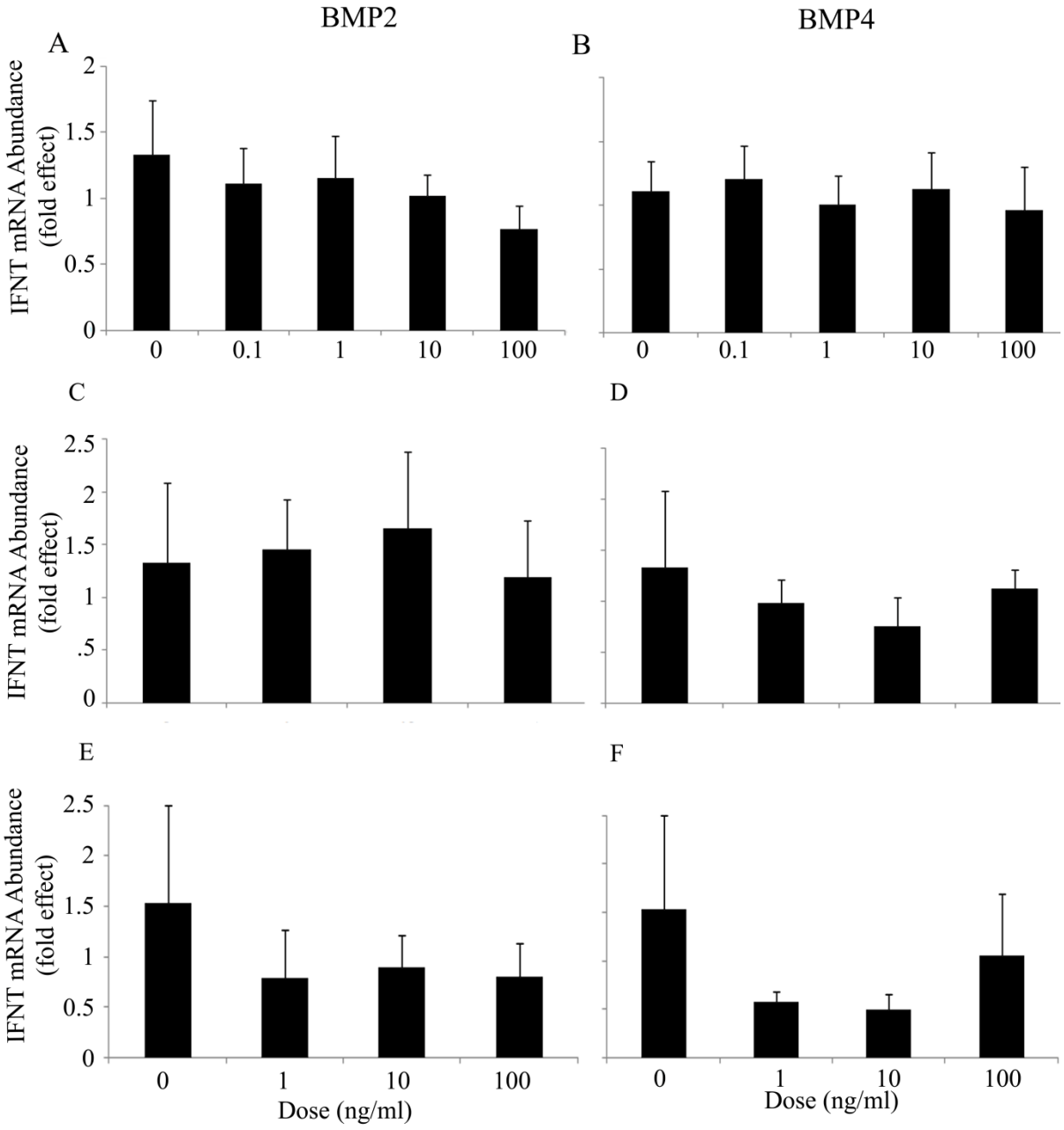

Figure 5-4. Effect of BMP2 or BMP4 supplementation on CT1 cell *IFNT* mRNA expression. Panels A, C and E are cells supplemented with varying doses of BMP2. Panels B, D and f are cells supplemented with varying doses of BMP4. Panels A and B are following 24 h of supplementation, panels C and D are after 96 h of supplementation and panels E and F are following 192 h of supplementation. No changes in *IFNT* mRNA expression was seen (n=5 replicates/treatment/time period)

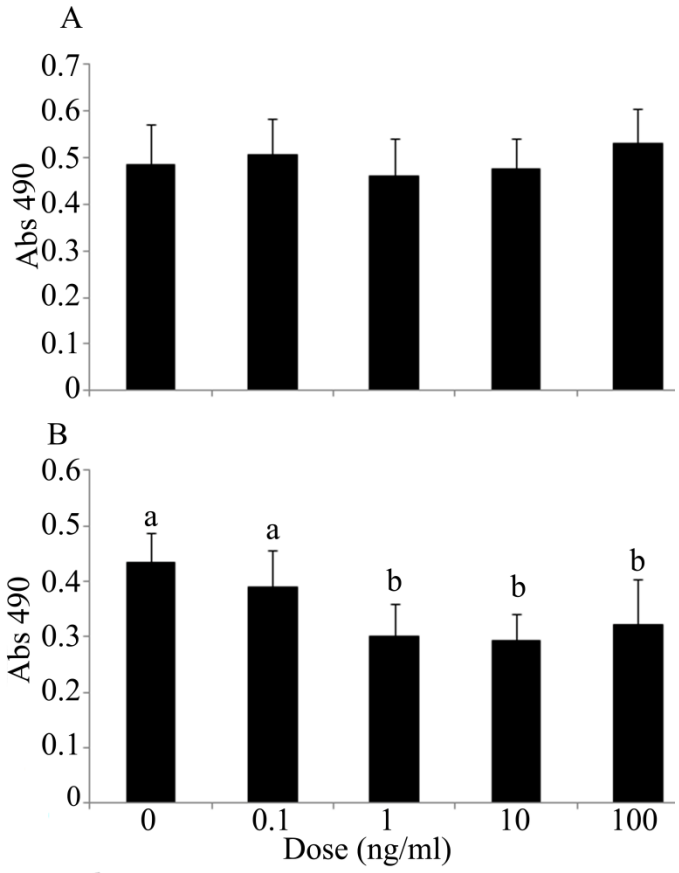

Figure 5-5. Effect of 48 h of BMP2 or BMP4 supplementation on numbers of CT1 cells. CT1 cells were supplemented with 0, 0.1, 1, 10 and 100 ng/ml of BMP2 or BMP4 for 48 h (n=4 replicates/ BMP). Cells were the submitted to the Titer 96 Aqueous One Solution Cell Proliferation Assay. BMP2 treatment did not affect the proliferation rate of CT1 cells. BMP4 supplementation caused a decrease in CT1 cell proliferation at 1, 10 and 100 ng/ml ($p<0.05$).

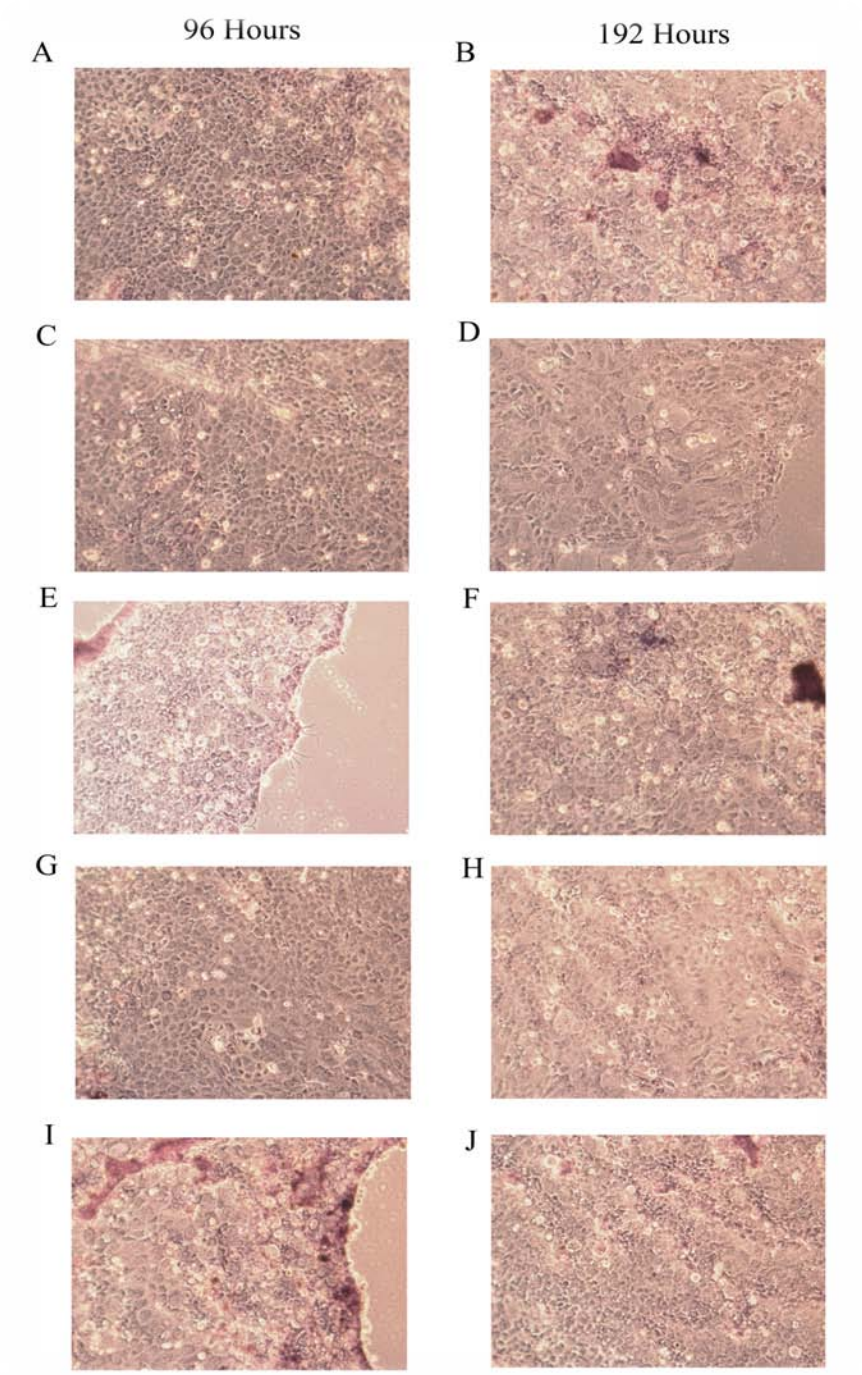

Figure 5-6. Effect of BMP2 and BMP4 treatment on alkaline phosphatase activity. A, C, E, G, and I are cells after 96 h of treatment and B, D, F, H and J are following 192 h of supplementation. A and B are control. C and D are cells treated with 10 ng/ml of BMP2. E and F are supplemented with 100 ng/ml BMP2. G and H are treated with 10 ng/ml of BMP4. I and J are treated with 100 ng/ml of BMP4. Darker areas of staining indicate positive alkaline phosphatase expression. Two wells per treatment and 2 replicates were preformed.

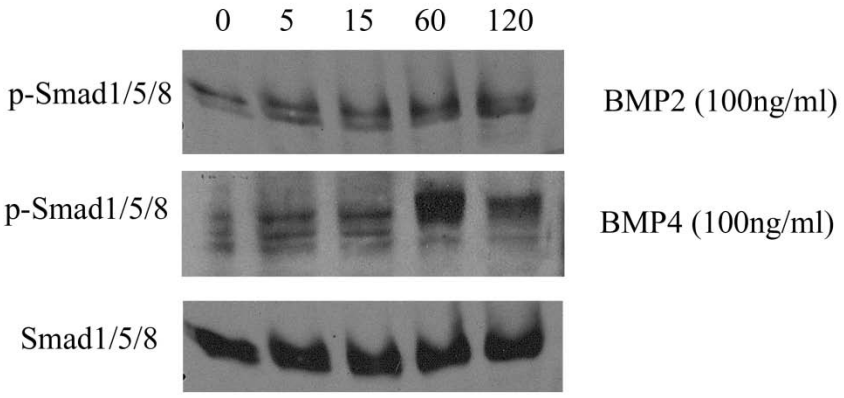

Figure 5-7. Phosphorylation of Smad 1/5/8 following BMP2 or BMP4 supplementation. CT1 cells were supplemented with 100 ng/ml of BMP2 or BMP4 for 0, 5, 15, 60, and 120 minutes and then submitted to western blot analysis for p-Smad1/5/8 and total Smad1/5/8 used as loading control. BMP2 supplementation did not cause an increase in the p-Smad1/5/8 present however endogenous BMP are activating Smad1/5/8. BMP4 treatment did cause an increase in p-Smad1/5/8 activation as seen at 60 min over endogenous activation. Total Smad1/5/8 was not different between samples.

CHAPTER 6
OVERALL DISCUSSION

Work presented here was directed at studying the basic mechanisms controlling trophoblast differentiation and function in the bovine placenta. Specifically, studies focused on developing new methods to study the formation of BNCs, examining the expression and function of several trophoblast differentiation factors in bovine placenta and elucidating the expression pattern and role of BMPs during early conceptus development.

The first project described a new method to examine BNC formation. Like others before us, we were unable to create BNCs in vitro and resorted instead to examining BNCs once they were formed *in vivo*. The FACS method utilized the hyperploidic nature of BNCs for cell enrichment. Using FACS to isolate enriched populations yielded 70-80% BNCs consistently. These cells could be used for mRNA and protein expression analysis as well as culture experiments.

While the method used to enrich BNCs is useful, being able to obtain even greater purity of BNCs may be possible. One way to improve the enrichment of BNCs with FACS would be to incorporate a BNC specific cell surface marker along with the nuclear content stain. Using the combination of nuclear content and cell surface marker during FACS would potentially increase the yield. The identification of a BNC specific cell surface marker would also allow the employment of other sorting techniques, such as Magnetic-activated cell sorting (MACS). Potential cell surface markers to examine include several integrins, such as integrin subunits α_6 and β_1. Integrins play an essential role in placental migration and implantation in rodents and humans, and several integrins have been localized by immunohistochemistry to the bovine placenta

[255, 259, 401]. Increasing the purity of the BNCs obtained would increase chances of deciphering changes between MNCs and BNCs. Also increased purity would allow the use of high through-put techniques such as global gene and protein analysis with a better efficiency.

It became evident from this work that BNCs cannot be maintained effectively in culture. BNCs maintain their morphology after three and a half days in culture but lose their BNC-specific features. This is consistent with observations made by others [191]. Studying BNCs *in vitro* is not a good model for elucidating mechanisms controlling trophoblast differentiation and function. One way to study BNCs *in vitro* would be to develop a system where BNC differentiation occurs. In order to develop this system, the mechanisms controlling BNC formation need to be understood.

In order to gain further understanding of how BNC development may be induced several putative differentiation factors were examined and found in mid-gestation bovine placentae (HAND1, MASH2, ID1, ID2, I-mfa, Stra13, GCM1, and E12/E47). Only *HAND1* mRNA expression was greater in BNC than MNC populations (fig 6-1). This difference was also observed at the protein level. This increase in HAND1 mRNA and protein expression in BNCs versus MNCs identifies HAND1 as a potential factor involved in BNC differentiation and function. HAND1 and GCM1 mRNA was absent in oTr and CT1 cell lines, while all other factors were present in amounts comparable to those found in BNC and MNC populations. To determine if HAND1 plays a role in BNC differentiation, it was over expressed in the oTr cell line. Overexpression did not cause a change in morphology; however expressed HAND1 protein was localized correctly to the nucleus.

Experiments indicate that HAND1 may play a role in BNC differentiation, but it cannot by itself induce BNC formation from trophectoderm cells. Rather, it is likely that other yet unidentified factors are over or underexpressed in the oTR cell line. It also may be necessary to use a different cell line or primary cell culture system. The cell line used, oTr, is thought to be a trophectoderm cell line produced from an elongated sheep conceptus. It does produce IFNT, but in very low levels. Also, the morphology of the oTR cell line is more like that of an endoderm or fibroblast cell line rather than what is typically observed in a pure trophoblast cell line. A potential cell line to develop is a mid-gestation bovine mononucleate trophoblast cell line. Such a cell line would enable the testing of potential differentiation factors in cells that are known to form BNCs.

Other avenues to pursue in regards to mechanisms controlling trophoblast differentiation include examining factors produced by both the conceptus and endometrium. Work examined the expression pattern and potential role of BMP2 and BMP4 in placental development and function. While BMP2/4 and their receptors were found in day 17 conceptus, endometrium and CT1 cells, the function of these factors remains unknown (fig 6-1). Localization of the expression of BMP2 and BMP4 in the endometrium and conceptus could provide greater insight into their function.

Not studied was the role of BMP4 in mesoderm formation in cattle. One model to test this hypothesis is an extended in-vitro embryo culture system. Experiments could examine the effects of BMP2 and 4 supplementation on mesoderm formation in the embryo by measuring the abundance of mesoderm marker, brachyury, in control and supplemented embryos. Immunohistochemistry could also be used to examine the formation of this layer in supplemented embryos.

Of interest is the role that the BMP inhibitor, Noggin, plays in trophoblast development. Noggin expression was present in the day 17 conceptus and endometrium but not in CT1 cells, potentially indicating a role for Noggin and the negative regulation of BMP signaling in trophectoderm differentiation and function. To test this hypothesis, further experiments are necessary to assess the function of Noggin in the trophectoderm cell line.

The continued study of basic trophoblast development, differentiation and function is necessary in order to develop methods to reduce pregnancy losses in cattle that occur due to the improper regulation of these events. Studies here identified HAND1 as a candidate for regulating BNC differentiation because it was differentially expressed between enriched populations of MNCs and BNCs. Although HAND1 alone did not induce BNC differentiation, future studies could focus on the combination of this factor with other potential differentiation regulators. Evaluated here was the expression of potential trophoblast regulators, BMP2 and BMP4. While both factors found are expressed by the conceptus and endometrium, their function is yet to be determined. Continued studies to identify the role that endogenous BMP2 and BMP4 have on trophoblast function could focus on inhibiting BMP2 and BMP4 signals and studying the effects. In conclusion, continued research on the basic biology controlling normal placental formation and attachment may illuminate the role of proper placentation pregnancy loss.

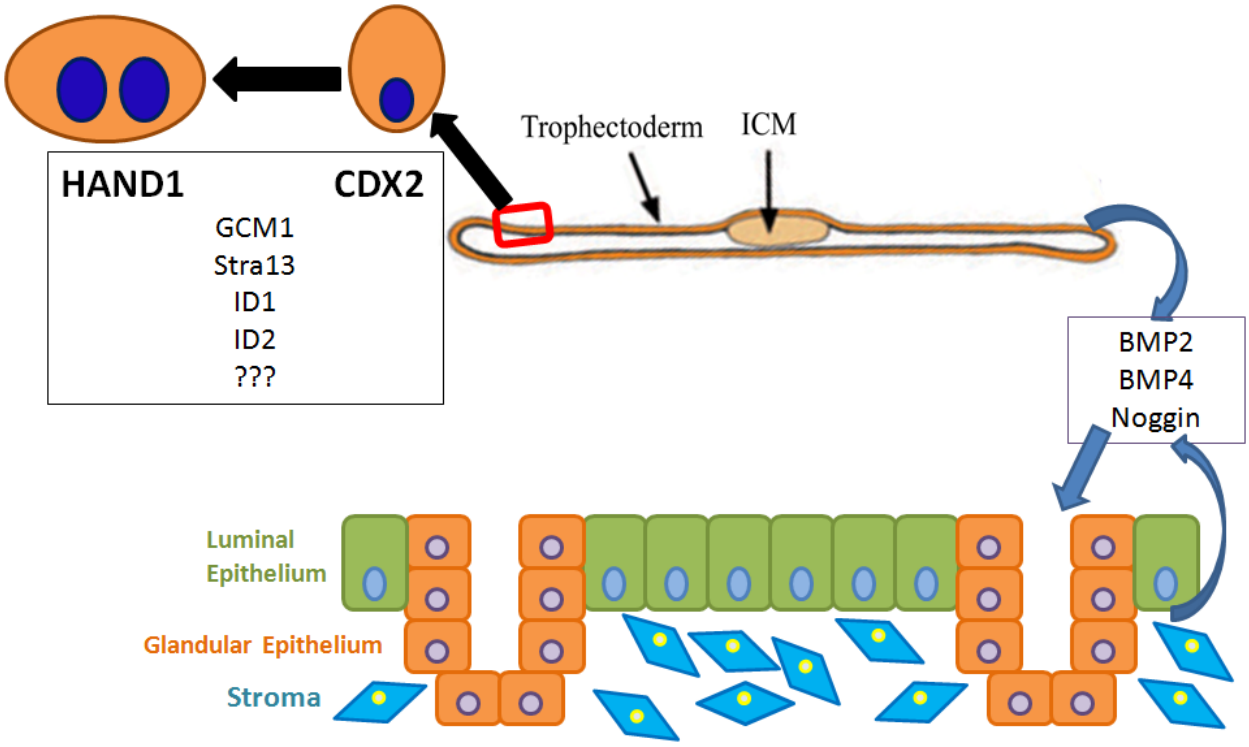

Figure 6-1. Summary of findings on factors effecting trophoblast cell development, differentiation and function. Several factors were examined for their potential role in BNC differentiation. The trophectoderm marker CDX2 was greater in MNCs versus BNCs. HAND1 was greater in BNCs versus MNCs indicating it may play a role in BNC differentiation. Other factors examines were not different between these cell types. BMP2 and BMP4 were found in the day 17 conceptus and endometrium while the BMP antagonist Noggin was found only in the day 17 conceptus. The role these factors play in conceptus and endometrium development needs to be further examined.

APPENDIX A
METHODS FOR FLUORESCENCE-ACTIVATED CELL SORTING (FACS) OF MID-GESTATION BOVINE PLACENTA

Materials

Dispase	BD Biosciences
DMEM with high glucose (4.5 g/l D-glucose)	Invitrogen Corp.
Dulbecco's phosphate-buffered saline (DPBS)	Invitrogen Corp.
Fetal Bovine serum (FBS)	Invitrogen Corp.
HEPES	Sigma
Pancreatin	Invitrogen Corp.
ProLong® Gold antifade reagent	Invitrogen Corp.
Vybrant® Dye Cycle™ Green	Invitrogen Corp.
16% ultra pure Paraformaldehyde	Polysciences Inc.

Tissue Collection

1. Obtain pregnant bovine uteri from the abattoir (Central Beef Industries L.L.C.; Center Hill, FL) and transport to the laboratory on ice.
2. Place uteri in appropriately sized basin and clean an incision area on the uterine horn ipsilateral to the corpus luteum with surgical scrub to remove any outside bacterial contamination.
3. Cut an incision through the uterine body and through the placental tissue all the way down to the fetus.
4. Remove several (5-6) placentomes, place in a clean petri dish, cover and take the tissue back to the laboratory.
5. Remove any unnecessary fetal membranes from the placentome.

6. Dissect away the cotyledonary tissue from the caruncular tissue; this can usually be achieved by carefully pulling the two tissues layers apart.
7. Wash the cotyledonary tissue twice in DPBS to wash away excess blood.
8. Cut tissue into smaller pieces (5-6 mm) and incubate in collection medium containing 25 units/ml Dispase and 0.625 mg/ml Pancreatin in a 50 ml conical tube at 37°C for 1 h under constant rotation (use the hybridization oven).
9. While the tissue is digesting, go back and measure the crown-rump length of the fetus to estimate gestational age as well as record the fetal sex.
10. Discard the left over tissue and fetus in the UF Meats lab awful room.

FACS Sample Preparation

11. Following tissue digestion, using a forceps take the large pieces of tissue and transfer into a new 50 ml conical tube.
12. Take the tissue homogenate and filter through a 200µm mesh.
13. Wash the large tissue pieces with collection medium and then transfer the large tissue pieces back to the original conical tube.
14. Repeat steps 12 and 13 twice.
15. Centrifuge the filtered tissue homogenate at 300 x g for 10 minutes at room temperature.
16. Resuspend cells in sort medium with 10µM Vybrant® Dye Cycle™ Green and incubate at 37°C for 30 minutes in the dark under constant rotation (use the hybridization oven).
17. Transport samples at 37°C to the University of Florida Interdisciplinary Center for Biotechnology Research Flow Cytometry laboratory (UF-ICBR; Gainesville, Fl).

Contact info: Neal Benson (nbenson@ufl.edu); recommend setting up flow appointments at least 2 weeks in advance.

FACS

18. Use the BD FACSAria™ cell sorting system (BD Biosciences) and FACS Diva software version 6.2.1 (BD Biosciences).
19. Before placing the placenta cell homogenate on the sorter dilute the sample at least in half with sort medium (you may need to dilute the sample further depending on the thickness of the sample). The sample needs to dilute enough to smoothly run the machine but if the sample is to dilute sorting will take a longer period of time.
20. Prepare collection tubes (2-15ml conical tubes with 3 mls collection medium) and label for MNC and BNC fractions.
21. Place diluted sample on the cell sorter and illuminate the cells with a 100mW laser emitting 488nM light.
22. Set thresholds at 20,000 of the forward light scatter and 5000 on green fluorescence (530 +/- 15nM) to eliminate excessive cell debris.
23. Plot the green fluorescence on a histogram and adjust the diploid peak to 50 on the linear scale of 0 to 255 using the photomultiplier voltage.
24. Once the diploid peak is established collect cells
    a. MNCs: cells that fall at the diploid peak
    b. BNCs: cells that have a fluorescence range of 2-to 4-times greater than the diploid peak
25. Approximately two million BNCs and six million MNCs can be collected in a three hour period.

26. Following FACS, centrifuge cells at 300 x g for 10 minutes at room temperature to remove FACs sheath fluid.
27. Resuspend cells in collection media.

Sorting Efficiency Analysis

28. Bring cells back to the laboratory.
29. Take a small aliquot of sorted cells and fix in 4% [w/v] paraformaldehyde for 15 minutes at room temperature

    Make 4% paraformaldehyde by mixing 1 vial 16% ultra pure Paraformaldehyde (10ml) with 30 ml PBS
30. Wash cells with DPBS twice
31. Mount cell onto glass slides using ProLong® Gold antifade reagent
32. View cells under phase-contrast and epifluorescence microscopy (nuclei will appear green) to determine the purity of sorted samples
33. Stored the remainder of the sorted sample appropriately for further use

Media Formulas

Collection Medium

DMEM with high glucose (4.5 g/l D-glucose)

10% [v/v] fetal bovine serum (FBS)

10mM HEPES

Sort Medium

Dulbecco's phosphate-buffered saline (DPBS)

5% FBS

APPENDIX B
STIMULATION OF IFNT BY FIBROBLAST GROWTH FACTORS IN THE BOVINE TROPHECTODERM CELL LINE, CT1

Introduction

For pregnancy to succeed the maternal unit must recognize the conceptus and maintain a uterine environment compatible for embryo survival. In ruminants, the maternal recognition of pregnancy hormone is interferon tau (IFNT), a protein produced by the trophectoderm prior to placental attachment to the uterine lining. IFNT prevents maternal rejection of the conceptus by blocking oxytocin receptor expression, thereby preventing pulsatile secretions of prostaglandin F2 alpha and luteloysis. It also induces several uterine proteins implicated in various pregnancy-regulatory functions [14, 402, 403].

Multiple intracellular and extracellular factors control *IFNT* gene expression. Intracellular factors include transcription factors Oct4 and Ets2 and signaling molecules MAPK and PKC [14]. Extra cellular factors include CSF2 [145], insulin growth factor I and II [404] and fibroblast growth factor 2 (FGF2). FGF2 increases IFNT gene and protein abundance in bovine trophectoderm [146]. FGF-2 localizes to luminar and glandular epithelium in the endometrium and is secreted into the uterine lumen [146, 405].

To date 23 FGFs have been identified along with 4 functional receptors with several isoforms [406]. FGF receptor 2b (FGFR2b) appears to have a function in the maternal uterine environment and has been localized to the epithelial lining of the endometrium in several species. Ligands for FGFR2b include FGF1, 7, and 10 [407] . FGFR2b localizes to the ovine uterine epithelium and mesoderm layer of the conceptus implicating FGF 1, 7, and 10 as potential players in maintaining pregnancy [408]. Our

laboratory identified FGFR2b as the FGFR2 receptor subtype present in bovine conceptus and the trophectoderm cell line CT1 [147].

FGF1 protein localizes to the trophectoderm and endometrial epithelium during mid pregnancy in cows [375]. In the ewe, FGF10 is expressed by the stromal endometrium and the developing embryonic mesoderm, a tissue layer juxtaposed to trophectoderm during conceptus elongation [408]. Current evidence indicates that FGF7 does not secrete it into the uterine lumen in ruminants [408]. However, FGF7 acts exclusively through FGFR2b [409], thereby implicating this receptor subtype in transducing the effects of this and potentially other FGFs on trophectoderm.

We hypothesize that FGFs 1, 7 and 10 increase IFNT expression. The goal of this work is to evaluate FGF1, 2, 7 and 10s ability to increase IFNT mRNA and protein levels.

Materials and Methods

Bovine Trophectoderm Cell (CT1) Culture

Cells were cultured as previously described [142, 146, 147] in DMEM with high glucose containing 10% fetal bovine serum and other supplements (100 uM non-essential amino acids, 2 mM glutamine, 2 mM sodium pyruvate, 55 µM β-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphoterin B; each from Invitrogen Corp.) on Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA) at 38.5°C in 5% CO2 in air.

IFNT mRNA Abundance

CT1 cells were seeded onto 12-well plates and allowed to attach for 48 h. Upon reaching ~50% confluence, cells were placed in fresh DMEM lacking FBS but containing other supplements plus a serum substitute (insulin/transferring/selenium;

ITS; Invitrogen Corp.). Following 24 h of serum free culture, cells were placed in treatments containing 0, 0.05, 0.5, 5, and 500 ng/ml of either rbFGF1 (R&D Systems; Minneapolis, MN), rbFGF2 (R&D Systems), rhFGF7 (R&D Systems) or rhFGF10 (Invitrogen Corp.) All treatments contained 50µg/ml carrier protein (BSA). Following 24 h of culture with treatment, tcRNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System with Trizol following manufactures guidelines (n=4 replicate experiments). TcRNA was stored at -80°C until further use.

All samples were incubated with RNase-free Dnase (New England Biolabs) as described above before RT with the High Capacity cDNA Archive Kit (Applied Biosystems Inc.). The abundance of *IFNT* and *18S* RNA (internal RNA loading control) in FGF1, FGF2, FGF7 and FGF10 treated CT1 samples were then determined by TaqMan based qRT-PCR. Primers and probes specific for *IFNT* [147] were synthesized (Applied Biosystems Inc.) and labeled with a fluorescent 5' 6-FAM reported dye and 3' TAMRA quencher. The *IFNT* probe was designed to recognize all know bovine and ovine *IFNT* isoforms [146, 147]. After an initial activation/denaturation step (50°C for 2 min; 95°C for 10 min), 40 cycles of a two-step amplification procedure (60°C for 1 min; 95°C for 15 s) was completed with TaqMan reagent (Applied Biosystems Inc.) and a 7300 Real-Time PCR System to quantify mRNA abundance. *18S* abundance was quantified using the *18S* RNA Control Reagent Kit (Applied Biosystems Inc.) containing a 5'-VIC-labeled probe with a 3'-6-carboxy-tetramethylrhodamine quencher. Each RNA sample was analyzed in triplicate (50 ng tcRNA). A negative control lacking reverse transcriptase was run for each sample to verify they were free from genomic DNA

contamination. The ΔC_t method was used to determine the abundance of *IFNT* transcripts relative to the *18S* RNA.

IFNT Antiviral Protein Assay

For a second study, CT1 cells were seeded onto 24 well plates coated in Matrigel™ and allowed to plate for 48 hours. Cells were allowed to reach 50% confluency and then placed in fresh medium lacking FBS as described above. After 24 h of serum free culture, cells were placed in treatments of 0, 0.5, 5, 50, or 500 ng/ml of rbFGF1, rbFGF2, rhFGF9 (R&D Systems) or rhFGF10 for 48 h. All treatments contained 50 µg/ml carrier protein (BSA). Medium samples were collected and frozen for future analysis. In order to control for cell number CT1 cells were submitted to the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) following medium collection. Absorbance was measured at 490 nm.

In order to determine the amount of biologically active IFNT, the collected medium samples were submitted to a cytopathic antiviral assay [146, 147, 410, 411]. Data values are expressed as ng/ml of biologically active IFNT found in conditioned media based on the standard rbIFNT (8.03×10^8 IU/ml). All values were corrected for cell number variability based on values obtained from the cell proliferation assay.

Statistical Analysis

Statistical analysis preformed using least squares analysis of variance (LS-ANOVA) using the generalized linear model (GLM) of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When analyzing qRT-PCR data, the ΔC_T values were used for analyses [146, 147] ΔC_T values were transformed to fold differences for illustration on graphs. Results are presented as arithmetic means ± SEM.

Results

FGF 1, 2, 7, and 10 Increase IFNT mRNA Abundance in CT1 Cells

Treatment of CT1 cells treated with 50 and 500 ng/ml of FGF1 (fig. B-1) increased ($P<0.05$) amounts of IFNT mRNA as compared to non-treated controls in a dose dependent manner. FGF2 increased ($P<0.05$) IFNT abundance at 5, 50, and 500 ng in a dose dependant manner. FGF7 increased ($P<0.05$) IFNT mRNA abundance (fig. B-1) in CT1 cells at concentrations of 50 and 500 ng/ml. FGF 10 (fig. B-1) increased ($P<0.05$) IFNT mRNA abundance at 500 ng/ml.

FGF 1, 2, 9, and 10 Increase IFNT Protein Abundance in CT1 Cells

Treatment of Ct-1 cells with 50 ng/ml of FGF1 (fig B-2) increased IFNT protein expression ($P<.05$). Treatment with of 500 ng/ml FGF1 increased IFNT concentration but there was no significant difference between controls and 50 ng/ml. FGF2 (fig B-2) treatment increased ($P<0.05$) the amount of biologically active IFNT protein at 50 and 500 ng/ml doses. Treatment of 500 ng/ml of FGF9 (fig B-2) significantly increased ($P<0.05$) IFNT protein. FGF10 (fig B-2) increased IFNT protein at 500 ng/ml ($P<.05$) and treatment at 50 ng/ml increased protein levels numerically but not significantly from controls.

Discussion

Early embryonic loss in dairy cattle accounts for a large portion of economic loss in the dairy industry [8]. For an embryo to be sustained early in pregnancy enough IFNT must be produced [14]. Few regulatory factors effecting IFNT regulation in ruminants have been determined. The present study describes the discovery of two factors, FGF 1 and 10, believed to be important in IFNT gene regulation both in the uterus and trophectoderm.

Previous work in the laboratory identified FGF 1, 7 and 10 mRNA expression in tissues [147]. FGF 1 expression occurs in conceptus, trophectoderm, and endometrium while FGF 10 expression occurred in conceptus and endometrium. FGF 7 expression occurred only in the endometrium, however was still of interest because it only can act through FGFR2b the receptor subtype the study was aimed at examining.

All FGFs increased *IFNT* mRNA abundance; however FGF 1 increased these levels at a lower treatment dose than other FGFs. FGF1 the same trends show for FGF 2 as seen in this and previously published work [146]. These two factors together could have more of an implication in increasing IFNT expression. FGF 7 showed that the FGFR2b subtype was present and functional in the Ct-1 bovine trophectoderm cell line because it increased IFNT mRNA abundance and can only work through that receptor. However we did not follow FGF 7 at the protein level because it localizes to the tunic muscularis of endometrial blood vessels in sheep and it is believed that it cannot make it into the uterine lumen and therefore act as a paracrine factor on the endometrium[408].

FGF1, 2, 9, and 10 treatment increased IFNT protein produced by the trophectoderm. However, efforts to locate transcripts for FGF9 in day 17 bovine conceptus or bovine endometrium were not effective. Thus from this work only FGF1 and 10 can be implicated in regulating IFNT production along with previously published results implicating FGF2 in this role as well [146]. FGF1 increased IFNT protein production at an early dose than FGF 10, a trend seen in the mRNA data as well.

FGF1 may increase IFNT production at an earlier dose because of the type of recombinant protein used. The FGF1 protein was a bovine recombinant while FGF10

was human. While bovine and human FGF10 share approximately 85% amino acid identity. This lower similarity may account for the need for a higher FGF dose. Also FGF1 does work through multiple receptors and may work through multiple ones to increase IFNT production while FGF 10 will not.

Results implicate FGF1 and 10 in potential IFNT regulation, but expression of FGF7 shows it may not come into contact with the trophectoderm thus preventing any effect it may have on IFNT production. Multiple FGFs may work in concert with each other to regulate IFNT and treatment with multiple FGFs may have an additive effect on IFNT production.

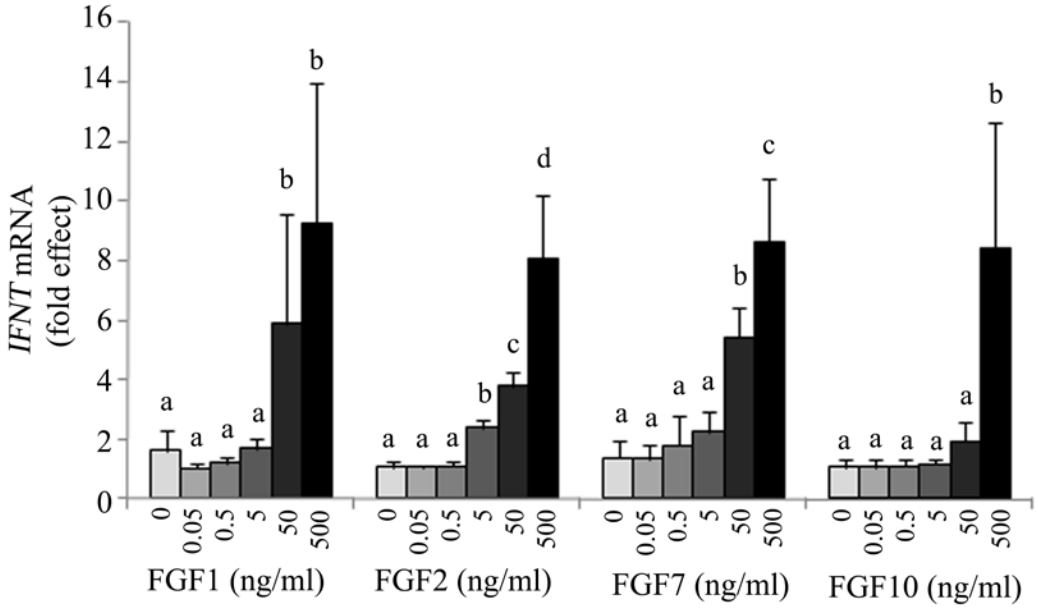

Figure B-1. Several FGFs increase IFNT mRNA abundance in a dose dependent manner. Cells were incubated in medium lacking serum and containing 0, 0.05, 0.5, 5, 50, and 500 ng/ml of rhFGF1, rbFGF2, rhFGF7 or rhFGF10. All cultures contained carrier protein (50µg/ml BSA). RNA was extracted 24 h post-treatment quantitative RT-PCR was used to determine IFNT mRNA abundance relative to an internal control (18s RNA). Changes in IFNT mRNA were analyzed (n=4) with Least Squares-Analysis of Variance using the General Linear Models Procedure of Statistical Analysis Software. Different superscripts within each panel represent differences ($P<0.05$).

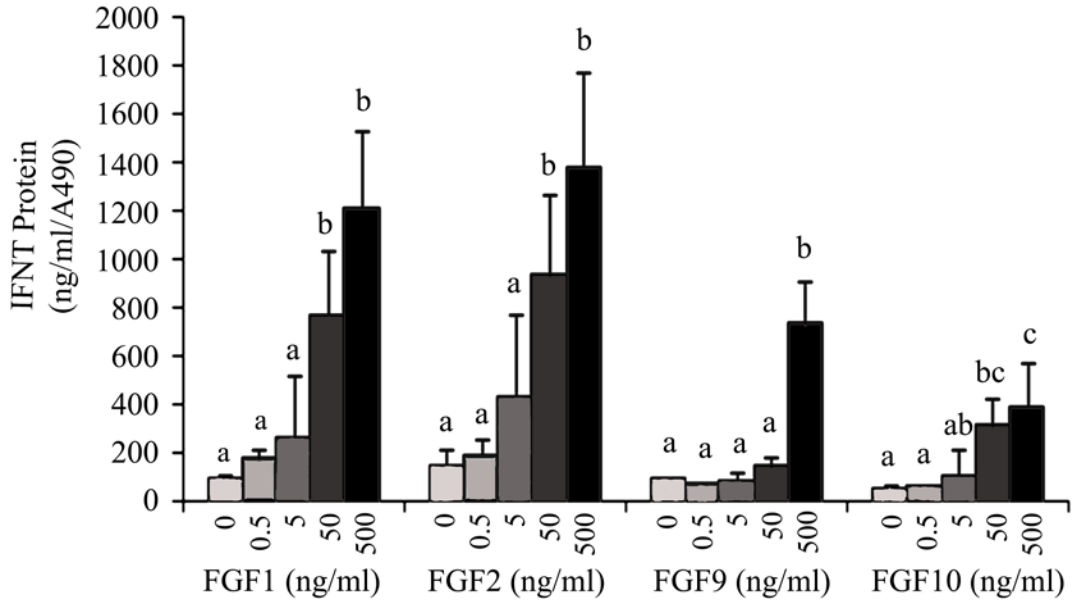

Figure B-2. Several FGFs increase IFNT protein secretion in CT1 cells. Cells were exposed to FGFs as described previously. Cell culture supernatant was collected 48 hours after start of incubation and antiviral assays were completed to quantify IFNT concentrations in conditioned medium. Cell density was measured using the CellTiter $96_{aqueous}$ One Solution Cell Proliferation Assay. Changes in IFNT protein concentration were analyzed (n=5) with LS-ANOVA. Different superscripts within each panel represent differences ($P<0.05$).

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BIOGRAPHICAL SKETCH

Kathleen Pennington grew up in Levittown, Pennsylvania just north of Philadelphia. She attended Queen of the Universe Elementary School through eighth grade and then moved to Neshaminy High School where she graduated in 2001. Following high school, Kathleen attended the University of Delaware where she majored in animal science with a Pre-Veterinary concentration. Kathleen graduated from the University of Delaware with a Bachelor of Science in the spring of 2005. While attending the University of Delaware, Kathleen took the opportunity to pursue undergraduate research which helped her to make the decision to pursue her doctorate. Kathleen began her PhD program in animal molecular and cellular biology in the Animal Sciences Department at the University of Florida in the fall of 2005 under the direction of Dr. Alan Ealy. Kathleen's research focus has been on maternal-fetal interactions during the first trimester of pregnancy in cattle, with particular interest on the signaling mechanisms controlling trophoblast cell development and function. Following her PhD Kathleen plans to pursue a postdoctoral position in the human biomedical field and focus on placental development and associated diseases.