THE ACTIONS OF FIBROBLAST GROWTH FACTORS DURING PERI-IMPLANTATION CONCEPTUS DEVELOPMENT IN CATTLE

By

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To my parents, sister and wife

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CDX2	Caudal Related Homeobox-2
CSF2	Colony-stimulating Factor 2
CT1	Cow Trophoblast Cell Line 1
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Cell Matrix
ERK	Extracellular Signal Regulated Kinase
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA family transcription factor 4
GCM1	Glial Cells Missing 1
HAND1	Heart and Neural Crest Derivatives-expressed protein 1
ID2	Inhibitor of Differentiation 2
IFNT	Interferon Tau
IGF	Insulin-like Growth Factor
IVP Embryo	In Vitro Produced Embryo
ITG	Integrin
ITS	Insulin-Transferrin-Selenium
LGALS	Galectin 15
MAPK	Mitogen Activated Protein Kinase
MASH2	Mammalian Achaete-Scute Homolog 2
MMP2	Matrix Metalloproteinase-2

NP-40	Nonidet-40
OCT4	Octamer-binding Transcription Factor 4
oTr1	Ovine Trophoblast Cell Line 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGE	Prostaglandin E
PI	Propidium Iodide
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAPK/JUN	Stress-activated protein kinase/c-Jun NH2-terminal kinase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLC	Solute Carrier Protein
SOX17	Sex Determining Region Y Box-17
SPP1	Secreted Phosphoprotein 1
TBST	Tris-Buffered Saline and Tween 20
YAP	Yes-Associated Protein

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In ruminants, the trophoblast product interferon tau (*IFNT*) acts as an anti-luteolytic hormone to maintain a pregnant state and promote uterine receptivity for subsequent embryo development and implantation. Defects in early embryo development and miscues between the embryo and uterus result in pregnancy failure in various species. Lactating dairy cattle are especially prone to early pregnancy losses and these pregnancy loss lead to low reproductive efficiency and substantial economic losses.

The fibroblast growth factor (FGF) family plays critical roles in regulating early embryonic development in mammals. In cattle and sheep, it is clear that several uterine-derived FGFs stimulate *IFNT* production in trophoblast cells. One of particular interest is FGF2. It is synthesized by epithelial cells and released into the uterine lumen in cyclic and pregnant animals. In the presence of a conceptus, FGF2 binds to its receptors on trophoblast cells and increases *IFNT* mRNA transcription and bioactive protein synthesis and release. Herein, three projects were completed to examine the signaling component that mediates FGF-induced *IFNT* production in bovine trophoblast cells and to discover additional biological roles of uterine- and conceptus-derived FGFs during bovine embryogenesis.

The first set of studies investigated the signaling pathways triggered by FGF2 to stimulate *IFNT* production. Using two bovine trophoblast cell lines and primary trophoblast outgrowths as model systems, the work revealed that ERK1/2 and p38MAPK are critical for basal expression of *IFNT*, while protein kinase C (PKC) delta mediates FGF2-induced *IFNT* expression.

Another set of studies revealed a new activity for FGF2 during bovine embryo development. Specifically, FGF2 promoted primitive endoderm formation in bovine blastocysts. Activation of FGF signaling or inhibition of FGFR kinase activity in blastocysts modulated early lineage marker expression.

A final set of experiments was completed to test the hypothesis that FGF2 or FGF10, a conceptus derived FGF, regulated trophoblast cell migration. FGF2 and FGF10 activated multiple MAPK pathways and the ERK1/2, p38 MAPK and SAPK/JNK signaling modules regulated FGF-mediated systems to stimulate trophoblast migration *in vitro*. Also, treatment of blastocysts or trophoblast cells with FGF2 modulated the expression of migration-related genes (*ITGA2B*, *ITGB6* and *MMP2*).

Taken together, current data suggest that FGF-dependent signals play crucial roles in conceptus growth and differentiation during pre- and peri-implantation development. Understanding the fundamental biology of embryo development at this stage will provide the basis to develop strategies to reduce early pregnancy loss.

CHAPTER 1
INTRODUCTION

Early pregnancy loss has a major influence on economic outcomes in farm animal operations. It is well documented that the modern dairy cow is sub-fertile in the US and other countries [1-4]. In lactating dairy cattle, a paltry 40% of initial pregnancies develop to term [5]. Among all the various reasons for pregnancy failure in cattle, embryonic death within the first month of pregnancy accounts for approximately 57% of all pregnancy losses [5, 6]. Furthermore, multiple reports estimated that as much as 70% to 80% of total embryonic loss occurs between days 8 to 17 of gestation [3, 5].

Pregnancy failures in dairy cattle have become progressively greater as intense selection for milk yield traits has been conducted over the past several decades [7]. Butler (1998) showed that the first service conception rate has declined from 65% to 40% over the past two decades in US dairy cattle [8]. Royal (2002) estimated that pregnancy rate to first service declined from 55.6 to 39.7% between 1975 and 1998 [9]. In the dairy industry, more than $500 of income from lifetime production is lost by each pregnancy failure [10] .

Developmental problems occurring during early pregnancy also may have significant economic implications later in gestation in dairy cattle. Although the initial pregnancy rates of cloned bovine embryos were similar to embryos derived from artificial inseminations, continued pregnancy loss occurred throughout gestation in cattle [11]. It has been suggested that pregnancy failure in middle or late gestation originates from miscues during peri-implantation development before day 21-25 of gestation [12, 13]. Based on these observations, several key development events, including

blastocyst formation, maternal recognition of pregnancy and early placental formation likely can have a significant impact on mid and late gestational success or failure.

Peri-implantation development in ruminants (cattle, sheep, goats, and deer) is dramatically different from rodents or primates. After hatching from the zona pellucida, ruminant blastocysts float freely in the uterine lumen and transform from a small, ovoid shape of less than 1 mm to a filamentous conceptus that can reach 40 cm in length over a period of approximately 10 days [14]. After trophoblast lineage specification, the blastocyst starts to express several factors, including *IFNT*, a type I interferon that acts as the signal for maternal recognition of pregnancy [15-17]. The level of *IFNT* mRNA in trophoblast cells and amount of *IFNT* protein released into the uterine lumen the increases significantly during the period of conceptus elongation [18]. Defects in conceptus elongation and miscues in *IFNT* production can, therefore, represent one reason for pregnancy loss in cattle [19].

Many growth factors and cytokines likely control conceptus. For example, providing bovine blastocysts with insulin-like growth factor-1 (IGF1) increased post-transfer survival under heat stress conditions [20]. Also, treatment of bovine trophoblast cells with colony-stimulating factor 2 (CSF2, previously knonw as GM-CSF or Granulocyte-macrophage Colony-stimulating Factor) enhanced trophoblast production of *IFNT* [21]. CSF2 supplementation to bovine in vitro produced (IVP) embryos improved pregnancy rates after transfer and limited late pregnancy losses [22]. In the ovine uterus, IGF2 and IGF binding protein 3 (IGFBP3) directly stimulate trophoblast cell proliferation and migration [22, 23].

Another group of important growth factors linked with successful peri-implantation development in cattle is the FGF family. The FGFs are composed of more than 19 members and associate with at least 4 main receptors with different ligand binding affinities and tissue specificities in mammal [24]. FGF signaling is essential for embryogenesis in several species. The mouse embryo begins producing FGF4 and its receptor, FGFR2, after the fifth blastomere division. FGF4 released by the undifferentiated inner cell mass stimulates trophoblast stem cell proliferation and regulates cell fate decisions [25-27]. A similar role of FGF4 during embryonic development has not been established in ruminants, but other FGFs certainly are important during early bovine development. In ruminants, FGF2 is present in uterine lumen throughout early pregnancy, and it is able to bind to its receptors and stimulate *IFNT* production by trophoblast cells while having little to no impact on trophoblast cell proliferation [28, 29]. Supplementation of bovine blastocysts and cultured trophoblast cells with different FGF1, FGF7 and FGF10 also stimulates *IFNT* production [30, 31]. In the ovine uterus, the synthesis of FGF10 by stromal cells is controlled by progesterone and FGF10 acts as a mediator of progesterone signaling to regulate conceptus development [32, 33]. The bovine conceptus produces FGF10, and FGF10 mRNA abundance increases dramatically when conceptus elongation occurs [30]. The mechanisms that underline FGF regulation of *IFNT* production and other cellular events during bovine pre- and peri-implantation remain unclear.

It is clear that uterine- and conceptus-derived growth factors, including various FGFs, impact embryo development in ways that promote conceptus development, differentiation and the establishment and maintenance of pregnancy. It is important,

therefore, to understand the specific developmental aspects of bovine embryogenesis affected by FGFs so that new strategies for reducing pregnancy losses can be conceived and tested. This dissertation work tested the hypothesis that FGF signaling plays a multifunctional role in regulating embryonic and trophoblast development during the first 2 weeks of gestation in cattle. Specifically, we propose that FGF2 and other FGFs function as key regulators of *IFNT* production, early lineage specification and trophoblast migration. A series of experiments was conducted to address three specific aims:

- Describe the signaling pathways that mediate FGF2-induced *IFNT* production in bovine trophoblast cells.
- Delineate the roles of FGF-dependent signaling for trophoblast and primitive endoderm lineage determination in bovine blastocysts.
- Elucidate how FGF-dependent signals impact integrin gene expression and trophoblast cell migration.

CHAPTER 2
LITERATURE REVIEW

Pre- and Peri-implantation Development

In mammals, preimplantation development refers to the period from fertilization to embryo implantation. The progression of this stage of development is marked by several important molecular events including maternal to zygote transition, blastocoel formation and early germ layer formation within the inner cell mass [34, 35]. The peri-implantation period of development encompasses the time from embryo release from the zona pellucida to the initiation of placenta formation [36, 37]. These complex and highly-regulated developmental processes are responsive to many factors present in uterine environment. Problems associated with embryo-uterine interactions are associated with high rates of pregnancy losses in various placental animals. Early embryo death is common to many species and it is part of selection events to ensure the survival of only most competent embryos. Meanwhile, undesired embryo loss frequently occurs in humans and other species. An overall goal of work in this field of study is to better understand the various problems that may occur during pre- and peri-implantation development so that schemes can be developed to minimize early embryonic pregnancy loss in humans and domesticated cattle, sheep, and pigs [3].

Conceptus Elongation in Ruminant Species

The mouse serves as the conventional mammalian model for studying preimplantation development because of its ease of propagation and maintenance and because techniques exist to genetically modifies this species. Genetic modification of cattle and other ruminants is more challenging and expensive. There is no unified scheme of embryogenesis that can be applied to all mammals, but several critical

cellular and molecular events occur in most mammalian species. For example, trophoblast cell linage specification and maintenance of a pluripotent inner cell mass is required for embryo implantation in all mammals. However, several aspects of peri-implantation development and gastrulation vary dramatically between species. The following sections of this review will describe the similarities and distinctions as they relate to bovine conceptus development.

Preimplantation development and genomic activation

Reproduction in mammalian species requires the merger of two parental haploid genomes by fertilization of the oocyte with the spermatozoa and activation of the new embryonic genome. Maternally stored mRNA and protein are loaded into oocytes during oogenesis to provide ample material for regulating embryo development for the first few blastomere divisions. After fertilization, maternal mRNA starts to degrade and must be replaced with newly synthesized mRNA derived from the embryonic genome. These new transcripts are especially important for establishing cell polarity, maintaining cell division rate, and specifying early embryo lineage segregation [38]. The timing of the maternal to zygote transition (MZT) in genome control over the newly formed embryo differs among species. This event occurs at 2-cell stage in mice, at the 4-cell or 8-cell stage in humans, and at the 8- to 16-cell stage in cattle [39].

Normal progression of embryonic development requires that MZT occur in a timely manner. Interestingly, one function of the new embryonic genome is to destroy remnants of the maternal genome. In the mouse, 90% of maternally-stored transcripts are degraded in 2-cell embryos [40]. Proteins responsible for mRNA stability in oocytes, for example MSY2, represent some of the initial targets of the new embryonic genome. [41]. Another function of the new embryonic genome is to restore specific transcripts

that were inadvertently destroyed after fertilization but are required for embryo development. A classic example of this activity is the fate of actin mRNA during early embryo development in mice. Around 90% of maternally-derived actin mRNA is degraded by the 2-cell stage, and since actin is indispensable for several basic cellular functions, it is readily transcribed from the embryonic genome as MZT is initiated [35]. The third function of the new embryonic genome is to generate new transcripts not produced by the maternal system that are required for embryonic programming. Interestingly, not all maternally-derived mRNAs are degraded at MZT and remain able to influence embryonic development after MZT is complete [42]. For example, maternally-derived *CDX2* plays an important role in cell allocation at the blastocyst stage [43].

Current data in mice suggest that two transient waves of transcription are observed during embryonic genome activation. Using globe gene expression analyses, Hamatani et al., (2004) found that two major transient waves of *de novo* transcription activation occurred after fertilization [44]. The first wave refers to the zygotic genome activation (ZGA), occurs between the 2- to 4-cell stages and leads to a robust expression of embryonic transcripts. The second wave, termed the mid-preimplantation gene activation (MGA), occurs at the 8-cell stage. The MGA results in the expression of gene products that are involved in blastomere polarity and morula compaction. In cattle, the major embryonic genome transcription occurs at 8 to 16-cell stage, although, a minor onset of gene transcription is detectable at earlier stages [45].

Cell differentiation and blastocyst formation

The hallmark of early embryonic development is the formation of a blastocyst with polarized epithelial cells called trophectoderm arranged along the outside border of the

embryo and pluripotent cells located inside the embryo termed inner cell mass (ICM). This initial lineage segregation begins at early morula stage when totipotent blastomere loses plasticity. Blastomere from 2-cell, 4-cell or 8-cell stage embryos can contribute to all tissues of the embryo [46] ,but lineage tracing experiments in mice reveal that after the late 8-cell stage, polarized cells located outside of an embryo contribute to trophectoderm lineage whereas unpolarized inside blastomere maintain their pluripotency [26, 47, 48].

Embryonic genome activation builds the foundation for cell differentiation and blastocoel formation. Intracellular and cell surface adhesion molecules that are transcribed after the second wave of embryonic genome activation play a critical role in the morphogenesis of the blastocyst and the appearance of the trophectoderm [49]. E-cadherin mediates the first cell adhesion event leading to individual cells lose their outlines termed embryo compaction in 8-cell mouse embryo or 32-cell bovine embryo [50]. Mouse embryos lacking E-cadherin fail to form organized blastocysts [51, 52]. Moreover, activation of protein kinase C (PKC) regulates E-cadherin localization and induces compaction in mouse embryos [53]. The specific PKC isoforms that control E-cadherin activation are undefined.

After compaction, the formation of tight junction complexes separates outside cells from cells located inside the embryo. Tight junctions are multimolecular membrane complexes that restrict paracellular diffusion and fluid movement. Two major types of proteins are required for these tight junctions; the tight junction peripheral membrane protein, zonula occludens and transmembrane protein, claudins [54]. The tight junction complex dictates the formation of the blastocoel cavity along with Na/K-ATPase. The

Na/K-ATPase serves as a Na^+ and K^+ transporter, and movement of Na^+ out of cell and transport of K^+ into the cell creates an ion gradient that promotes fluid accumulation [55]. It is interesting to note that embryo compaction coincides with the onset of cytoplasmic and microvillus polarity in blastomere. Recent advances in preimplantation biology have linked cell position and the expression of key transcription factors that are gateways of cell fate commitment. For example, the knockdown of Pard6b expression, one component of Par-aPKC (atypical PKC) complex, leads to the cavitation failure and abnormal distribution of tight junctions [56].

The segregation of trophectoderm during early embryogenesis is imperative since mammals have viviparous placentae, which requires nutrition and gas exchange between the fetal and maternal units (placenta and uterus, respectively). Trophectoderm gives rise to all the tissue types needed to form the outer layers of the placenta. The blastocoel is important for providing room for embryo rearrangement during gastrulation and embryo morphogenesis [57]. Eventually the blastocoel will become the fluid-filled component of the yolk sac.

Conceptus elongation and germ layer formation

One unique feature of development in ruminants is conceptus elongation (a conceptus contains embryo/fetus and associated extraembryonic membranes) [58]. In rodents, humans, and many other mammals, conceptuses begin attaching to uterine epithelial cells immediately after hatching from the zona pellucida. However, in ruminants, the free-floating conceptus transition from an ovoid to spherical and finally to a filamentous shape prior to uterine attachment [59, 60]. The hatched blastocyst is spherical and ranges in diameter from 0.125 to 0.5 mm around days 8 to 11 of pregnancy. At approximately 14, bovine blastocyst begins elongating and ranges in

length from 0.85 to 2.5 cm [61]. By day 17, conceptuses usually occupy most of one uterine horn (15-30 cm) and at day 21 the trophoblast finally begins attaching to uterine caruncular and intercaruncular areas. Conceptus elongation is required for the establishment of pregnancy in ruminants, and significant insights into understanding of this developmental puzzle have been made in last decades. However, the molecular basis of conceptus elongation and early cell patterning remains to be completely understood [59].

The ontogeny of gastrulation differs between cattle and mice. In rodents, gastrulation occurs right after embryo implantation [19]. These events occur more slowly in cattle [62]. A distinctive trophectoderm usually is evident at day 7 after fertilization. The generation of primitive endoderm usually begins on day 9, and then the emergence of primitive ectoderm occurs around day 12. Primitive mesoderm forms around day 14 within the epiblast [14, 63]. Changes in trophoblast also occur around this period of development in ruminants. Specifically, the polar trophectoderm that covers the epiblast, called Rauber's layer, is degenerated around day 14. This results in the embryonic disk becoming exposed to the uterine lumen [63]. The gastrulation marker, *brachyury*, which encodes a mesoderm-specific transcription factor, is detectable in embryonic discs and these nascent mesoderm cells is close to the basal membrane of epiblast in day 15 bovine conceptus [64]. On day 21, visible somites and several germ layers appear within the primitive steak. The neural groove, neuroectoderm, mesoderm and amnion are present at this time [65] .

Embryo Implantation and Early Placentation

Embryo implantation is a two-way communication between a competent blastocyst and a receptive uterus. Irrespective of the differences in peri-implantation development,

Enders and Schlafke suggested that the process of implantation could be dissected into three steps: apposition, adhesion and penetration [66]. Apposition refers to the step when trophoblast cells become juxtaposed to the uterine luminal epithelial cells. Adhesion involves the attachment of the trophoblast cells and uterine epithelium mediated by adhesion molecules such as integrins. The final step of implantation is penetration. During invasive implantation in humans and mice, trophoblast cell penetration is coupled by the stromal cell differentiation and loss of epithelial cells, a process known as deciduation. Penetration does not occur in species that contain relatively noninvasive placentae such as bovine and sheep. Instead, a syncytial structure is formed by the fusion of trophoblast and luminal epithelium [36, 67].

Blastocyst activation for implantation in mice

The mouse embryo escapes from zona pellucida on day 3.5 and immediately initiates the process of implantation under normal conditions [68]. Progesterone and estrogen from corpus luteum are master regulators of embryo implantation. That activation of the mouse embryo for implantation is dependent on an estrogen signal, and blocking this estrogen signal can induce blastocyst dormancy in rodents and several other species [69] . Attachment of the blastocyst to the uterine lining induces stromal cell deciduation and trophoblast cell differentiation, which ensure the production of prolactin and prolactin-related proteins from decidual cells. These factors prevent corpus luteum regression [70].

Blastocyst attachment is initiated by adhesion molecules. Heparin Binding EGF (HB-EGF) is the first identified marker that initiates embryo implantation in mice. The expression of HB-EGF by uterus epithelium is dependent the presence of blastocysts [71]. HB-EGF interacts with its receptors ErbB1 and ErbB4 on blastocysts to promote

trophoblast transition to an adhesive stage [72]. Interestingly, blastocysts also produce HB-EGF, and exposure of blastocyst-size beads soaked with HB-EGF induces HB-EGF in uterine epithelium, suggesting this signal auto-amplifies itself during embryo attachment [73]. In the human, in addition to containing a conserved HB-EGF system, attachment is mediated by L-selectin and various integrin subunits [74, 75].

It is interesting to note that several molecules not directly involved with cell adhesion play active roles in regulating blastocyst implantation. For example, inactivation of the Wnt /β-catenin signaling system in early mouse embryos does not affect blastocyst formation but blocks implantation [76]. Emerging evidences in mouse models and human clinical work reveal the importance of cannobiniods and its receptor signaling in pregnancy, especially in the process of embryo implantation [77]. Cannobiniod released from the uterus interacts with its receptor (CB1 and CB2) to control embryo activation for implantation [78]. The involvement of these molecules in embryo implantation in other species, including ruminants, awaits investigation.

Embryo implantation in ruminant species

In ruminants, the extended post-hatching development that occurs before attachment and adhesion requires that mechanisms controlling cell attachment be blocked. This occurs by the extended presence of anti-adhesive agents. One primary component to the anti-adhesive nature of the uterus during early pregnancy is the production of mucins. Secretion of mucins and specifically MUC1 occurs within the endometrial epithelium until day 17 of pregnancy, and as MUC1 concentrations decrease, trophoblast cells initiate adhesion with the uterine epithelium [79]. The conceptus also plays an active role in preparing for uterine adhesion. Global gene expression changes were examined on days 7, 14 and 21 by microarray. Between

days 7 and 14 the predominant gene expression changes related to cytoskeleton remodeling and cell metabolism, reflecting the dramatic morphological changes that occur during blastocyst elongation. However, between days 14 and 21 the expression of many genes associated with cell adhesion and extracellular matrix (ECM) including alpha2 collagen, MMPs, several integrins and integrin binding proteins were increased [80].

The formation of focal adhesions at the maternal-fetal interface during embryo implantation seems to be controlled by integrin signaling [81-83]. In ruminants, a recent study found that multiple integrin subunits such as *ITGAV*, *ITGA4*, *ITGA5* and their binding proteins *LGALS15* and *SPP1* were present in luminal epithelium and trophoblast surfaces, indicating the assembly of focal adhesion components along maternal-trophoblast interfaces [84].

The major types of placentation in different species

The major function of the placenta is to ensure the efficient gas and nutrient exchange between fetus and mother. The mission is accomplished by remarkable growth and terminal differentiation of trophoblast cells so that they may support embryonic and fetal development. Establishment of an efficient placenta is vital for pregnancy success. For instance, in women, the ratio of fetal to placental weight increases more than 40 fold as the gestation continues towards term [37, 85]. The placenta in most mammals falls into three major categories: hemochorial, endotheliochorial and epitheliochorial [86, 87].

Hemochorial placentae are found in most primates and rodents. In this type of placenta, trophoblast cells invade into the maternal uterine arteries and directly embed in maternal blood. Endotheliochorial placenta refers to the placenta that present in most

carnivores and some other species like elephants. In an endotheliochorial placenta, the trophoblast is in direct contact with the endothelium of the uterine blood vessels. In epitheliochorial placentae, trophoblast cells are noninvasive or show limited invasion. This type of placenta is found in most even-toed ungulates such as ruminant and pig. The epitheliochorial placenta is so called because trophoblast is directly connected with uterine epithelium. The initiation of bovine placentation occurs when trophoblast starts to be in touch with uterine epithelium and fetal blood vessels are evident about 33 days of gestation [88].

Despite of difference types of placentation, most placental specific genes are conserved between rodents and cattle. For example, there are 1217 genes exclusively expressed in placentae in mammals, 1000 of these genes are present in both mice and cattle [89]. However, the expression pattern and regulatory network of particular genes within placental tissues may vary in different animals because of mutations in transcription binding sites or insertion of cis-regulatory modules [90]. This likely led to the diversity in early lineage determination and placentation among species.

Molecular Regulation of Early Lineage Specification

The adaption of viviparity in placental mammals involves the appearance of the chorioallantois placenta and a short-lived yolk sac [91]. The trophectoderm and primitive endoderm are two early cell lineages that contribute to these extraembryonic tissues. The term “trophoblast” is derived from the Greek term, *trephein* (to feed), [92]. Primitive endoderm give rises to parietal and visceral endoderm in mice [93, 94]. A general scheme of lineage segregation is described in Fig 2-1B.

Specification of Trophoblast Cell Lineage

As discussed previously, the first lineage specification involves the emergence of polarized epithelial cell layer termed trophectoderm. There is a debate about whether cell fate is already decided by the 2- to 4-cell stage in mice, but it is clear that embryo compaction and cell polarity at the 8- to 16-cell stage leads to redistribution of cytoplasm and several key factors that determine the fate of different blastomeres [43, 95-97].

Asymmetric cell division and cell polarity

After cell adhesion is evidence and blastomeres reorganizes into a compact morula, cell quickly polarizes with cell nuclei relocate to basolateral area [98] and the membrane protein Par3, Par6, aPKC move to the apical domain [99-101]. Interestingly, polarity protein Par1 becomes localized to the basolateral region of a blastomere [101]. As in other epithelial or polarized cells, the functions of these polarity proteins have been implicated in the formation of tight junctions and undergoing asymmetric cell divisions [102, 103]. In preimplantation embryo, cell polarity is important because it affects cell localization and lineage decision. Down-regulation aPKC in a random blastomere from a 4-cell embryo directs daughter cells of this blastomere into the inside part of the embryo [100, 104]. Once cell polarization is established, outside cells become trophectoderm, while inside cells contribute to the inner cell mass with distinguishable gene expression (Fig 2-1A).

Molecular control of trophectoderm specification

Several key transcriptional regulators dictate trophoblast committal in mammals. In mice, trophectoderm specification is regulated by caudal type homeobox 2 (*CDX2*). *CDX2* mRNA localizes at the apical region of 8-cell blastomeres, and as asymmetrical

cleavage occurs, the progeny containing *CDX2* is destined to become trophoblast cells whereas the inner-dividing cells do not [43]. *CDX2* is not required for cell polarity [105, 106] or E-cadherin mediated cell adhesion events, indicating that E-cadherin directs blastomere allocation independent of early lineage specification [105]. However, *CDX2* is essential for blastocyst survival. Mouse embryos lacking *CDX2* form blastocysts but fail to implant. The epithelial features of trophoblast are lost in these *CDX2*-null embryos. Moreover, *CDX2*-null blastomeres continue to produce pluripotent genes, including *OCT4* and *NANOG*, [107]. Forced expression of *CDX2* will induce pluripotent cells to become trophoblast cells [108] .

Upstream mediators of *CDX2* have been partially identified in the mouse. Two independent groups have established a role for *TEAD4*, a transcription factor from TEA domain family. *TEAD4* null embryos are preimplantation lethal, do not form blastocysts [109, 110] and up-regulate *CDX2* expression. The regulation of *TEAD4* expression is controlled by the transcription factor Yes-associated protein 1 (YAP) [111]. *YAP*, a *TEAD4* coactivator, is phosphorylated YAP and actively transported out of the nuclei of blastomeres within the inner regions of embryos. By contrast, YAP is maintained within the nucleus of cells found along the outer region of embryos, consistent with the concept that these cells will produce *CDX2* in response to *TEAD4* stimulation and become committed to trophectoderm. The kinase responsible for YAP phosphorylation may be Hippo pathway [111-113].

The GATA family transcription factor, *GATA3*, is controlled by TEAD4 and plays a role in trophectoderm lineage segregation [114]. In the preimplantation mouse embryo, *GATA3* is first expressed at the 4-cell stage and its expression continues in cells from

trophectoderm lineage. Deletion of *GATA3* in trophoblast stem cell leads to reduced levels of *CDX2* transcripts [115]. *GATA3* acts independently of *CDX2* and controls its own sets of genes in trophoblast stem cells [114]. ERK2 also plays a role in trophectoderm specification. ERK2 becomes localized in the apical membrane of 8-cell embryo before embryo compaction and is distributed into daughter cells with different concentrations. For example, cells located outside receive higher dose of ERK2 and eventually allocate to trophectoderm. Activation of the upstream signal Ras-MAPK promotes trophectoderm formation in mouse blastocysts [116]. It is unclear whether ERK2 controls *GATA3* and *CDX2* expression.

CDX2 is detectable in bovine blastocysts and the elongating conceptus, but the essential role of trophoblast lineage specifies in cattle has not been examined using loss of function experiments. Retrospective studies suggest that *CDX2* expression is lower in blastocysts that produced a failed pregnancy following transfer to synchronized hosts [117, 118]. Further experiments are needed to explore the mechanisms that drive trophoblast differentiation and other extra-embryonic lineage formation in cattle.

Trophoblast cell differentiation

Gene targeting experiments have generated a long list of genes that are critical for trophoblast cell function, although they are not necessarily needed for the first lineage specification. Most of these genes maintain the proliferation of a trophoblast stem population or induce differentiation. In rodents, trophoblast stem cells differentiate into different cell types including trophoblast giant cells, spongiotrophoblast, and syncytiotrophoblast cells [119].

After trophectoderm specification occurs, this tissue layer from a quiescent state into a rapid proliferation state. ICM-derived FGF4 maintains the polar trophoblast cells,

those cells in close proximity to the inner cell mass in a proliferative, undifferentiated state because of begin acted upon by [27]. Genetic ablation of FGF4 or its receptor, FGFR2, results in proliferation defects and loss of proliferative trophoblast cells [120]. Similarly, trophoblast cells readily differentiate in culture once separated from the inner cell mass, and this outcome can be blocked by supplementing FGF4. Deprivation of FGF4 induces trophoblast stem cell differentiation to trophoblast giant cells. It is proposed that cell cycle regulator CDK1 is required for FGF4-deprived trophoblast stem cell differentiation [121].

Trophoblast differentiation is regulated, in part by basic helix loop helix (bHLH) transcription factors in mice. Several of these factors can override FGF4 to induce differentiation in cultured trophoblast cells. *HAND1* over-expression promotes giant cell formation in FGF4-treated trophoblast cells while *GCM1* forces cell cycle exit and directs trophoblast stem cell towards forming syncytiotrophoblast cells [119]. *MASH2*, an inhibitor of *HAND1*, promotes trophoblast proliferation in the absence of FGF4. There also appears to be other mechanisms that control trophoblast differentiation in rodents. As an example, retinoic acid promotes giant cell formation in mouse trophoblast stem cells colonies [122].

The involvement of these systems in regulating bovine trophoblast differentiation is less clear. In cattle and other ruminants, a differentiated cell known as the binucleate cells (BNCs, also called the trophoblast giant cell) can be detected beginning on day 16-17 of gestation [123]. These cells serve as hormone-secreting cells and control angiogenesis [124]. Several of the major transcription factors that direct trophoblast giant cell formation in rodents (*HAND1*, *MASH2*, *ID2*) also are abundant in bovine BNCs

[62, 125], but the precise functions of these factors in bovine trophoblast development still needs clarification.

Molecular Regulation of Primitive Endoderm Development

Several key transcription factors govern the differentiation of pluripotent cells in the inner cell mass. In general, transcription factors *OCT4* and *NANOG* controls the maintenance of pluripotent epiblast cells, *GATA4* and *GATA6* plays a central role in directing the primitive endoderm lineages. Direct or indirect interactions of these transcription factors determine the fate of early lineages.

Pluripotent stem cell self-renewal

The first segregated inner cell mass cells can give rise to all tissues in fetus and , therefore, often are called pluripotent cells. Pluripotency requires the transcription factors *OCT4* and *NANOG*. Other transcription factors, notably *SOX2* and *c-MYC* are involved with *NANOG* and control over maintenance of epiblast self-renewal [126].

The POU transcription factor *OCT4* is the first transcription factor found to dictate the formation of the inner cell mass. Without *OCT4*, embryo fails to form a pluripotent inner cell mass; instead, these inside cells express markers for trophectoderm [127]. Trophoblast cells from *OCT4*- null embryos cannot be maintained in vivo because they lack FGF4 production. The scrutiny of *OCT4* responsive genes in early embryo reveals that *OCT4* and *CDX2* mutually inhibit each other. Dominant expression of *OCT4* in the inner cell mass turns off trophectoderm specific genes [108]. *OCT4* expression is evident in all blastomeres at late morula stage and it becomes restricted to the inner cell mass when a blastocyst forms. In embryonic stem (ES) cells, *OCT4* acts in a dose dependent manner to control cell fate [128]. Repression of *OCT4* results in trophoblast

cell differentiation whereas, over-expression *OCT4* by 2-fold induces endoderm and mesoderm differentiation.

NANOG is specifically expressed in undifferentiated cells in early blastocysts and it is required for the maintenance of pluripotency in mice and likely other mammals. Similar to *OCT4*, *NANOG* is specifically expressed in the inner cell mass, later in the epiblast [126]. At the late blastocyst stage in mice, *NANOG* expression is restricted to a population of undifferentiated cells that likely are the progeny for the epiblast. Deletion of *NANOG* results in embryonic lethal. Although *NANOG*-null embryos develop into morphologically normal blastocysts that induce decidualization, these embryos fail to form a proper epiblast and fail soon after implantation is initiated. It was originally thought that down-regulation of *NANOG* resulted in primitive endoderm development in mice blastocysts [129]. A recent report reveals that *NANOG* knockout embryos lose the potential to self-renew and differentiate into endoderm [130].

Attempts have been made by generations of animal scientists to generate stem cells in large animals; however, lines that have a convincing resemblance to mouse stem cell lines are still not available [131]. Part of the reasons is that basic knowledge about epiblast development and differentiation is missing. For example, *OCT4* is present in trophoblast cells in elongating bovine conceptus, suggesting that regulatory network for pluripotency may be different [62, 132].

Primitive endoderm specification

Similar to other early lineages, the derivation and differentiation of primitive endoderm is also controlled by several key transcription factors. At the blastocyst stage in mice, a population of the inner cell mass loses its pluripotency and begins expressing *GATA6* and *GATA4*. At this stage, *NANOG* positive cells and *GATA6* positive cells co-

exist in blastocysts and exhibit a "Salt and Pepper" staining pattern when using dual immunofluorescence labeling [26]. These two populations of cells are already pre-patterned and cannot switch their fates no matter what kind of niche is provided.

The zinc-finger containing transcription factors *GATA4* and *GATA6* are master regulators of primitive endoderm development. In early mouse embryos, *GATA6* and *GATA4* expression is restricted to the primitive endoderm and its derivatives, visceral endoderm and parietal endoderm [133]. *GATA4*-knockout embryos produce a post-implantation lethal phenotype, and embryos contain defects in endoderm development [134]. Similarly, *GATA6*-null embryos die at day 5.5 due to the absence of a functional extraembryonic endoderm [135]. Ectopic expression of *GATA6* and *GATA4* in ES cells is sufficient to direct ES cells to differentiate into primitive endoderm [136, 137]. Mouse ES cells contain a subset of cells that are negative for *NANOG* but positive for *GATA6*. Forced expression of *NANOG* can repress *GATA6* and increase homogeneity of ES cells [138]. Also, *GATA4* or *GATA6* null ES cells cannot form primitive endoderm in vitro [139]. Although both are essential, *GATA4* and *GATA6* have distinctive roles during primitive endoderm development. *GATA4* is required for cell arrangement and positioning while *GATA*6 primarily functions as a sensor to receive extracellular signals, such as retinoic acid [139].

An FGF signal may play an important role in establishing primitive endoderm in the mouse blastocyst and ES cells. Embryos lacking FGFR2 cannot form a functional primitive endoderm [140]. Data from ES cells suggested that an FGF4-dependent signal induces primitive endoderm formation *in vitro* by repressing *NANOG* expression [141, 142]. This certainly appears to be the case in mouse blastocysts. Manipulation of

FGF signaling either by providing blastocysts with high concentrations of recombinant FGF4 or inhibiting its key downstream signaling (e.g. Grb2 or ERK-MAPK) can alter cell fates of inner cell mass cells. For example, FGF4 promotes primitive endoderm formation while inhibition of the FGF-dependent signal increases the population of epiblast and enhances the ground state of pluripotency [26, 142-144].

Primitive endoderm development is a complicated process involves cell movement, apoptosis and proliferation. Additional growth factor signaling is required to control the fate of primitive endoderm segregation and differentiation. Platelet-derived growth factor receptor alpha (PDGFRa) has been identified exclusively in primitive endoderm lineages in blastocysts, and PDGF signaling controls proliferation of primitive endoderm cells [104, 145].

Taken together, *NANOG* and potential other pluripotent factors repress the activation of *GATA6*, *GATA4* and other transcriptional regulators of differentiation, therefore maintaining 'stemness' of epiblast cells [146]. In contrast, two of GATA family transcription factors drive the appearance and maintenance of primitive endoderm lineages in blastocyst or in ES cell.

Primitive endoderm development in ruminant

In rodents, primitive endoderm gives rise to parietal endoderm and visceral endoderm, two morphologically different lineages that form yolk sac and play important role in regulation of embryonic patterning formation [147]. In rodents, these two layers form the egg cylinder, which provides structural support for placentation and secretes factors that regulate epiblast differentiation [93].

In cattle and other ruminants studied, primitive endoderm formation begins after hatching and a completed layer can be identified in the early elongating conceptus. The

formation of a yolk sac is marked by the development of a visceral endoderm layer, which occurs around day 16 post fertilization [148]. The yolk sac is ventral to the embryonic disc and covered by trophoblast cells in the filamentous conceptus. When gastrulation occurs, nascent mesoderm cells are adjacent to visceral endoderm and form vascular structures [149]. The functions of the yolk sac include facilitation of nutrient transport between the trophectoderm and mesoderm vascular network and production of several proteins, including alpha-fetoprotein, which directs early blood cell formation [148]. The yolk sac begins to be degenerated when placentation occurs and a chorioallantosis takes over nutrient transportation [149].

Primitive endoderm-like cells can maintain similar cellular morphology for extended periods *in vitro*. Bovine blastocysts can form a primitive endoderm layer in an extended culture system containing feeder layer-conditioned medium [150-152]. However, the lineage markers for primitive endoderm and the cues that direct primitive endoderm development and survival in bovine remain largely unknown. One study suggests that *GATA6* exists in bovine blastocysts [153]. The molecular basis of this part of development biology is important because problems in primitive endoderm development and yolk sac formation likely represent one type of developmental miscues that contribute to pregnancy failures in cattle. .

Since genetic manipulation of embryo is difficult to achieve in domestic animals, much of our current knowledge about placental abnormalities in ruminant has been completed by examining placental mutations. A high degree of placental defects exist in bovine embryos derived from somatic cell nuclear transfer (SCNT) [154, 155]. SCNT blastocysts have aberrant FGFR expression, indicating potential lineage segregation

problems [12]. Indeed, pregnancy loss frequently occurs in these embryos, and this may be caused by impaired trophoblast differentiation and yolk sac formation [156-158].

In summary, the yolk sac plays an indispensable role in facilitating nutrient exchange and regulating embryo differentiation. Key transcription factors mainly *CDX2*, *NANOG* and *GATA4/GATA6* controls the early lineage segregation of trophoblast, epiblast and primitive endoderm. Although the role of these factors and some of their upstream regulators have been elucidated in rodent models, such invaluable knowledge is missing in cattle and other ruminant species.

Control of Conceptus Development and Interferon tau Expression

Maternal recognition of pregnancy refers to the biological pathways used by the conceptus to modify the maternal reproductive system so pregnancy can be maintained beyond the length of a normal estrous cycle. In various species where this phenomenon has been studied, these signals also induce uterine receptivity of pregnancy, specifically by increasing uterine blood supply, modifying the maternal immune system. Progesterone from corpus luteum is the master regulator of these events [159]. In ruminant species, elongating conceptus produce a hormone that prevents corpus luteum regression, thus maintaining a uterine environment for the conceptus to continue to develop.

Maternal Recognition of Pregnancy in Ruminants

A groundbreaking finding in peri-implantation embryo biology in ruminants was the identification of interferon tau (*IFNT*) as the maternal recognition of pregnancy factor. This factor initially was named trophoblastin, protein-X and trophoblast protein-1 [160]. In the 1960s, embryo transfer and removal experiments revealed a phenomenon that trophoblast cells from elongating conceptus but not from mature placenta produce a

compound to extend the lifespan of the corpus luteum [161, 162]. In 1982 this secreted protein released by the peri-implantation ovine conceptus was identified at the University of Florida [163]. The existence of bovine trophoblast protein-1 was confirmed soon after [164]. Trophoblast protein-1 shows high homology with type I interferon (IFN), 65% of its amino acids are identical to interferon alpha [165, 166], and functionally, *IFNT* possesses antiviral, anti-proliferation activities like other type I IFNs. Because of these similarities, this protein is now referred to as trophoblast interferon, or interferon-tau [15].

The primary function of *IFNT* is to prevent corpus luteum regression. However, recent data has uncovered additional functions of *IFNT* in the female reproductive system. *IFNT* is specifically expressed in trophoblast cells and current information suggest that growth factors and cytokines secreted by the uterine endometrium play a critical role in promoting transient *IFNT* production during conceptus elongation.

The function of interferon tau during early pregnancy

IFNT is the major protein secreted by mononuclear trophoblast cells in elongating conceptuses. In ruminants, the structural and functional regression of the CL, an event known as luteolysis, is mediated by the pulsatile release of prostaglandin F2α (PGF2α) [167]. PGF2α is synthesized in luminal and glandular epithelium and transferred to ovaries through countercurrent exchange in response to pituitary- and luteal-derived oxytocin (OXY) [168]. In pregnant animals, conceptus production of *IFNT* prevents this OXY-induced release of PGF2α and thus protects the CL from regression [169].

IFNT achieves its effects on the uterus by interacting with specific receptors found on the endometrial epithelium. Binding of *IFNT* to these type I interferon receptors (IFNR), which also react with many other Type I IFNs, triggers the JAK/STAT signaling

pathway, which modifies the expression level of numerous genes, and specifically those known as interferon stimulated genes (ISGs) [170]. In sheep, the presence of a conceptus or intrauterine injection of recombinant *IFNT* also prevents the transcription of estrogen receptor and oxytocin receptor, key components of PGF2α synthesis machinery [171, 172]. In bovine endometrium, the effect of *IFNT* depends not only on inhibition of PGF2α release by endometrium but also on promotion of PGE2, a luteoprotective compound [173].

Recent advances in large scale transcriptome analysis led to the discovery of many novel *IFNT* responsive genes in the ruminant endometrium and have suggested that various additional activities of *IFNT* likely exist during early pregnancy. Specifically, *IFNT* directs the transcription of several genes associated with angiogenesis, hypoxia and adhesion [174, 175]. For instance, *IFNT* induced expression of *WNT7A* functions as a mediator of trophectoderm proliferation and potentially promotes binucleate cell formation [176]. Importantly, *IFNT* facilitates the nutrient input to the conceptus from uterine endometrium by selectively increasing the expression of glycogenesis enzymes as well as glucose and amino acids transporters like *SLC2A* and *SLC5A11* [177, 178].

Additional mechanisms independent of this paracrine effect have been proposed. During pregnancy, the *IFNT*-mediated antiviral activity of uterine vein blood increases dramatically. Infusion of recombinant *IFNT* in uterine vein of nonpregnant sheep induces interferon stimulated genes and delays corpus luteum regression. These data support the idea that *IFNT* is released into uterine vein and acts in an endocrine manner to modulate the ovarian expression of ISGs to prevent luteolysis [179, 180].

In summary, *IFNT* plays an indispensable role in mediating conceptus and maternal interactions early in pregnancy. *IFNT* directly and indirectly modulates genes required for prevention of corpus luteum regression in ruminants. Also, it is important to note that *IFNT* regulates key transcripts that, in turn, promote conceptus development and implantation.

Transcriptional regulation of interferon tau production

The expression pattern of *IFNT* is unique in several aspects. First, *IFNT* transcripts are first detected in the trophectoderm of blastocysts and are evident in peri-attachment trophoblast cells but not in other fetal tissues [181]. Second, *IFNT* demonstrates a dynamic expression pattern. Conceptus secretion of *IFNT* is maximal on d 16-17 when elongation occurs. *IFNT* secretion ceases when placentation takes place around day 21 [182]. Lastly, *IFNT* genes are not induced by exposure to viruses or other pathogens like most other IFNs. Indeed, the *IFNT* promoter lacks functional viral responsive elements [183]. Instead, the 5' flank region is unique among IFN genes, and provides *IFNT* with the specific ability to be expressed early during early embryonic development in ruminants [2, 51, 54]. *IFNT* is only present in ruminants and there is no clear homolog in mice or human.

The mechanisms that govern *IFNT* expression have been partially elucidated. The first discovered transcription factor essential for *IFNT* expression was *ETS2. ETS2* is specifically expressed by trophoblast cells during early development. In mice, elimination of *ETS2* activity results in defects in extraembryonic tissue and failure of ectoplacental cone development [184]. Further analysis revealed that *ETS2* function is essential to maintain the trophoblast stem cell population in mice [185]. *ETS2* is also a trophoblast-specific transcript in the elongated bovine conceptus [62]. Bovine *IFNT*

genes contain a conserved Ets binding domain, which allows *ETS2* to enhance *IFNT* transcription [186]. EST2 is essential for *IFNT* expression because deletion of *ETS2* binding motif eliminates *IFNT* promoter activity in vitro. Interestingly, some *IFNT* gene isoforms that lack the *ETS2* binding site show minimal expression and replacement of this region with a functional promoter sequence can rescue those genes [187].

Another transcription factor known to interact with the *IFNT* promoter is AP1 (c-fos and c-Jun). Although the exact binding site for AP1 remains controversial, ectopic expression of c-fos and c-Jun can enhance *IFNT* transcription [188]. In the ovine embryo, c-fos and c-jun protein accumulates in trophoblast cells and the expression pattern of these transcription factor subunits is coincident with *IFNT* expression [189].

Not surprisingly, transcription factors essential for trophoblast lineage specification play an important role in regulating *IFNT* production. In ovine trophoblast cells, *CDX2* binds to the *IFNT* promoter region and enhances *IFNT* transcription [190]. Usually the transcription of *IFNT* only takes place in trophoblast cells; however, endogenous *IFNT* can be induced in MDBK cells by over-expression of *CDX2*, suggesting that *CDX2* is one of the core drivers that control *IFNT* transcription [191]. Interestingly, *GATA3* also appears to be a key player in *IFNT* transcription. Over-expression of a *GATA3* construct alone does not increases *IFNT* production, but co-transfection with *ETS2* and *CDX2* synergistically enhances *IFNT* promoter activity and mRNA abundance in bovine trophoblast cells [192]. Another trophoblast-specifying factor, *DLX3*, regulates *IFNT* expression. *DLX3*, a transcription factor essential for trophoblast differentiation and labyrinthine layer formation in mice has been recently identified to control *IFNT*

transcription in bovine trophoblast cells [193, 194]. The putative binding region for *DLX3* in bovine *IFNT* gene is overlapped by the *ETS2* site.

In contrast to transcription factors permissive for *IFNT* expression in trophoblast cells, *OCT4* negatively regulates *IFNT* gene transcription. *OCT4* is detectable in bovine trophectoderm at the blastocyst stage and trophoblast expression of *OCT4* continues in spherical and ovoid conceptuses [62, 195]. Ectopic expression of *OCT4* inhibits *ETS2*-mediated increase in *IFNT* promoter activity but it does not bind to the DNA directly. *OCT4* forms a complex with *ETS2* through binding of the POU domain of *OCT4* to a site adjacent to *ETS2* DNA binding domain [196]. These observations indicate that one of the reasons that *OCT4* remains in trophoblast cells is to sequester *ETS2*-induced *IFNT* transcription for a few days until the conceptus begins to elongate.

Signaling pathways controlling interferon tau production

Several extracellular regulated signaling systems have been linked with regulating *IFNT* expression. Most signaling work in this area have been completed in two human carcinoma cell lines, JEG or JAR cells, or in the mouse NIH3T3 cell line. Ras-MAPK is likely the key regulator of *IFNT* gene transcription. Treatment of NIH3T3 cells with CSF2 quickly stimulates ERK1/2 phosphorylation, and these kinase cascades mediate CSF2-induced increases in *IFNT* promoter activity [197]. The potential targets for ERK1/2 in trophoblast likely include *CDX2* and *ETS2*. In mice trophoblast stem cells, FGF4 directly activate ERK1/2 to enhance *CDX2* expression [198]. Whether MAPK-dependent *CDX2* directly involves in *IFNT* regulation remains unsolved. Also, ERK1/2 phosphorylates the Thr72 site on *ETS2*, and thereby dramatically increases this factor's activity to stimulate *IFNT* transcription [197].

PKA is involved with mediating *IFNT* promoter activity. *ETS2* is not a direct target for PKA but it apparently synergizes with PKA. In CT1 cells, cAMP response element-binding protein-binding protein (CBP)/p300, the known substrate for PKA, binds to the proximal promoter region of *IFNT* gene [199].

Treatment of day 16 ovine conceptuses with phorbol ester, PMA significantly increased *IFNT* mRNA abundance, suggesting that a PKC-dependent pathway is involved with regulating *IFNT* transcription [200]. Eleven PKC family are categorized into three subgroups of protein kinases based on structural similarities and conserved responsive elements [201]. PKC members are present in early mice trophectoderm and several appear important for controlling embryo compaction and blastocoel formation [202, 203]. The PKC isoform involved with embryo development or *IFNT* gene expression remained discovered until recently, and this will be described in more detail in chapter 3.

Regulation of Pre- and Peri-development by Growth Factors

In cattle, the independence of preimplantation development *in vitro* is not limitless, but rather it is clear that current culture conditions cannot fully support conceptus development beyond the blastocyst stage. It was originally thought that the structural support by uterus is the key mediator for the continuation of embryo development *in vitro*. This is partially true because limited trophoblast elongation can be induced when hatched blastocysts were placed in an agarose gel tunnel filled with growth medium containing serum and glucose [60, 151]. However, a normal, healthy looking primitive streak could not be observed in these elongating embryos. These experiments suggest that uterine secretions are needed for epiblast differentiation and normal conceptus elongation.

Further support for the contention that uterine-derived factors are required for pre-implantation conceptus development is observed by using uterine gland knockout models [204]. In ewes, exposure to neonatal progesterone alters uterine morphology and impairs the uterine glands development [205, 206]. Morphological normal blastocysts can be recovered in these animals, but pregnancy cannot be established mainly because of severe defects in the process of conceptus elongation. Several key factors that regulate conceptus development have been identified using this model. To follow is an overview of uterine and conceptus factors that regulate various aspects of pre- and peri-implantation conceptus development.

Factors shown beneficial effects on preimplantation development

Growth factors help embryo development *in vitro* or *in vivo* through different ways. For example, some factors are known for their mitogenic effects on trophoblast cells while others are involved in preventing cell death or controlling trophoblast migration. Numerous growth factors and cytokines have been investigated in different in vitro systems for these and other activities.

The insulin growth factor (IGF) family promotes bovine embryo development in vitro. IGF system is a complex family including two ligands IGF1and IGF2, binding proteins (IGFBP1-6) and type I receptor. Supplementation of in vitro produced bovine embryos with IGF1 increases the blastocyst rates and total cell numbers of blastocysts [207]. This action is mediated by increasing the number of cells committed to trophectoderm lineage and reducing cell apoptosis [208]. IGF1 also protects embryos from stress. IGF1 increased thermotolerance of embryos *in vitro* [209]. and promoted the survival rates after transfer to cows during summer months [20].

Another uterine-derived factor, CSF2, which initially was described as a cytokine has also been shown pivotal for embryo implantation and placental formation [210]. Bovine embryos exposed to CSF2 from day 5 to 7 post fertilization had improved developmental competency as recorded by higher pregnancy rates and lower pregnancy loss after embryo transfer [22]. Further data suggested that this factor modulates early germ layer differentiation and likely provides an important survival signal to the inner cell mass.

Other factors that promote preimplantation development include members of the EGF family of proteins [208] as well as TGF-beta1, LIF and FGF2 [211]. These growth factors accelerate embryo development alone or show a synergistic effect when providing embryos with the combination of two or three factors [211, 212]. Providing in vitro produced embryos with various growth factors that are present in either oviduct fluid or uterus lumen activate key molecular pathways that are important for embryo development [213].

Regulation of trophoblast cell migration

The rapid growth and remodeling events that relate to conceptus elongation are not fully understood, but epithelial cell migration is central to development and tissue remodeling in various tissues and undoubtedly is involved with controlling elongation in ruminant conceptuses. Directional migration is controlled by various extracellular molecules in other cell systems [214]. Changes in extracellular environment such as the gradient of growth factors and cytokines or ECM alter actin cytoskeleton dynamics and cell adhesion. Cells express various adhesion receptors; however, integrins are vitally important transmembrane receptors that sense and integrate ECM and cytoskeleton signals during epithelial cell migration in various cells, including trophoblast

cells [215, 216]. For example, disruption of an integrin-actin linkage compromises cell adhesion and migration [217]. In ruminants, integrin systems are also actively involved in modulating the migratory ability of trophoblast cells.

Several uterine factors have been linked with controlling trophoblast migration. One of the best known examples of this is the effects noted for galectin 15 (LGALS15). This protein is synthesized by uterine epithelial cells and is released into the uterus lumen where it interacts with trophoblast cells. LGALS15 directly promotes the assembly of integrin-actin complexes and stimulates ovine trophoblast cell migration [218]. Another factor, SPP1 (also known as osteopontin), is a common binding partner for several integrins, and its presence also mediates the cell migration [219]. Integrin and ECM dynamics also potentially mediate the migration of bovine trophoblast giant cells in bovine placenta because a descriptive study demonstrated those bovine trophoblast giant cells were immunoreactive for integrin subunits (alpha 6, alpha 2 and beta 1) and laminin, the major protein of basal membrane at the maternal-fetal interface [220]. Although a series of experiments were carried out to examine the expression profiling of integrins in bovine blastocysts and elongating conceptus [221, 222], because of the complexity of integrin family, the major integrin subunits responsible for post-hatching development and trophoblast cell migration is inconclusive.

The IGF and their binding proteins increase trophoblast migration in vitro. IGF2 and its binding partner IGFBP1 are both present in uterus lumen, and treatment of ovine trophoblast cells with recombinant IGF2 or IGFBP1 increases trophoblast cell migration in vitro [223]. Interestingly, IGFBP-1 concentrations are elevated during conceptus elongation in cattle, suggestive of its potential role in mediating elongation in utero. The

signaling systems involved with this activity also have been examined. Blocking ERK1/2 or p38 MAPK phosphorylation prevents IGF2 induced cell migration, suggesting that ERK1/2 and p38 MAPK mediated systems control trophoblast cell migration [23]. Immunohistochemistry work revealed that ERK1/2 and p38 MAPK are activated in trophoblast cells during elongation [223]. Further studies that attempt to uncover the additional underlying mechanisms controlling trophoblast cell migration will be invaluable because this kind of knowledge will enhance our understanding of conceptus elongation and potentially will provides additional clues of how normal conceptus development progresses in ruminants.

Uterine derived factors stimulate *IFNT* production

Another well studied but far from complete area of research is describing how the uterus mediates *IFNT* production. We hypothesize that optimal production of *IFNT* requires specific uterine secreted factors. The bases for this argument can be found in several studies. Firstly, co-culturing bovine blastocysts with uterine flushing from cyclic animals in the late luteal phase increases *IFNT* production [224]. Furthermore, treatment of bovine primary trophoblast outgrowths or isolated trophoblast cell lines with specific growth factors or cytokines found in the uterine lumen enhances *IFNT* mRNA and protein abundance [197]. Also, conceptus growth and *IFNT* secretion is compromised in conceptuses exposed to a uterus devoid of uterine glands, the primary producers of uterine histotrophic agents [204, 225]. Lastly, several extracellular-regulated signals described previously in this literature review activate signaling factors that control the *IFNT* transcription. For example, PKC and ERK dependent systems are controlled by CSF2 to increase *IFNT* production in ovine trophectoderm and human choriocarcinoma cells transected with *IFNT* promoter reporter constructs [197, 226].

The fact that other growth factors and cytokines stimulate *IFNT* promoter activity or *IFNT* production by trophoblast cells indicates that more than a signal signaling mechanism is involved. A good example is that the day 13 ovine whole conceptus is cultured in serum free medium. In this embryo, neither IGF1 or IGF2 alone is able to induced *IFNT* production but the combination of those two factors significantly enhances *IFNT* secretion [227]. CSF2 also promotes *IFNT* production by primary trophoblast cells from elongated conceptuses or isolated trophoblast cell lines [21, 226]. The cellular mechanisms that mediate CSF2 induced *IFNT* expression is under discussion because experiments using ovine primary trophoblast cells or investigating *IFNT* promoter activity in a non-trophoblast cell line result in different conclusion. For example, CSF2 induced *IFNT* in ovine trophoblast cells is mediated by a PKC-dependent system, but in a different study, the authors conclude that RAS-MAPK is essential for CSF2-induced *IFNT* promoter activity [197, 200, 226].

Roles for FGFs in mediating conceptus development in ruminants

As described earlier, FGF4 plays a pivotal role in regulating early embryo development in mice. The inner cell mass derived FGF4 binds to its trophectoderm specific receptor FGFR2 to regulate trophoblast stem cell proliferation [27, 152]. Most recently, a new role of FGF signal in promoting primitive endoderm lineage commitment has been described [152]. In bovine, it is not clear whether FGF4 is produced by blastocyst and this factor plays a similar role in controlling trophoblast cell proliferation, however, considerable evidence suggests that FGF family proteins are important regulators of pre- and peri-implantation conceptus development.

FGFs are a family of growth factors that have a wide variety of biology effects including angiogenesis, cell proliferation, migration and maintenance the pluripotency of

human ES cells [228]. The first FGF to be identified in this regards was FGF2 or basic FGF. Specifically, the luminal and glandular epithelium of pregnancy and cyclic cows is positive for FGF2 transcripts, and uterine lumen contains immunoreactive FGF2 protein. The FGFs that present in uterus lumen is not limited to FGF2, FGF10 is synthesized by stromal cells and the amount of FGF10 secreted increases with the rising of progesterone level [32]. It is thought that FGF10 works as a mediator of progesterone during the peri-implantation period to control conceptus growth and development, although the exact function of FGF10 is unclear [32]. Bovine blastocyst and elongation conceptus itself is also a source of FGFs in embryos [28]. FGF2, FGF4, FGF7 and FGF10 transcripts are all present in bovine blastocysts, ovoid and elongating conceptus [12, 29, 30, 229].

Multiple FGF receptors exist on bovine trophoblast cells. Four functional FGF receptors (FGFR1-4) have been identified in mammalian species [230]. FGF receptor contains of an extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain of FGF receptor consists of three immunoglobulin-like domains and the third IgG-like domain of FGFR1-3 is encoded by alternate mRNA splicing and creates two different isoforms (IIIb, IIIc) [231, 232]. Various FGFRs and isoforms show distinct tissue specific expression pattern and provide different recognition sites for FGF ligands. Bovine blastocysts, elongating conceptuses, and trophoblast giant cells all contain FGF receptors including a trophoblast-specific FGFR2b [30, 233]. This receptor subtype also has been identified in ovine conceptuses [32].

The most potent effect of FGFs is to stimulate *IFNT* production by bovine trophoblast cells. Providing bovine trophoblast cells or *in vitro* produced blastocysts with as low as 1 ng/ml FGF2 increases *IFNT* mRNA and protein abundance. Interestingly, the amount of immunoreactive FGF2 in uterus increases days 12-13 after estrus in both cyclic and pregnant ewe, associated with conceptus elongation and maximal production of *IFNT* [29]. It also is worth mentioning that FGF2 has little to no effect on trophoblast cell proliferation, suggesting that intracellular pathways distinct from those used to control mitogenesis are being triggered by FGF2 other than mitogenic mechanism [28, 31].

In conclusion, several lines of evidences support the model that emphasizes the significant role that growth factors play in regulating conceptus development and *IFNT* production, however, further experiments are absolutely required to provide critical information about how these growth factors. It is of particular important to understand how FGF2 regulates *IFNT* expression in bovine trophoblast cells and whether this relationship is necessary for maternal recognition of pregnancy in ruminants. Also special attention should be paid to uncover the other actions of FGF signaling during pre- and peri-implantation development in cattle and other economically important ruminant species.

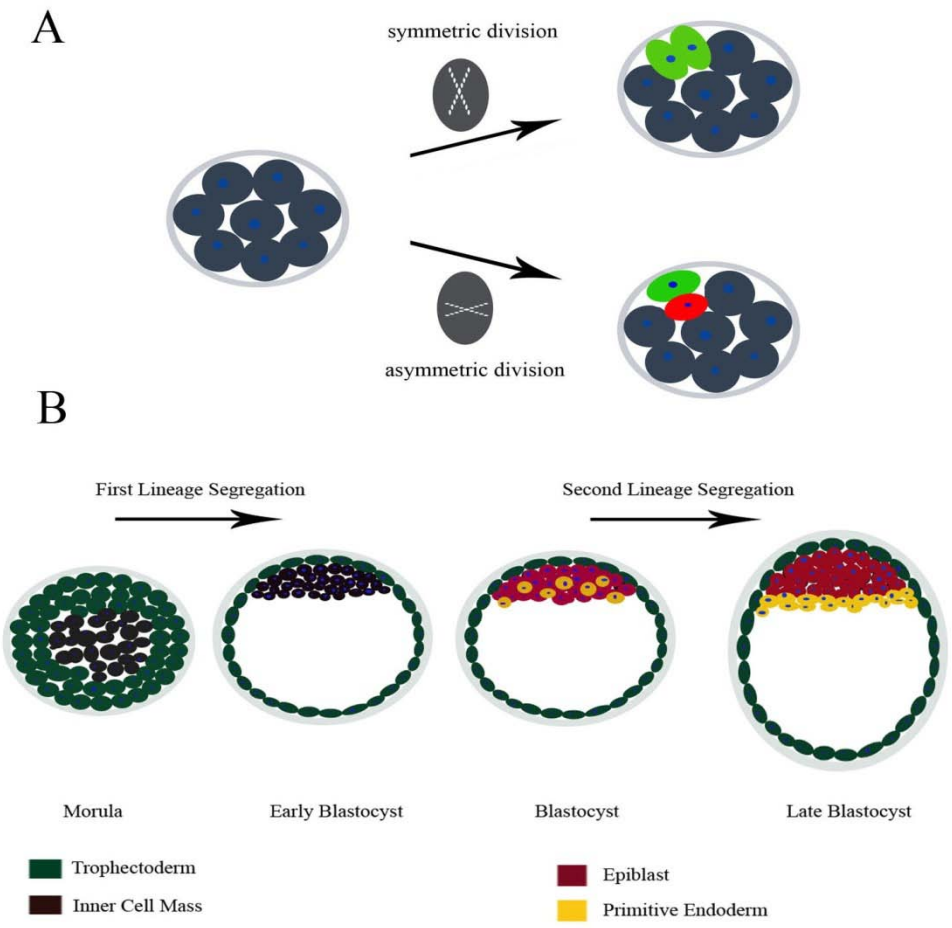

Figure 2-1. Early lineage segregation during preimplantation development. At the late 8-cell stage, after compaction blastomere undergoes two types of cell division, symmetric and asymmetric cell division. A blastomere with asymmetric division gives to one outside cell and another inside cell. The cell located outside the embryo eventually forms trophectoderm, while inside blastomeres contribute to inner cell mass. In the new formed blastocyst, cell with the inner cell mass start to differentiate to epiblast and primitive endoderm.

CHAPTER 3
PROTEIN KINASE C DELTA MEDIATES FIBROLBAST GROWTH FACTOR-2-INDUCED INTERFERON TAU EXPRESSION IN BOVINE TROPHOBLAST

Interferon tau (*IFNT*) is the trophectoderm-secreted factor responsible for establishing and maintaining early pregnancy in cattle, sheep, goats, deer and likely other ruminants [181, 234]. *IFNT* is best known as an antiluteolytic agent; it prevents luteolysis by limiting the pulsatile release of endometrial prostaglandin (PG) F2α production [181, 234]. *IFNT* also stimulates endometrial PGE2 synthesis, a putative luteotrophic agent [173, 235]. More recently *IFNT* has been linked to additional activities, such as inducing uterine factors that promote conceptus development [174, 236, 237], increasing endometrial glucose and amino acid transporter expression [177, 238], regulating uterine and systemic immune responses to pregnancy [239, 240], and impacting gene expression in the corpus luteum (CL) [180, 241]. It is not surprising that miscues in *IFNT* production and action are linked with pregnancy failures in cattle [5, 242, 243].

Dynamic changes in *IFNT* production occur as the conceptus develops and elongates prior to uterine attachment. Bovine embryos begin producing *IFNT* at the morula and blastocyst stages coincident with trophoblast lineage specification [244, 245]. The relative abundance of *IFNT* transcripts and protein increases dramatically at days (d) 14-15 of pregnancy as conceptus elongation begins [246, 247]. Expression decreases abruptly as trophectoderm attaches to the uterine epithelium on or after d 21 of pregnancy [246, 248]. Exposure to viruses or other pathogens do not impact *IFNT* expression as they do for most other Type I IFNs [249, 250]. Instead, *IFNT* transcription is controlled developmentally by at least two trophoblast-specifying transcription factors

(*CDX2* and *DLX3*) and by *ETS2*, a member of a family of transcriptional regulators involved with various cellular activities [186, 190, 194, 197].

Over the past several years it has become clear that several fibroblast growth factors (FGFs) regulate *IFNT* production in bovine trophoblast cells and blastocysts [28, 30, 31]. Multiple FGFs exist in mammals, and most serve as paracrine-acting regulators of proliferation, differentiation, morphogenesis and angiogenesis during embryonic, fetal and post-natal development [251]. Several FGFs are produced by the bovine conceptus and uterus prior to implantation. Most uterine-derived FGFs are produced within the endometrial stroma or smooth muscle layers surrounding local blood vessels [32, 252] and may not reach the uterine lumen to influence conceptus development before implantation. However, FGF2 is produced by luminal and glandular epithelium and released into the uterine lumen throughout early pregnancy in sheep and cattle [28, 29]. Elevated quantities of FGF2 protein are released into the uterine lumen coincident with conceptus elongation and maximal *IFNT* production in sheep [29]. Bovine conceptuses also produce several FGFs, most notably FGF2 and FGF10, prior to implantation [30].

Several FGF receptors (FGFRs) also exist in bovine and ovine conceptuses [29, 30]. Four genes encode tyrosine kinase receptors (FGFR1-4) [251, 253], and transcripts for each FGFR exists in ovine and bovine conceptuses at the blastocyst stage (d 7-8 post-insemination) and thereafter [29, 30]. It is unclear how FGFs regulate *IFNT* production. *ETS2* activity is crucial for *IFNT* transcription, and maximal *ETS2* activity requires tyrosine phosphorylation within its pointed-domain. A Ras-mediated MAPK pathway controls this phosphorylation event [186, 197]. Protein kinases A and C

(PKA and PKC, respectively) also regulate *IFNT* transcription. Stimulating PKA activity with dibutyryl (db) cAMP increases *IFNT* promoter/enhancer activity [199]. Similarly, exposure to phorbol ester, a diacylglycerol (DAG) mimic and PKC activator, increases *IFNT* mRNA concentrations in ovine conceptus explants and stimulates *IFNT* promoter/enhancer activity in various human cell lines [254, 255].

The overall goal of this study was to describe the signaling mechanisms used by FGF2 to regulate *IFNT* production. This report provides evidence that PKC-delta, a member of the novel PKC subfamily, mediates *IFNT* mRNA abundance during early pregnancy in bovine trophectoderm. PKCs are grouped within three subfamilies based on structural similarities and commonality of responses to various stimuli [201]. Classical PKCs (α, βI, βII, γ) are activated by various stimuli, including Ca^{2+}, DAG and phorbol esters whereas novel PKCs (δ, θ, ε, η) are not Ca^{2+} dependent but are responsive to DAG and phorbol esters. The third subfamily, termed atypical PKCs, represents lipid-dependent kinases that are not controlled by Ca^{2+} or phorbol esters. Identifying a role for PKC-delta in bovine trophoblast cells provides new insight into the mechanisms controlling early conceptus development in this species and offers another function for this signal transducer molecule.

Materials and Methods

Materials

Dulbecco's modified essential medium containing high glucose (DMEM), Opti-MEM, fetal bovine serum (FBS), insulin/transferring/selenium solution (ITS), cell culture supplements, PCR primers, siRNAs, Trizol, PureLink Total RNA Purification System, SuperScript III First Stand Synthesis kit, ThermalAce DNA Polymerase, pCR-Blunt TOPO vector and Cy3-labeled control siRNA were purchased from Invitrogen Corp.

(Carlsbad, CA, USA). HiPerFect siRNA transfection reagent was purchased from Qiagen Sciences (MD, USA). Matrigel™ was purchased from BD Biosciences (San Jose, CA, USA). RNase-free DNase was purchased from New England Biolabs (Ipswich, MA). Pharmacological inhibitors for MEK (U0126 and PD98059), p38 MAPK (SB203580), PKCs (calphostin C, Gö6976, rottlerin), phorbol 12-myristate 13-acetate (PMA) and 4α-PMA were purchased from EMD Chemicals (Gibbstown, NJ, USA). The FGFR inhibitor, PD173074 was purchased from Stemgent (Cambridge, MA, USA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The High Capacity cDNA Reverse Transcription kit and SYBR® Green Detector System were purchased from Applied Biosystems Inc. (Foster City, CA, USA). Polyvinylidene Difluoride (PVDF) (Immobilon-P) membrane was purchased from Millipore Co. (Bedford, MA, USA). Enhanced chemiluminescence (ECL) western blot detection system was purchased from Amersham (GE Healthcare, USA). The Cell Titer 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega Corp., (Madison, WI, USA). Recombinant human IFN-α standard was purchased from EMD Biosciences (Gibbstown, NJ, USA). Reagents and materials for superovulation and conceptus collections were purchased from Agtech Inc. (Waukesha, WI). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA).

Trophoblast Cell Culture

The CT1 and Vivot bovine trophoblast cell lines were isolated by Talbot *et. al.* [256, 257]. The CT1 line was derived from an *in vitro*-produced bovine blastocyst whereas the Vivot line was developed from an *in vivo*-derived bovine blastocyst [256, 257]. Each cell line was propagated without a feeder-cell as described previously [28]

on Matrigel-coated plates in DMEM containing 10% [v/v] FBS, 100 µM non-essential amino acids, 55µM β-mercaptoethanol and antibiotic-antimycotic (100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B) at 38.5°C with 5% CO2 in air. Cells were passaged manually by separating them from plates with a cell scraper and dissociating them into small clumps with repeated dissociation through a 20-ga needle. Cells were seeded onto 12- or 24-well plates containing Matrigel. After 3 d, medium was removed and replaced with DMEM lacking FBS but containing all other supplements and ITS (10 µg/ml insulin, 5.5 µg/ml transferrin and 6.7 ng/ml sodium selenium). After 20-22 h, pharmacological inhibitors or vehicle (≤0.01% [v/v] DMSO) was added. After 2 h, medium was replaced and cells were supplemented with 50 ng/ml boFGF2, 100 nM PMA or vehicle (1% w/v BSA) in fresh serum-free medium. 4α-PMA, an inactive phorbol ester, served as a control for PMA in some studies. After 22-24 h exposure to FGF2 or PMA, total cellular (tc) RNA was extracted using Trizol and the PureLink Micro-to-Midi Total RNA Purification System.

Primary bovine trophoblast outgrowths were generated from *in vitro*-produced bovine embryos as described previously with modifications (40). On d 8 post-fertilization, individual expanded blastocysts were placed in Matrigel-coated wells of 48-well plates with DMEM containing 10% FBS, 100 µM non-essential amino acids, 55 µM β-mercaptoethanol and antibiotic-antimycotic. A morphological assessment of was completed on d 15 post-IVF (monolayers of tightly packed cells with prominent nuclei and numerous secretory granules; dome formation were evident in some outgrowths). On d 20 post-fertilization, trophoblast outgrowths were scraped and dissociated manually. Outgrowths were pooled together within each replicate study (n=3 replicates)

and used to seed 12-well plates containing Matrigel. After 4 to 5 d, outgrowths were serum starved and treatments were supplemented as described for CT1 and Vivot cells.

Quantitative Real Time RT-PCR

TcRNA concentration and purity was determined using a NanoDrop Spectrophotometer (Thermo Scientific). Samples (10 ng tcRNA; ≥ 1.8 A_{260}/A_{280} ratio) were incubated with RNase-free DNase for 30 min at 37°C. After heat-inactivation the DNase (75°C for 10 min), RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit and random hexamers. Primers (200 nM) specific for *IFNT, PRKCD* (gene abbreviation for PKC-delta), *PRKCQ* (gene abbreviation for PKC-theta) or *18S* (internal control) (Table 3-1) were used in combination with a SYBR® Green Detector System and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to quantify gene target gene abundance [28, 30]. A dissociation curve analysis (60-95°C) was used to verify the amplification of a single product. Each sample was completed in triplicate reactions. A fourth reaction lacking reverse transcriptase was included to control for genomic DNA contamination. Standard curves generated from serial dilutions of bovine conceptus tcRNA collected on d 17 of pregnancy [30] were used to determine *IFNT* primer efficiency (94%). The comparative threshold cycle (C_T) method was used to quantify mRNA abundance [28]. 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.

Western Blot Analyses

CT1 cells were seeded onto 6-well plates containing Matrigel. After 3 d, medium was removed and replaced with DMEM lacking FBS but containing all other supplements and ITS. After 20-22 h, pharmacological inhibitors or carrier only was

added. After 2 h, medium was removed and replaced with fresh serum-free medium containing 50 ng/ml boFGF2 or 100 nM PMA. Cells were collected either immediately before (time 0) or at several time-points after FGF2 and PMA supplementation. Cells were rinsed in 0.1 M PBS [pH 7.4] and dissolved using NP-40 buffer (20 mM Tris HCl pH 8, 137mM NaCl, 20 mM EDTA, 1% [v/v] NP40) supplemented with protease and phosphatase inhibitor cocktails. Cell lysates were sonicated for 15s and centrifuged (10 min at 10,000 x g). Protein concentrations of supernatants were determined using a BCA Protein Assay.

In one study, CT1 cells were cultured in serum-free medium without ITS to examine whether the inclusion of these factors affected ERK1/2 phosphorylation status. In another study, CT1 cells were treated with 100 nM PD173074, an FGFR kinase inhibitor [258] or its carrier only (0.01% [v/v] DMSO) during serum starvation and FGF2 challenge to examine whether endogenous FGFs impacted ERK1/2 activity. This concentration of inhibitor effectively blocked FGF2 induced increases in *IFNT* mRNA abundance in CT1 cells without altering apoptosis rate (M. Ozawa and A. D. Ealy, Unpublished observations).

Identical amounts of protein (15 to 35 µg depending on the study) were loaded and electrophoresed in 10 or 12% [w/v] SDS-PAGE gels. Samples were electrotransferred onto 0.45µm PVDF using a semi-dry membrane blotter (Amersham Biosciences). Membranes were blocked with 5% [w/v] nonfat dry milk in TBST (50mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% [v/v] Tween-20), then incubated overnight at 4°C with total (pan) or phospho-specific ERK1/2 (T202/Y204) (1:2000), p38 MAPK (1:1000) or PKC delta Y311 (1:1000) in TBST containing 3% [w/v] BSA. Horseradish

peroxidase-conjugated anti-rabbit IgG, ECL and exposure to BioMax film were used to visualize reactive bands. After detection, membranes were washed with TBST and incubated in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris HCl pH 6.8) at 50°C for 30 min with gentle shaking. For ERK and p38-MAPK studies, blots were blocked and used again so that total and phospho-specific bands could be visualized on the same blot. For PKC-delta antiserum studies, blots were blocked and incubated in anti-α-tubulin (1:3000). Three independent Western blots generated from different CT1 cultures were completed for each study. Representative blots were photographed for presentation.

Antiviral Activity

CT1 cells were serum-starved for 24 h and then were treated with 5 μM rottlerin or carrier only (0.01% DMSO) for 2 h. Medium was exchanged and cells were cultured in serum-free medium containing either FGF2 (0 or 50 ng/ml) or PMA (0 or 100 nM), depending on the study. After 48 h, conditioned medium was collected and stored at -80°C. Viable cell number was determined by using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay. Absorbance at 490 nm was recorded using SpectraMax® 340PC384 (Molecular Devices, Sunnyvale, CA, USA).

The amount of biologically active *IFNT* in CT1-conditioned medium was determined by using an antiviral assay as described previously [29]. The ability of conditioned medium samples to inhibit vesicular stomatitis virus-induced death of Madin-Darby bovine kidney cells by 50% was compared with the activity of recombinant human IFNα standard (3.84×10^8 IU antiviral activity per mg protein). The antiviral activity of conditioned medium was adjusted based on this standard to represent the IU

activity/ml of sample. Data also were adjusted to account for potential variations in cell number by normalizing for cell titer readings (A_{490}).

Bovine Conceptus Collection and End-point RT-PCR

All animal studies were performed in accordance with guidelines and with the approval of the Institutional Animal Care and Use at the University of Florida (UF). Conceptuses were harvested on d 14 and 17 post-insemination from non-lactating Holstein cows housed at the UF Dairy Unit (Hague, FL, USA). Conceptuses were either collected nonsurgically by transcervical uterine flushing (d 14) or after cows were slaughtered at the UF Meats Laboratory as described previously [30]. Approximately half of the d 14 conceptuses collected were ovoid. The remaining d 14 conceptuses were elongated and ranged from 0.5 to 3 cm in length. All d 17 conceptuses were filamentous and ranged from 5 to 45 cm in length. From 3 to 5 conceptuses were pooled together and snap-frozen in liquid nitrogen. Each of the d 14 samples contained at least one ovoid and one elongating conceptus. Corpora lutea (CL; n=3) were collected from ovaries of non-superovulated cows at slaughter. All samples were stored at -80°C until RNA isolation using Trizol reagent.

For endpoint RT-PCR analysis, tcRNA (500 ng) from CT1cells, d 14 and 17 conceptuses and CLs were incubated in RNase-free DNase as described previously. Reverse transcription was completed using the SuperScript III First Stand Synthesis kit and random hexamers according to manufacturer's instructions. PCR amplification was completed with primers for *PRKCD*, *PRKCQ* or *ACTB* (Table 3-1) using ThermalAce DNA Polymerase (35 cycles of 95 °C for 1 min, 58-60°C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10 min). Products were electrophoresed in a 1.5% [w/v] agarose gel containing ethidium bromide (100 ng/ml) and visualized under ultraviolet

light. Amplicons were cloned into the pCR-Blunt TOPO vector, and inserts were sequenced to verify correctness of amplification at the UF DNA Sequencing Core Facility.

RNA-interference

Three small, interfering RNA (siRNA) duplexes were used (Table 3-1). siRNA#1 was examined previously in a bovine endothelial cell line [259]. siRNAs#2 and #3 were designed by using the BLOCK-iT RNAi Designer Program (Invitrogen Corp.) based on the *Bos taurus* sequence for *PRKCD* in GenBank (GeneID: 505708). Cy3-labeled control siRNA served as the negative control.

For transfection, Vivot cells were scraped off plates at 30-40% confluence in Opti-MEM and passed through a 22-ga needle 3-5 times before being placed onto Matrigel-coated 24-well plates (100 µl volume/well). A siRNA cocktail containing 25 nM of each siRNA was combined with 4.5 µl HiPerFect siRNA transfection reagent in 100 µl Opti-MEM and incubated at room temperature for 10 min before mixing with Vivot cells. Opti-MEM and siRNA reagents were removed after 9 h and replaced with DMEM containing 10% FBS and other additives. For qRT-PCR studies, cells were serum-starved after 2 d in culture as described previously and exposed to 50 ng/ml FGF2 or vehicle only for 24 h before tcRNA extraction. For Western blot analysis, cells were cultured for 3 d before collecting protein lysates.

Statistical Analyses

All analyses were performed by least-squares ANOVA using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Fold differences of real-time analyses were log-transformed to remove heterogeneity of variances [28, 30]. Differences between individual means were contrasted with pair-

wise analysis (PDIFF [probability of difference] analysis in SAS). Results are presented as arithmetic means ± SEM.

Results

ERK1/2- and p38 MAPK-dependent Regulation of *IFNT* Expression

Ras-mediated pathways regulate *IFNT* transcription [197], and the objective of the first set of studies was to determine if ERK and/or p38-MAPK pathways control constitutive and FGF2-dependent increases in *IFNT* mRNA levels in bovine trophoblast cells. CT1 cells were exposed to pharmacological inhibitors of MEK (10 μM U0126) and p38 MAPK (25 μM SB203580) for 2 h; medium was then changed and replaced with fresh serum-free medium supplemented with 0 or 50 ng/ml FGF2 for 22-24 h. This duration of FGF2 treatment was chosen based on outcomes of preliminary studies. In those studies FGF2 responses could first be realized after 8 to 12 h of FGF2 treatment but were maximal after 24 h exposure to FGF2 (Data not shown).

Constitutive phosphorylation of ERK1/2 was evident in CT1 cells, and addition of FGF2 did not influence ERK1/2 phosphorylation status (Fig. 3-1A). Supplementation with U0126 abolished constitutive ERK1/2 phosphorylation, and no ERK1/2 phosphorylation was observed in the first 2 h after providing FGF2 (Fig. 3-1A). Studies were not completed to examine if ERK1/2 phosphorylation was interrupted for more than 2 h. Supplementation with U0126 had a major impact on the relative abundance of *IFNT* mRNA in CT1 cells (Fig. 3-1B). As seen in previous reports [28, 30, 31], FGF2 increased ($P<0.05$) the relative abundance of *IFNT* mRNA in the absence of MEK inhibitor. Exposure to the MEK inhibitor reduced ($P<0.05$) *IFNT* mRNA levels below that of the control value in cells supplemented with 0 or 50 ng/ml FGF2.

The phosphorylation status of p38-MAPK was affected by FGF2 (Fig. 3-1C). Specifically, increases in phospho-p38-MAPK abundance were detected within 5 min of FGF2 treatment. This effect was transient, and p38-MAPK phosphorylation status returned to baseline levels within 60 min. Exposure to SB203580 prevented FGF2-induced phosphorylation of p38 MAPK (Fig. 3-1C). However, modifying p38-MAPK phosphorylation status did not affect FGF2-induced *IFNT* mRNA concentrations in CT1 cells (Fig. 3-1D). Increases in *IFNT* abundance ($P<0.05$) were evident regardless of whether the p38-MAPK inhibitor was provided. In the absence of FGF2 this inhibitor reduced ($P<0.05$) basal *IFNT* concentrations (Fig. 3-1D).

PKC-dependent Regulation of *IFNT* Expression

A separate set of studies was completed to determine if FGF2 utilized PKC-dependent systems to regulate *IFNT* abundance in bovine trophoblast cells. Initially, a series of pharmacological PKC inhibitors and mimics were examined.

In the first study, supplementing medium with the pan-PKC inhibitor, calphostin C, at a concentration suitable for blocking most PKC isoforms (5 μM) [260] for 2 h prevented FGF2 from increasing *IFNT* abundance (Fig. 3-2A). Constitutive concentrations of *IFNT* were not affected by calphostin C treatment, but FGF2 induction of *IFNT* was blocked ($P<0.05$) in cells exposed to calphostin C.

In a follow-up study, PMA, a phorbol ester commonly used to activate PKC pathway, was used to determine if FGF2 effects in CT1 cells could be replicated by DAG activation (Fig. 3-2B). Supplementation with 100 nM PMA for 22-24 h increased ($P<0.05$) *IFNT* abundance in CT1 cells to the same degree as FGF2 (Fig. 3-3B). Combining FGF2 and PMA did not have additive or synergistic effects on *IFNT* concentrations.

A subsequent set of studies was completed to define the PKC subtype utilized by FGF2 and PMA in CT1 cells. Two pharmacological inhibitors were tested; Gö6976, an inhibitor of classical PKCs (α, β, γ) [261], and rottlerin, an inhibitor of PKC-delta and PKC-theta [262-264] (Fig. 3-3). Pre-treatment with Gö6976 for 2 h at a concentration effective at inhibiting classical PKC isoforms in other cells (5 μM) did not affect basal, FGF2- or PMA-induced *IFNT* levels in CT1 cells (Fig. 3-3A&B). By contrast, FGF2 and PMA responses were inhibited ($P<0.05$) after a 2 h exposure to rottlerin (Fig. 3-3C&D). Rottlerin did not affect constitutive *IFNT* concentrations. Also, rottlerin had no effect on tcRNA quality and quantity, *18S* RNA concentrations or rate of apoptosis (as determined by TUNEL analysis) (data not shown).

A subsequent study was completed to determine if rottlerin also affected the amount of *IFNT* protein secreted into conditioned CT1 medium after FGF2 or PMA treatment (Fig. 3-4). *IFNT* protein concentration was determined by examining the antiviral activity of conditioned medium. Both FGF2 and PMA increased ($P<0.001$) the antiviral activity of CT1 conditioned-medium (Fig. 3-4 A & B). Furthermore, pre-incubation with rottlerin prevented the FGF2 and PMA effects without affecting basal antiviral activities (Fig. 3-4A & B).

The FGF2- and rottlerin-dependent effects on *IFNT* mRNA concentrations were extended to Vivot cells and primary trophoblast outgrowths to examine whether the effects noted were also apparent in other cell models for bovine trophoblast. Both trophoblast cell systems responded similarly to FGF2 and rottlerin (Fig. 3-5 A & B). Exposure to FGF2 increased ($P<0.05$) *IFNT* abundance, and this effect was abolished ($P<0.05$) with prior exposure to rottlerin.

PKC-delta Expression and Activation in Trophectoderm

A series of studies were completed to identify the PKC target for rottlerin in CT1 cells and verify that FGF2 activates this target. Initially the presence or absence of PKC-delta and PKC-theta was determined by using end-point RT-PCR (Fig. 3-6A). PKC-delta amplicons were identified in CT1 cells, d 14 and 17 bovine conceptuses, and CL (positive control). In other work, PKC-delta mRNA also was identified in Vivot cells, bovine blastocysts and bovine primary trophoblast outgrowths (data not shown).

PKC-theta mRNA was detected in CT1 cells, d 14 and 17 conceptuses and CL (Fig.3-6A), but the intensity of this product was lower than that for PKC-delta in CT1 and d 17 conceptuses. When using qRT-PCR, the relative amounts of PKC-delta mRNA were 89.8±7.3 fold greater than the concentration of PKC-theta in CT1 cells.

The ability of FGF2 to mediate PKC-delta activity was examined by determining if FGF2 affected the phosphorylation status of PKC-delta (Fig. 3-6B). FGF2 supplementation increased phosphorylation of Y311within 30 min and sustained this effect for at least 2 h in CT1 cells. This phosphorylation event is important for PKC-delta activation in several cell systems [265]. Also, PMA mimicked the effects of FGF2 and increased Y311 phosphorylation status in CT1 cells (Fig. 3-6B).

Impact of PKC-delta Knockdown on Vivot Cell Responsiveness to FGF2

RNAi was used to establish the involvement of PKC-delta in mediating FGF2 effects on *IFNT* production (Fig. 3-7). Sufficient amounts of siRNA could not be transfected into CT1 cells to impact overall PKC-delta mRNA abundance (data not shown). However, Vivot cells could be transfected well enough to reduce PKC-delta concentrations by approximately 70% when using a cocktail of three PKC-delta siRNAs (Fig. 3-7A). PKC-delta protein abundance also was decreased in Vivot cells exposed to

the PKC-delta siRNA cocktail (Fig. 3-7B). The PKC-delta siRNA cocktail did not affect constitutive concentrations of *IFNT*. However, cells containing the PKC-delta siRNA were less able to respond to FGF2 than those containing the control siRNA (Fig. 3-7). FGF2 supplementation increased ($P<0.05$) *IFNT* abundance in cells containing the control siRNA but not in cells containing the PKC-delta siRNA. The *IFNT* response in these cells was not statistically different from the FGF2- and non-FGF2-treated controls.

PKC-delta Regulates *IFNT* Expression through ERK1/2-independent Pathways

PKC-delta regulates the activity of various downstream signaling molecules, including ERK1/2 [266, 267]. Previous findings indicated that FGF2 supplementation did not increase ERK1/2 phosphorylation status in CT1 cells (Fig. 3-1), and a set of follow-up studies were completed to determine whether ERK1/2 was required for PKC-delta-mediated effects on *IFNT* abundance (Fig. 3-8). The first study was designed to examine whether rottlerin treatment altered ERK1/2 phosphorylation status in CT1 cells (Fig. 3-8A). No change in the phosphorylation status of ERK1/2 was evident after 2 h exposure to rottlerin.

A subsequent study was completed to determine if ITS serum substitute used in the serum-free medium formulation affected ERK1/2 phosphorylation status in CT1 cells. Cells cultured in serum-free medium without ITS for 24 h still exhibited pronounced basal phosphor-ERK1/2 and remained unresponsive to FGF2 challenge (Fig. 3-8B; left panel). A subset of CT1 cells also were treated with 100 nM PD173074, a pharmacological inhibitor of FGFRs, before exposure to FGF2 (Fig. 3-8B; right panel). No changes in phosphorylation status were detected before or after FGF2 challenge in cells exposed to this inhibitor.

Since slight modifications in ERK1/2 phosphorylation status may go unnoticed in Western blot analyses, the ERK1/2 inhibitor, PD98059 (25 nM), also was used to examine whether ERK1/2 is required for FGF2 effects on CT1 cells (Fig. 3-8C). Unlike the MEK inhibitor, U0126, which targets both active and inactive MEK1/2, PD98059 functions primarily to prevent phosphorylation of inactive MEK1/2 [268]. Exposure to this inhibitor did not affect basal levels of ERK1/2 phosphorylation (Fig. 3-8C). Exposing CT1 cells to this inhibitor before supplementing FGF2 did not affect constitutive or FGF2-induced *IFNT* concentrations (Fig. 3-8D) indicating that ERK1/2 likely was not required for exerting FGF2 effects on *IFNT* abundance.

Discussion

Work presented here provides evidence that PKCs, and specifically PKC-delta, mediate FGF2-induced production of *IFNT*. Measurement of *IFNT* mRNA abundance was used to describe these changes. The relative abundance of *IFNT* mRNA is directly associated with *IFNT* protein concentrations in trophoblast cell lysates and conditioned medium, this is consistent with previous reports [28, 30, 269, 270]. No direct evidence that FGF2 affected *IFNT* transcription was provided in this study. *IFNT* promoter/enhancer reporter constructs were not used because the bovine trophoblast cell lines used here were difficult to transfect with calcium phosphate precipitation and commercially available lipid- and amine-based transient transfection reagents (Yang and Ealy; Unpublished observations). Unpublished work from this laboratory determined that maximal increases in *IFNT* abundance were evident after 6 to 8 h exposure to FGFs, suggesting that FGF2 and other FGFs manipulate *IFNT* transcription rates. However, it remains possible that FGF2 may also control *IFNT* concentrations by mediating RNA stability. Additional work is required to describe the precise

transcriptional and post-transcriptional steps influenced by FGF2 in bovine trophoblast cells.

Various PKCs are linked to pre-implantation embryo development. From seven to ten PKC isotypes are expressed in mouse embryos [43, 202, 271, 272]. Various PKC isoforms also are expressed in human placentae [273]. In this work, PKC-dependent regulation of *IFNT* abundance initially was established by inhibiting PKC activity with calphostin C and stimulating PKC activity with PMA. Basal concentrations of *IFNT* were unaffected by calphostin C supplementation, indicating that low endogenous PKC activity exists in these cells, but this inhibitor prevented FGF2 from increasing *IFNT* levels in bovine cells. PMA supplementation stimulated *IFNT* mRNA and protein production in CT1 cells. Others have linked PKC activity with *IFNT* production in ovine trophectoderm. Adding PMA increased *IFNT* concentrations and calphostin C treatment abolished CSF2-mediated increases in *IFNT* concentrations in ovine conceptus explants [254]. In addition, reporter plasmids containing 5' UTR regions of the *IFNT* promoter/enhancer were stimulated in JEG3 human choriocarcinoma cells treated with PMA [255, 274].

Additional PKC inhibitors were used to identify the specific PKC isoform employed by FGF2 to control *IFNT* expression in bovine trophoblast cells. Atypical PKCs were not examined since these kinases are not responsive to PMA and other phorbol esters [201]. Exposing CT1, Vivot and bovine trophoblast outgrowths to the PKC-delta inhibitor, rottlerin blunted FGF2-induced increases in *IFNT* levels. Rottlerin also blocked FGF2 and PMA from increasing *IFNT* protein concentrations in conditioned medium. Rottlerin effectively inhibits PKC-delta in other cell types at concentrations used in this

work (effective range: 3-6 μM) [263]. Preliminary studies found that rottlerin was partially effective at blocking FGF2 responses in CT1 cells at 1 μM but the 5 μM treatment provided full inhibition of this response. Rottlerin also inhibits other kinases. Notably, it blocks the activity of PKC-theta, another novel PKC, when used at 10-20 μM [264, 275]. PKC-theta expression is less widespread throughout tissues and primarily is studied in regards to platelet activation and T-cell function [201]. It is present in CT1 cells, but its level of expression is substantially less than PKC-delta. Exposing CT1 cells to Gö6976, an inhibitor of classical PKC molecules (α, β, γ), did not affect the ability of FGF2 to stimulate *IFNT* levels in CT1 cells. A single concentration of this inhibitor was examined herein (5 μM), and although this concentration of inhibitor blocked classic PKC activity in other cells [261], no attempts were made in the present work to verify its effectiveness in CT1 cells. Therefore, the role of classic PKCs on *IFNT* gene expression regulation cannot be discounted based on the present findings.

The involvement of PKC-delta in FGF2-mediated effects on trophoblast cells was confirmed by RNAi. Difficulties in obtaining high-efficiency transfection precluded using CT1 cells for siRNA studies. However, a contemporary trophoblast cell line derived from an *in vivo*-generated blastocyst (Vivot line) could be transfected with moderate efficiency, and reductions in PKC-delta mRNA and protein concentrations were associated with diminished FGF2 effects on *IFNT* concentrations. The siRNA treatment did not generate a complete loss in FGF2-induced effects in Vivot cells. This likely reflects limitations in the knockdown efficiency of PKC-delta mRNA and PKC-delta protein. Both were still evident after transfection. It remains possible that additional PKCs, such as PKC-theta, may also be involved with mediating FGF2 actions, but

observing a partial reduction in FGF2-mediated events after *PKC-DELTA* siRNA treatment provides conclusive evidence that PKC-delta is one of the signaling molecules involved with this process.

PKC-delta is a widely-expressed, multifunctional kinase that is best known as a regulator of apoptosis [276, 277]. However, it also can promote cell survival in some instances [276, 277]. Moreover, it also contains apposing activities in regards to cell proliferation and tumorigenesis [276, 278]. There are several reports describing how PKC-delta regulates peri-implantation embryo development and trophoblast function. PKC-delta is detected throughout early embryo development in mice [271]. It associates with the spindle apparatus during meiosis and localizes to the nucleus during initial cleavage events where it regulates mRNA processing [62, 279]. At the blastocyst stage, PKC-delta localizes primarily to the plasma membrane in trophoblast cells and is a central regulator of tight junction formation and ion transport during blastocoel formation [202, 203].

The PMA responses observed in this work are consistent with previous descriptions of PMA effects on ovine *IFNT* transcription [254, 255, 274, 280]. Both PMA and FGF2 likely affect *IFNT* abundance in bovine trophoblast cells by acting through PKC-delta. No additive or synergistic effects were noted upon FGF2 and PMA co-treatment, and rottlerin effectively blocked the ability of both FGF2 and PMA to stimulate *IFNT* levels in various bovine trophoblast cell lines. However, PKC-delta probably is not the only signaling molecule impacted by PMA in trophoblast cells. Phorbol esters are best known for their ability to stimulate DAG-mediated events, but many of these agents, including PMA, interact with non-PKC targets [201]. This is especially germane

when considering previous work using JEG3 cells to study *IFNT* promoter/enhancer activity. JEG3 cells lack PKC-delta [281] (unpublished observations by authors), yet PMA treatment stimulated promoter/enhancer activity in this and other human cell lines [255, 274, 280]. Therefore, it appears that PMA acts on PKC-delta to impact *IFNT* transcription and/or RNA stability in homologous cell systems but acts on different signaling molecules in heterogonous systems to regulate *IFNT* expression.

PKC-delta can impact a variety of downstream signaling molecules. There are several examples of FGF-induced PKC-delta activation events that subsequently impact ERK1/2 activity [266, 267]. Activation of ERK1/2 did not appear to be required for FGF2 to increase *IFNT* abundance in this work. Rottlerin did not influence ERK1/2 phosphorylation status. Also, preventing ERK1/2 phosphorylation with PD98059, an inhibitor that limits phosphorylation of ERK1/2 without affecting the activity of previously phosphorylated ERK1/2, did not affect the ability of FGF2 to increase *IFNT* levels. However, abolishing all of the active ERK1/2 in CT1 cells with U0126 (including constitutively active molecules) dramatically decreased *IFNT* abundance, and FGF2 supplementation was not effective at increasing *IFNT* abundance in the absence of activated ERK1/2. This suggests that ERK1/2 is requisite for basal *IFNT* transcription. It must be noted, however, that FGF2 studies were completed over a 22-24 h time period whereas effects of the inhibitors on ERK1/2 activity were only examined for the first 2 h of this period. Therefore, the possibility that FGF2 affected ERK1/2 activity after 2 h from the beginning of FGF2 treatment cannot be discounted. It also remains unknown how long the inhibitors blocked ERK1/2 activity in these studies. Therefore, ERK1/2 does not appear to be directly involved with transducing the FGF2 signal, but these

molecules do appear to be essential for basal responses in CT1 cells. The direct control of *IFNT* transcription by Ras-dependent pathways has been reported by others [197]. In that work MAPK-dependent systems impacted *ETS2* activity. It also is possible that Ras-MAPK affects other transcriptional regulators of *IFNT*. In mice, MAPK controls *CDX2* expression in preimplantation embryos [116]. This trophectoderm specification factor is a central component of *IFNT* promoter/enhancer activity [190].

It was interesting to note that CT1 cells and Vivot cells (data not shown) contained ample amounts of phosphorylated ERK1/2 prior to FGF2 administration. Constitutive phosphorylation of ERK1/2 also exists in mouse trophoblast cells, and ERK1/2 activity is essential for trophoblast development and differentiation in this species [116, 282, 283]. Endogenous ERK1/2 activity could not be reduced by removing the serum substitute from cultures (insulin/transferrin, selenium). Also, blocking endogenous FGFs with a pharmacological inhibitor to FGFRs did not influence constitutive phosphorylation of ERK1/2.

An association between p38 MAPK and *IFNT* abundance has not been described until now. FGF2 induced a rapid and transient phosphorylation of p38 MAPK. Blocking this induction did not impact the ability of FGF2 to increase *IFNT* levels. However, *IFNT* concentrations were decreased in non-FGF2 treated cells. It remains uncertain whether this outcome resulted from the specific interference with a p38 MAPK-dependent signaling module targeting *IFNT* transcription or whether an indirect or nonspecific mechanism caused this response. The importance of p38 MAPK during early placental development is established in mice where loss of p38 MAPK function is an embryonic-lethal phenotype characterized with spongiotrophoblast defects [284]. Also, p38 MAPK

is recruited by FGF4 for maintenance of trophoblast stem cells in mice [285]. Thus, it is reasonable to suspect that p38 MAPK is important for trophoblast gene expression in bovids, but the specific targets of this kinase remain unknown.

To conclude, based on the present work, it is reasonable to propose that PKC-delta acts as a signaling module for uterine- and conceptus-derived FGFs and potentially other molecules in peri-attachment bovine conceptuses. Several uterine-derived factors, including multiple FGFs can increase *IFNT* production in ovine and bovine trophoblast cells. Moreover, several FGFs are produced by conceptuses throughout pre- and peri-attachment development coincident with maximal *IFNT* production [30]. This work provides new insight into how uterine- and conceptus-derived factors impact conceptus development and gene expression during pre- and peri-attachment development. Further exploration is needed to understand the full extent of PKC-delta activities during peri-attachment development in bovine conceptuses.

Table 3-1. Primers and siRNA oligo sequences

Primer/ siRNA oligo	Sequence (5'-3')
PRKCD forward*	AACTGGGACCTACGGCAAG
PRKCD reserve*	TGCAGAAGAGGTGGGTGAGA
PRKCQ forward*	TCCAGTTGAAATTGGTCTCC
PRKCQ reverse*	GCACTCCACGTCATCGTCCA
PRKCD siRNA#1 sense	GGUUCAAGGUUUAUAACUA
PRKCD siRNA#1 antisense	UAGUUAUAAACCUUGAACGG
PRKCD siRNA#2 sense	AGAAAUGCAUCGACAAGAU
PRKCD siRNA#2 antisense	AUCUUGUUGAUGCAUUUCU
PRKCD siRNA#3 sense	GCUGCCAUCCACAAGAAAU
PRKCD siRNA#3 antisense	AUUUCUUGUGGAUGGCAGC

*Primer sets were used for qRT-PCR and end-point RT-PCR.
PRKCD GeneID: 505708. *PRKCQ* GeneID: 505901
Other primer sets used are referenced elsewhere [30].

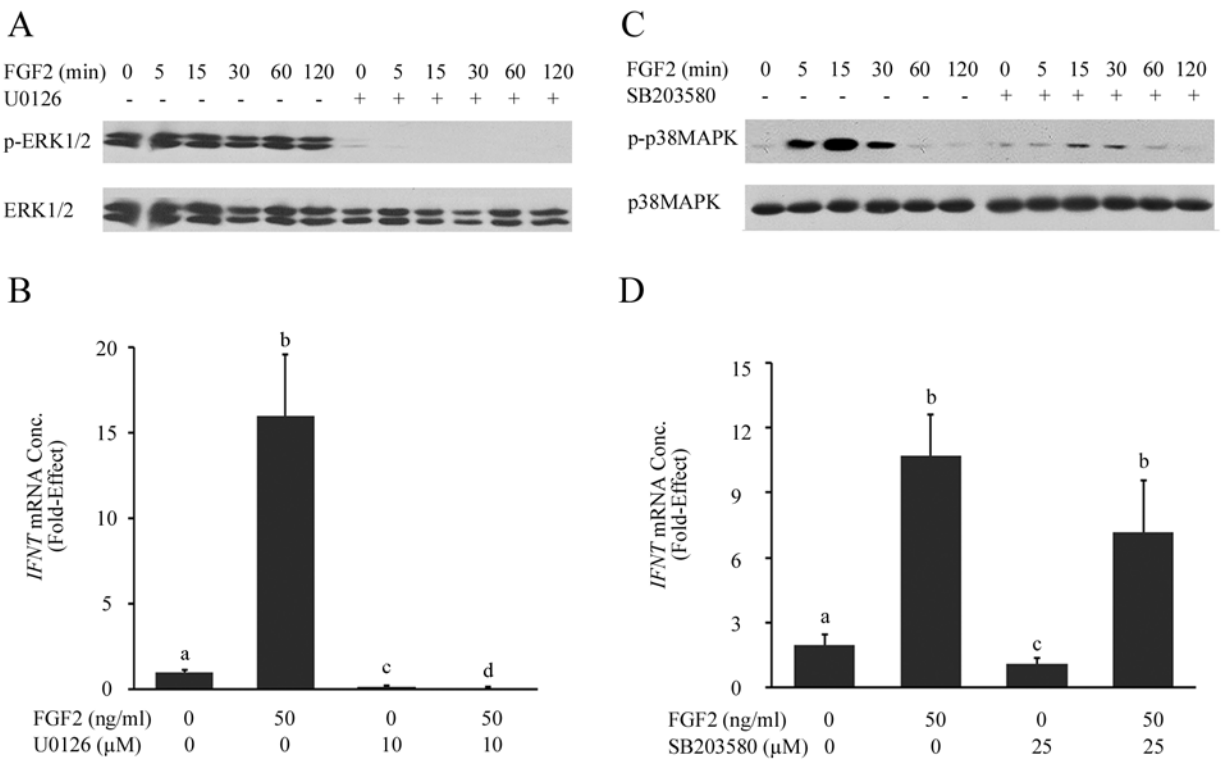

Figure 3-1. The dependence of constitutive and FGF2-induced *IFNT* expression by ERK1/2 and p38 MAPK. *Panel A:* Western blot analysis was completed to determine whether FGF2 affected ERK1/2 phosphorylation status and to assess how U0126 (10 μM) affected ERK1/2 phosphorylation status. Cells were exposed to inhibitors or DMSO (control) for 2 h, then were harvested either immediately before FGF2 supplementation (time 0) or at specific times after FGF2 treatment. Estimates of phosphorylated (p) ERK1/2 and total ERK1/2 concentrations were determined using Western blotting. Three replicate studies were completed and representative blots are provided. *Panel B:* The effect of MEK inhibition on basal- and FGF2-induced *IFNT* abundance was examined by exposing CT-1 cells to each inhibitor for 2 h, changing medium and incubating in the presence or absence of FGF2 for 24 h. tcRNA was isolated and qRT-PCR was used to determine the relative abundance of *IFNT*. *18S* RNA was used as the internal control. *Panel C:* Western blot analysis was completed on CT1 cell lysates exposed to FGF2 in the presence or absence of the p38 MAPK inhibitor, SB203580 (25μM) using the same experimental approaches as described previously. *Panel D:* The effect of p38 MAPK inhibition on basal- and FGF2-induced *IFNT* abundance was examined as described previously. In both sets of studies, qRT-PCR data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies). Differences ($P<0.05$) are denoted within each panel with different superscripts.

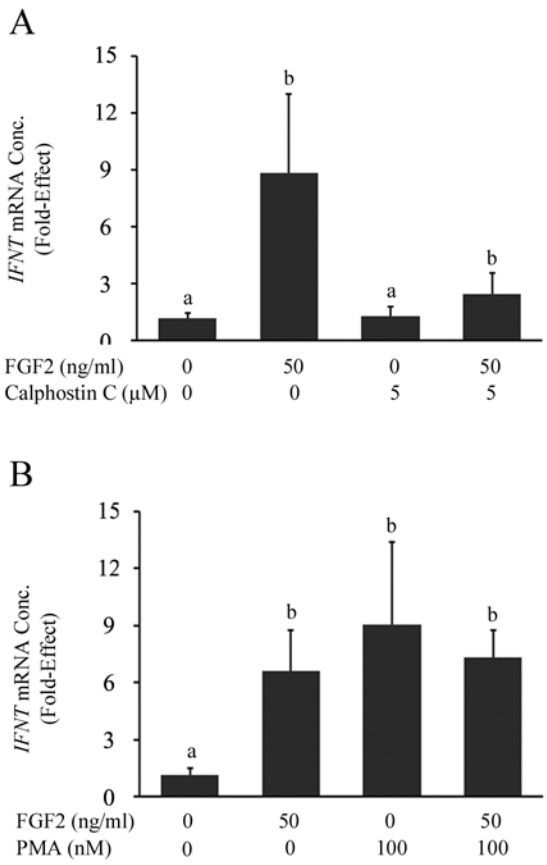

Figure 3-2. PKC-dependent systems regulate *IFNT* mRNA levels in CT1 cells. *Panel A*: The role of PKC in regulating FGF2-dependent increases in *IFNT* mRNA levels was examined by exposing CT1 cells to calphostin C (0.5 μM) or its carrier (DMSO) for 2 h. Cells were then incubated with or without FGF2 for 24 h. *Panel B*: A separate experiment was completed to determine if exposure to PMA mimicked the effect of FGF2 in CT1 cells. Cells were incubated in the presence or absence of FGF2 and/or PMA for 24 h. In both experiments, tcRNA was isolated at the end of the incubation period and qRT-PCR was used to determine the relative abundance of *IFNT* mRNA. 18S RNA was used as the internal control. Data are represented as mean fold-differences ± SEM from the control value (n=4 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

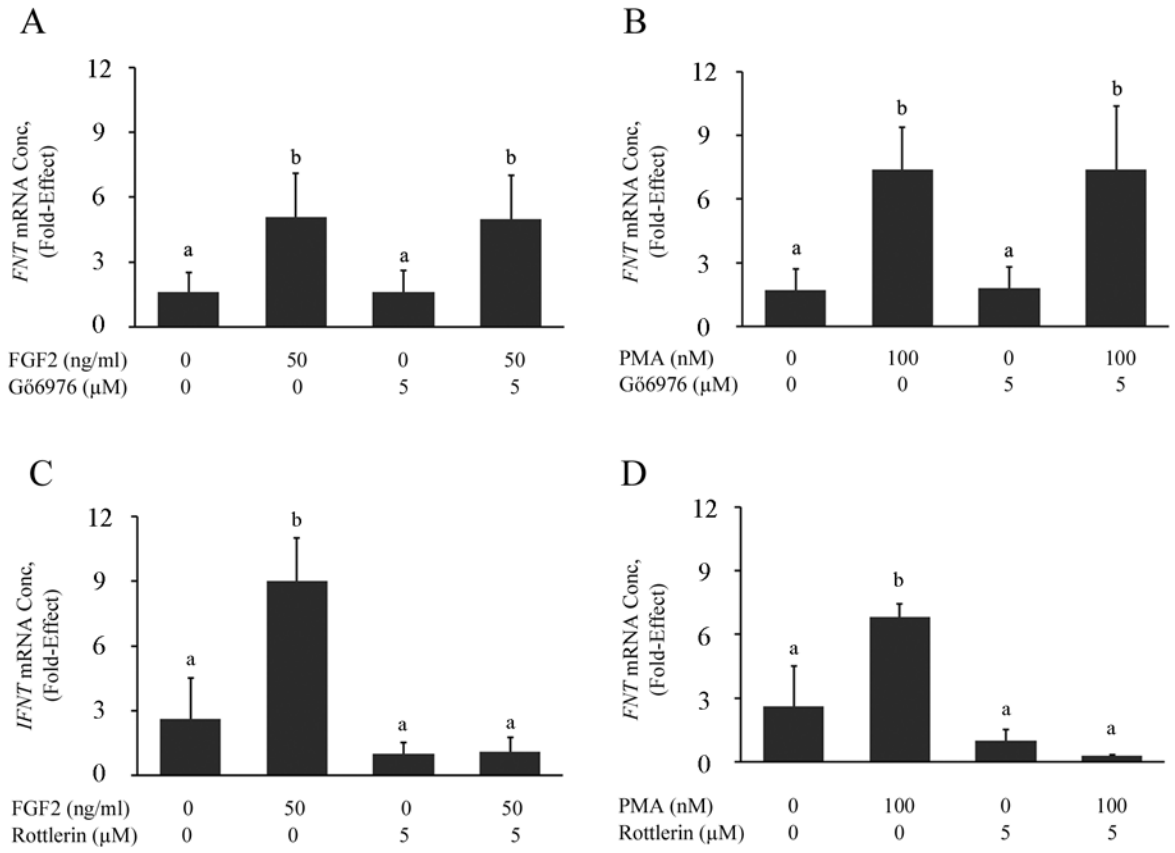

Figure 3-3. Examination of how isoform-specific PKC inhibitors affect FGF2-induced *IFNT* expression in CT1 cells. A series of experiments were completed in CT1 cells to determine whether an inhibitor of classical PKCs (Gö6976; 5μM) or PKC-delta (rottlerin 5μM) influence FGF2- and PMA-dependent increases in *IFNT* levels. Cells were provided inhibitors for 2 h, and then medium was replaced with medium containing or lacking FGF2 or PMA. *Panel A*: treatments included Gö6976 and FGF2; *Panel B*: treatments included Gö6976 and PMA; *Panel C*: treatments include rottlerin and FGF2; *Panel D*: treatments include rottlerin and PMA. After 24 h exposure to FGF2 or PMA, tcRNA was isolated and qRT-PCR was used to determine the relative abundance of *IFNT*. *18S* RNA was used as the internal control. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

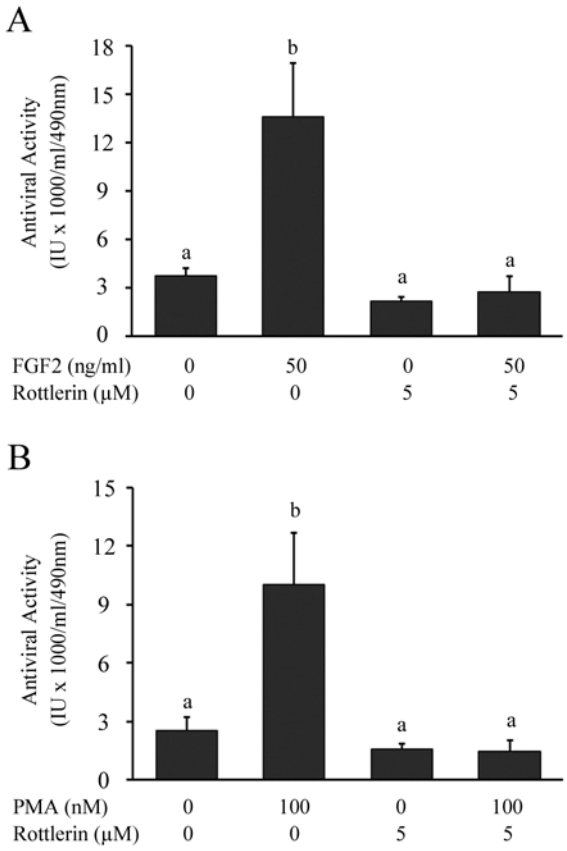

Figure 3-4. Rottlerin prevents FGF2 or PMA from increasing *IFNT* protein concentrations in conditioned CT1 medium**.** CT1 cells were serum-starved for 24 h then were treated with 5 μM rottlerin or vehicle (DMSO). After 2 h, medium was removed and replaced with medium containing 0 or 50ng/ml FGF2 (*Panel A*) or with 0 or 10 nM PMA (*Panel B*). After 48 h, conditioned medium was harvested and antiviral assays were used to determine *IFNT* secretion. Antiviral results (IU/ml conditioned medium) were normalized based on viable cell number (measuring the amount of tetrazolium oxidation; A_{490} readings). Data are represented as mean IU/ml/A_{490} ± SEM (n=5 replicate studies within each panel). Differences ($P<0.01$) are denoted within each panel with different superscripts.

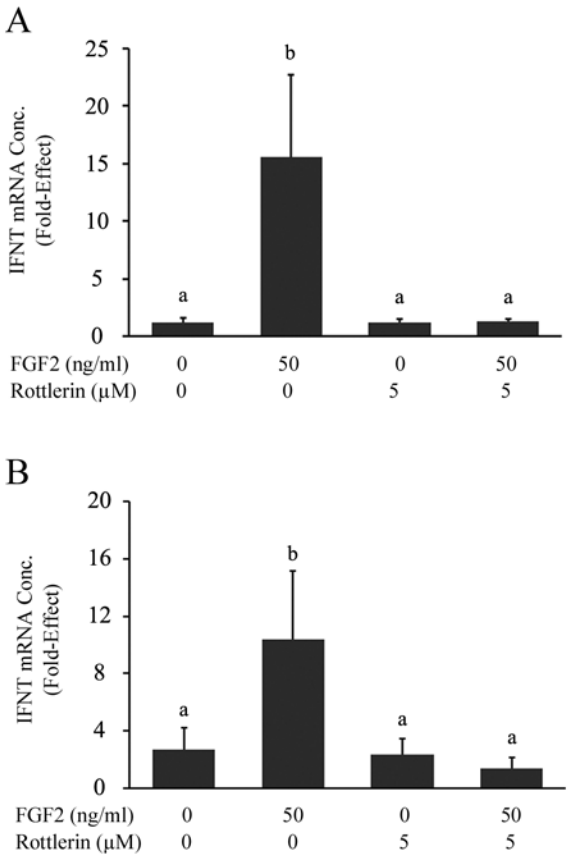

Figure 3-5. Rottlerin prevents FGF2 from increasing *IFNT* mRNA levels in two additional bovine trophoblast cell systems. Vivot cells (*Panel A*) and primary trophoblast outgrowths (*Panel B*) were exposed to rottlerin or its carrier control for 2 h, then cultured were exposed to FGF2 or BSA-control. After 24 h, tcRNA was isolated and qRT-PCR was used to determine the relative abundance of *IFNT* mRNA. 18S RNA was used as the internal control. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). Differences ($P<0.01$) are denoted within each panel with different superscripts.

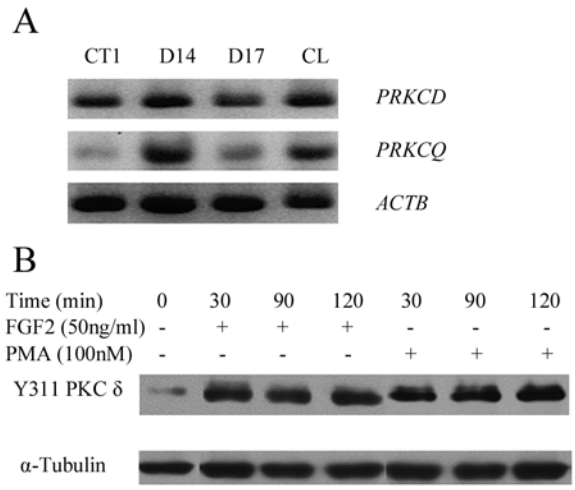

Figure 3-6. The expression and activation of PKC-delta in bovine trophoblast cells. *Panel A*: End-point RT-PCR was used to establish that PKC-delta (PRKCD) and PKC-theta (PRKCQ) mRNA was evident in CT1 cells and elongating bovine conceptuses. tcRNA was collected from CT1 cells, d 14 and 17 conceptus, and bovine CLs (positive control). PCR products were electrophoresed on an agarose gel and visualized with ethidium bromide staining. No amplified products were detected in non-reverse transcribed tcRNA samples (data not shown). *Panel B:* The ability of FGF2 and PMA to affect PKC-delta phosphorylation status at Y311 was examined by Western blot analysis. CT1 cell lysates were collected either immediately before (time 0) or at specific periods after treatment with 50 ng/ml FGF2 or 100 nM PMA. Lysates were electrophoresed, blotted onto PVDF membrane and immunoblotted with antibodies recognizing phosphorylated Y311 within PKC-delta. Three replicate studies were completed and a representative blot is provided. A single immunoreactive band of the correct molecular mass was observed in all blots. Blots were stripped and reused to detect the relative amounts of α-tubulin to ensure appropriate sample loading.

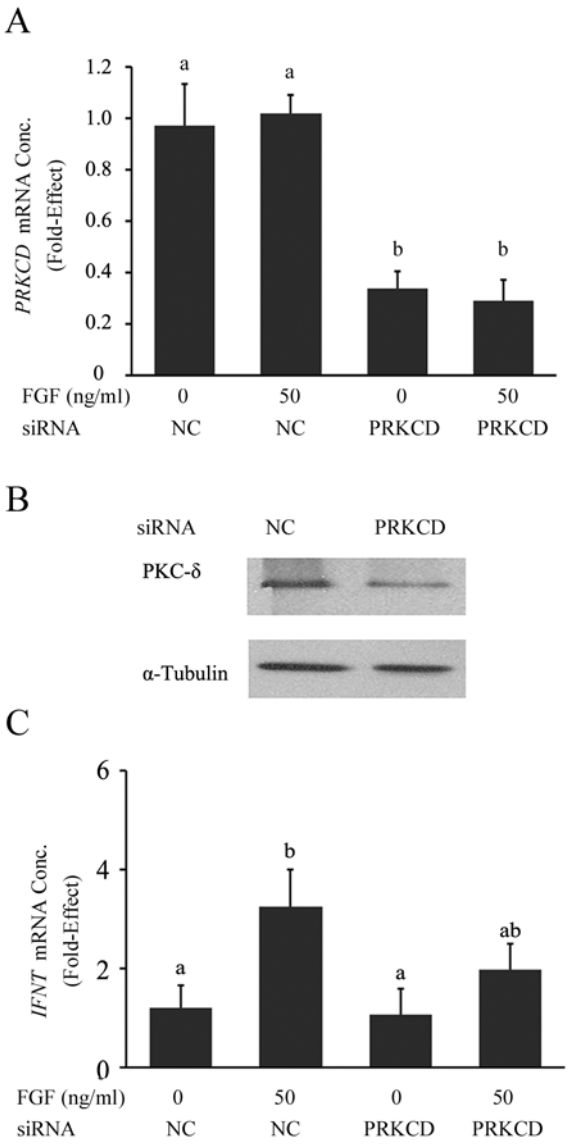

Figure 3-7. siRNA knockdown of PKC-delta mRNA and protein in trophoblast cells impacts the ability of FGF2 to increase *IFNT* mRNA levels. *Panel A:* The relative abundance of PKC-delta mRNA (*PRKCD)* was determined. At 60 h post-transfection, cells were cultured in the presence or absence of FGF2 for 24 h. tcRNA was isolated and used for qRT-PCR. *Panel B:* Relative amounts of PKC-delta were determined by Western blot analysis. *Panel C:* The relative abundance of *IFNT* mRNA was determined after FGF2 challenge. At 60 h post-transfection, cells were cultured in the presence or absence of FGF2 for 24 h. tcRNA was isolated and used for qRT-PCR. *18S* RNA was used as the internal control. Data are represented as mean fold-differences ± SEM from the control value (n=4 replicate studies). In Panels A & C, differences ($P<0.05$) are denoted within each panel with different superscripts.

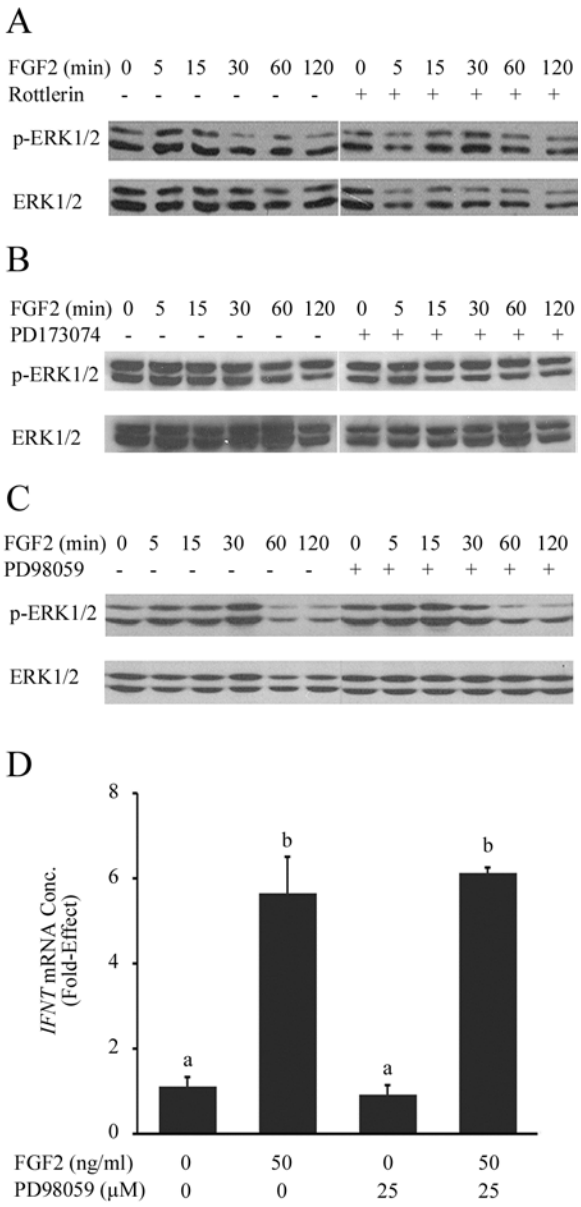

Figure 3-8. ERK1/2-dependent systems are not required for FGF2-dependent increases in *IFNT* mRNA abundance. *Panel A*: Western blot analysis of ERK1/2 phosphorylation status affected by rottlerin (5 μM). *Panel B*: Western blot analysis of ERK1/2 phosphorylation status in serum-free medium without ITS in the presence or absence of FGFR inhibitor PD173074 (100nM). *Panel C*: Western blot analysis ERK1/2 phosphorylation in the presence of PD98059 (25μM). *Panel D*: The effect of MEK inhibition on basal- and FGF2-induced *IFNT* mRNA abundance was examined by qRT-PCR. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies). Differences ($P<0.05$) are denoted within each panel with different superscripts. For western blot analysis, three replicate studies were completed and a representative blot is provided.

CHAPTER 4
PRIMITIVE ENDODERM DEVELOPMENT IS STIMULATED BY FIBROBLAST GROWTH FACTOR 2 IN BOVINE BLASTOCYSTS

Early conceptus development in ruminants is notably different from rodents, primates and many other species because of the prolonged pre- and peri-implantation development occurring in these species. Bovine and ovine blastocysts hatch from the zona pellucida between days 8 and 10 post-fertilization and remain free-floating for another week or more [59, 286]. These conceptuses begin elongating during the second and third weeks of pregnancy due to the reorganization and proliferation of trophectoderm and occupy nearly the entire length of one uterine horn before attaching to the uterine lining on or after day 17 and 19 in sheep and cattle, respectively [67, 287]. This rapid trophectoderm development maximizes early placental surface area interactions with the uterus and ensures the production of sufficient amounts of interferon-tau (IFNT), the maternal recognition of pregnancy factor in these species [18, 288]. Formation of the three primary germ layers and gastrulation also occurs as bovine and ovine conceptuses float freely in the uterine lumen. Primitive endoderm is evident between day 8 and 10 post-fertilization, primitive ectoderm emerges around day 12 and primitive mesoderm forms on day 14 to 16 [14, 63, 289]. It is not surprising, therefore that miscues in germ cell generation, hypoblast/epiblast formation and gastrulation are linked with early pregnancy failures in cattle [59, 290]. Early pregnancy failures are common in domesticated ruminants, and especially in lactating dairy cattle, where from 25 to 50% of pregnancies are lost within the first 6 weeks of gestation [5].

After blastocyst formation and specification of trophectoderm has occurred, the inner cell mass (ICM) develops into distinct primitive endoderm and epiblast lineages. The epiblast is a pluripotent lineage that forms additional extraembryonic and embryonic

tissues. The primitive endoderm is an extraembryonic lineage that forms the hypoblast and the inner layer of the yolk sac [291, 292]. Little is known about primitive endoderm formation in ruminants. Bovine primitive endoderm cultures can be established from bovine blastocysts after their attachment to feeder cells [150, 293], but no information exists to explain how their formation and growth is controlled. The lineage specification of primitive endoderm has been examined to a greater extent in mice. In this species, determination of primitive endoderm and epiblast fates is not solely defined by their location within the inner cell mass, but rather also is controlled by differential expression of specific transcription factors. Cells that become epiblast express high levels of *NANOG* whereas cells that segregate to primitive endoderm express high levels of *GATA4* and *GATA6* [141, 294, 295]. *GATA4* and *GATA6* are essential for normal primitive endoderm development. Targeted disruption of *GATA4* or *GATA6* expression blocks primitive endoderm formation in ES cells [296, 297]. Also, ectopic expression of *GATA4* or *GATA6* induces ES cell differentiation to the primitive endoderm lineage [136, 137]. Roles of *GATA4* and *GATA6* are not clear in the bovine conceptus, but immunoreactive *GATA6* localizes to specific cells within the ICM of bovine and porcine blastocysts, suggesting this factor may also be involved with controlling primitive endoderm/epiblast fate determination in these species [153].

There is good evidence that fibroblast growth factor (FGF) signaling and MAPK activation play key roles in initiating primitive endoderm formation in mouse embryos. Embryos that lack ICM-derived FGF4, its cognate receptor FGFR2, or the receptor adapter protein Grb2 do not form primitive endoderm [298-301]. Overexpression of a dominant-negative FGFR in ES cells also prevents primitive endoderm formation in ES

cells [302]. FGF signaling through Grb2 induces primitive endoderm formation via GATA factor activation. Grb2 mutant embryos do not produce *GATA6* and all ICM cells become *NANOG*-positive EPI [294]. Also, inhibition of FGFR- or MAPK-activation using specific pharmacological inhibitors prevents *GATA6* expression and primitive endoderm formation in mouse blastocysts whereas supplementation with FGF4 increases the number of *GATA6*-positive primitive endoderm cells in the ICM [303].

Although similar work has not been completed in cattle and other ruminants to establish a linkage between FGF/MAPK signals and primitive endoderm development, several FGFs are produced in bovine embryos during early pregnancy. Transcripts for FGF4 are detected in bovine blastocysts, and levels of this transcript are reduced in cloned bovine embryos [12, 229], which suffer from extensive post-transfer pregnancy failure [11]. Another FGF, FGF2 or basic FGF, also is expressed in bovine blastocysts [28, 30, 229]. FGF2 also is produced by endometrial epithelium and detectable quantities of this FGF can be detected in the uterine lumen throughout the pre- and peri-implantation period in ewes and cattle [28, 29]. FGF2 and FGF4 utilize many of the same FGFR isotypes (e.g. FGFR1IIIc, R2IIIc, R3IIIc), and the commercial availability of bovine recombinant FGF2 permits the exploration of how this and other FGFs may control early fate determination in bovine embryos. Work outlined in this study describes how supplementation with FGF2 promotes primitive endoderm lineage emergence and examines how this activity may be controlled in bovine pre-implantation embryos.

Materials and Methods

Materials

All cell culture reagents, including Dulbecco's modified essential medium containing high glucose (DMEM) and Fetal Bovine Serum (FBS), PCR primers and EdU Cell Proliferation Assay were purchased from Invitrogen Corp. (Carlsbad, CA). Synthetic Oviduct Fluid (SOF) medium was purchased as a custom formulation from the Specialty Media Division of Millipore Corp. (Billerica, MA). Bovine recombinant FGF2 and Matrigel^{TM} Basement Membrane Matrix was purchased from BD Biosciences (San Jose, California). The PicoPure ^{TM} RNA isolation kit was purchased from MDS Analytical Technologies (Sunnyvale, CA). RNase-free DNase was purchased from New England Biolabs (Ipswich, MA). The High Capacity cDNA Reverse Transcription kit and SybrGreen Detector System were purchased from Applied Biosystems Inc. (Foster City, CA). 4'-6-diamidino-2-phenylindo (DAPI) was from Invitrogen Mouse monoclonal Anti-*CDX2* antibody was purchased from BioGenex (San Ramon, CA, USA). Rabbit polyclonal Anti-*GATA4* antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).FITC labeled Anti-Mouse IgG was from Abcam (Cambridge, MA, USA), Alexa Fluor 555 labeled anti-rabbit IgG and HRP labeled anti-rabbit IgG were from Cell Signaling Technology (Beverly, MA, USA). Goat anti-transferrin antiserum was purchased by Sigma-Aldrich Inc. (St. Louis, MO). The chemical inhibitor for FGFR (PD173074) was purchased from EMD Chemicals (Gibbstown, NJ). The proteinase and phosphatase inhibitors cocktails, BCA Protein Assay and BioMax film were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Polyvinylidene Difluoride (PVDF) (Immobilon-P) membrane was purchased from Millipore Co. (Bedford, MA). The

enhanced chemiluminescence (ECL) Western blot detection system was purchased from Amersham (GE Healthcare, USA).

In Vitro Production of Bovine Embryos

In vitro produced (IVP) bovine blastocysts were generated using maturation, fertilization and culture procedures described previously [22, 209] using bovine ovaries collected from a local slaughterhouse (Center Hill, FL) and transferred in 0.9% (w/v) NaCl at room temperature. Matured oocytes were fertilized with 1×10^6 Percoll-purified spermatozoa from frozen-thawed semen collected from three bulls. Different bulls were used throughout the studies. After fertilization, putative zygotes were denuded by vortexing and placed in groups of 20 to 30 in 50 µl drop of SOF at 38.5°C in a humidified atmosphere containing 5% oxygen (5% [v/v] O_2, 5% [v/v] CO_2, 90% [v/v] N_2). On specific days post-fertilization embryos were collected based on morphology (morula; regular, expanded, hatched blastocyst).

For one study, non-expanded (Day 7) or expanded (Day 8) blastocysts (n=10/50 µl drop) were exposed to 50 ng/ml FGF2 or 1 µM FGFR inhibitor (PD173074) in DMEM containing 5% FBS and other supplements (100 µM non-essential amino acids, 55µM β-mercaptoethanol and 250 U/ml antibiotic-antimycotic) at 38.5°C in a 5% oxygen environment as described previously [31]. After 24 h, RNA was isolated using the PicoPure isolation kit.

Blastocyst Outgrowth Culture

On day 8 post-fertilization, non-expanded and expanded blastocysts were placed individually into Matrigel-coated 48-wells plate (0.75 cm2) in 500 µl DMEM containing 5%FBS and other supplements described earlier at 38.5°C in a 5% oxygen environment. Medium was changed on days 13 and 15 post-fertilization (day 5 and 7

after beginning individual culture) without disrupting blastocysts that had attached to the matrix. For embryos that had not attached by days 13 or 15, approximately half of the medium was exchanged on each day.

In one study different concentrations of FGF2 (0.5, 5, 50 ng/ml in DMEM containing 1% [w/v] BSA) or controls (carrier only) was provided at the beginning of culture on day 8 post-fertilization. On day 13 and 15 post-fertilization each blastocyst was assessed under phase contrast microscopy and for its attachment and viability status (floating/unattached; attached; outgrowth formation; degenerating).

Propagation of Primary Trophoblast and Primitive Endoderm Cultures

Outgrowths were generated from individual blastocysts as described in the previous section. Outgrowths formed by day 15 were cultured for an additional 5 days to obtain sufficient cells for RNA isolation. To establish pure cell lines, single putative trophoblast or primitive endoderm colony was treated with trypsin and cultured several passages. Primitive endoderm cells continued to maintain trophoblast specific morphology such as dome formation within the cell monolayer, large nuclei and numerous secretory granules, while primitive endoderm cells showed similar morphology as mouse XEN cells, an extraembryonic endoderm cell line isolated from mouse blastocyst (Fig 4-1) [137, 304].

RNA Isolation and Quantitative RT-PCR

Total cellular RNA (tcRNA) was isolated from individual trophoblast and primitive endoderm colonies and from groups of embryos (n=10-12) by using the PicoPure RNA isolation kit. Quality and concentration of tcRNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Samples were incubated with RNase-free DNase for 30 min at 37°C. After heat inactivating the DNase (75°C for 10 min), RNA

was reverse transcribed using the High Capacity cDNA Reverse Transcription kit and random hexamers. The SybrGreen Detection System was used in combination with primer pairs (200 nM) depicted in Table 4-1 and a 7300 Real Time PCR System (Applied Biosystems) to quantify the relative abundance of specific transcripts. Suitable primer efficiencies (>94%) were verified for each primer pair before their use. The same amount of tcRNA was used for each sample within each study. *GAPDH* mRNA level was used to normalize values. *GAPDH* mRNA abundance did not change based on cell type or treatment (data not shown). A dissociation curve analysis (60-95°C) was used to verify the amplification of a single product. Each sample was completed in triplicate reactions. A fourth reaction lacking reverse transcriptase was included to control for genomic DNA contamination. The comparative threshold cycle (C_T) method was used to quantify mRNA abundance [30].

Immunofluorescence Microscopy

Primary trophoblast and primitive endoderm cells were fixed with 4% [w/v] paraformaldehyde for 15 min, permeabilized with 0.5% [v/v] Triton X-100 in 0.01 M PBS [pH 7.4] for 30 min and blocked with 10% [v/v] goat serum for 60 min. Primary antibodies used included rabbit anti-*GATA4* (1:200 in 10% [v/v] goat serum) and mouse anti-*CDX2* (ready to use in PBS). Secondary antibodies (1:500 in PBS) were conjugated to FITC (goat-anti rabbit) or AlexaFluor 555 (goat anti-mouse). Nuclei were labeled with 4'-6-diamidino-2-phenylindole (DAPI). Immunoreactive complexes were visualized with an Eclipse TE2000-U inverted microscope (Nikon, Lewisville, TX) equipped with an X-Cite 120 epifluorescence illumination system (EXFO; Mississauga. Ontario, Canada). Images were captured with a Nikon DXM-1200F digital camera and assembled with NIS-Elements Software (Nikon).

Bovine blastocysts were fixed and stained with *CDX2* and *GATA4* antibodies as described above. Immunoreactive signals were visualized with an Olympus IX81-DSU Spinning Disk Confocal Microscope (Center Valley, PA, USA). Images were captured with a Hamamatsu C4742-80-12AG Monochrome CCD Camera (Hamamatsu Corporation, Bridgewater, NJ) and assembled with SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA).

Western Blot Analyses

The primitive endoderm and trophoblast outgrowths (n=3 for each) were seeded onto Matrigel-coated plates (25 mm diameter). After 3 days cellular protein was extracted and SDS-PAGE and Western blotting was completed as described previously (Chapter 3). In brief, cells were dissolved using NP-40 buffer (20 mM Tris HCl pH8, 137 mM NaCl, 20 mM EDTA, 1% [v/v] NP40) supplemented with protease and phosphatase inhibitor cocktails. Cell lysates were sonicated and protein concentrations of supernatants were determined using a BCA Protein Assay. Protein (20 µg) was loaded and separated on 10% [w/v] SDS-PAGE gels and electrotransferred onto 0.45µm PVDF. Membranes were blocked with 5% [w/v] nonfat dry milk in TBST (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% [v/v] Tween-20) and incubated with anti-transferrin antiserum described previously [256]. Horseradish peroxidase-conjugated anti-rabbit IgG was used in combination with ECL to visualize reactive bands after exposure to BioMax film. After detection, membranes were stripped and α-tubulin was detected (1:3000 dilution in 3% nonfat dry milk) to serve as a loading control. Three independent western blots generated from independent primitive endoderm or TE cultures were completed.

Proliferation Assay

The mitogenic index of trophoblast and primitive endoderm colonies was determined using the Click-iT^{TM} EdU (5-ethynyl-2'-deoxyuridine) Cell Proliferation Assay. Cells were seeded on Matrigel-coated plates (25 mm diameter). At 40-50% confluence, cells were serum-starved for 12 h and then FGF2 was added (0, 5 or 50 ng/ml). After 11 h, EdU reagent was added (20 μM) and cells were incubated for 60 min before fixation in 4% paraformaldehyde in PBS for 15 min. EdU-positive cells were determined by reaction with Alexa Fluor 488 azide. All nuclei were visualized after staining with Hoechst 33342 (10 μM). Total and EdU-positive nuclei were counted in 5 representative fields (about 100cells/field) in each well using NIS-Elements Software (Nikon) after capture on an Eclipse TE2000-U inverted microscope equipped with an X-Cite 120 epifluorescence illumination system and Nikon DXM-1200F digital camera (described previously).

Statistical Analyses

All analyses were performed by least-squares ANOVA using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between individual means were compared using pairwise comparisons (PDIFF [probability of difference] analysis in SAS). For qRT-PCR, $-\Delta C_T$ values were used for the statistical analyses and results were presented as fold differences from the control expression value [28, 30]. Differences between individual means were contrasted with pair-wise analysis (PDIFF [probability of difference] analysis in SAS). Results are presented as arithmetic means ± SEM. A *P*-value ≤ 0.05 was considered significant.

Results

Effect of FGF2 on Blastocyst Outgrowth Formation

The primary objectives of the first study were to determine the proficiency of generating outgrowths from bovine blastocysts and to determine whether FGF2 supplementation impacts their rate of formation. A majority of the outgrowths began forming between Days 13 and 15 post-IVF. The overall percentage of outgrowth formation averaged 61.56± 2.97% at Day 13 and increased ($P<0.05$) to 78.52 ±6.06% at Day 15 post-IVF. The percentage of attached blastocysts that had not formed outgrowths and the percentage of unattached blastocysts were greater ($P<0.05$) at Day 13 than Day 15 (62.65±4.63% vs. 73.27±4.7% for unattached blastocysts; 71.98±5.18% vs. 80.91±4.66% for attached blastocysts), suggesting that blastocysts still were actively attaching and forming new outgrowths beyond Day 13. No new outgrowths formed after Day 15. Lastly, the incidence of observing degenerated embryos was similar at Days 13 and 15 (average 6.58±2.28% over both days).

The effects of FGF2 supplementation on outgrowth formation at day 13 and 15 post-IVF is provided in Table 4-2. Treatment with 0.5, 5 or 50 ng/ml FGF2 did not affect the percentage of blastocysts that formed outgrowths on days 13 and 15 when compared with non-treated controls. FGF2 treatment also did not affect have a substantial effect on the percentage of attached blastocysts that had not formed outgrowths.

Effect of FGF2 on Trophoblast and Primitive Endoderm Outgrowth Formation

Examination of outgrowths revealed striking difference in response to FGF2 supplementation (Table 4-3). In the absence of FGF2 nearly all of the outgrowths (78/79) contained trophoblast cells based on a morphological assessment (small,

cobblestone-like cells containing prominent nuclei and small cytoplasmic areas) (Fig. 4-1A). The remaining outgrowth contained a preponderance of cells that were larger than trophoblast cells, polyhedral in shape and were less densely arranged that trophoblast outgrowth (Fig. 4-1B). The morphology of these cells suggested they were primitive endoderm [256, 293]. Enumeration of the trophoblast outgrowth and primitive endoderm colonies in response to FGF2 is summarized in Table 4-2 and 4-3.

FGF2 altered the incidence of trophoblast and primitive endoderm outgrowth formation (Fig. 4-3). Treatment with 0.5 ng/ml FGF2 on day 8 post-IVF caused a numerical but statistically non-significant increase ($P=0.1196$) in the percentage of outgrowths containing primitive endoderm at day 13. A greater ($P<0.05$) proportion of primitive endoderm outgrowths were observed at day 15 in blastocysts treated with 0.5 ng/ml FGF2. Treatment with 5 or 50 ng/ml FGF2 produced more profound effects on the percentage of primitive endoderm outgrowths, and both FGF2 treatments increased ($P<0.05$) the percentage of primitive endoderm outgrowth when compared with 0 and 0.5 ng/ml FGF2 treatments at day 13 and 15.

Concurrent reductions in the percentage of outgrowths containing trophoblast cells and devoid of primitive endoderm cells were noted in FGF2-treated blastocysts forming outgrowths (Table 4-3). Reductions ($P<0.05$) in the percentage of trophoblast-only outgrowths were evident at day 13 in cultures treated with 5 or 50 ng/ml FGF2 and at day 15 in cultures containing 0.5, 5 or 50 ng/ml FGF2. No examples of outgrowths containing substantial amounts of both primitive endoderm and trophoblast cells were detected at day 15 and thereafter (see following work). The primitive endoderm-containing outgrowths rapidly overran cultures that initially contained both cell types,

and by day 15 few to no trophoblast cells were evident in primitive endoderm outgrowths (data not shown).

A follow-up study was completed to verify that the non-trophoblast cells identified in this work were primitive endoderm cells. Several primitive endoderm and trophoblast outgrowths were combined (n=5-10 outgrowths/group; 3 separate groups) and processed for western blot assessment of transferrin production, a primitive endoderm-specific product [256, 293]. Immunoreactive transferrin was detected in each of the 3 groups of primitive endoderm outgrowths and was absent in each of the protein lysates from trophoblast outgrowths (Fig. 4-1C).

Profiling of Lineage Marker in Primitive Endoderm and Trophoblast Outgrowths and IVP Embryos

Several transcriptional regulators that serve as lineage markers for primitive endoderm, trophectoderm and pluripotency in rodents and the human were examined to further verify primitive endoderm and trophoblast phenotypes and examine the similarities and distinctions in lineage marker expression in the bovid. In the first study, tcRNA was extracted and qRT-PCR was completed to examine the relative abundance of lineage markers in a subset of primitive endoderm and trophoblast outgrowths (n=6) cultured until Day 20 post-IVF (Fig. 4-2).

The putative primitive endoderm-specifying genes, *GATA4*, *GATA6*, and *SOX17* were examined for their lineage specification in bovine primitive endoderm and trophoblast cells. *GATA4* mRNA was readily detected in all primitive endoderm outgrowths and was not detectable in trophoblast cells. *GATA6* mRNA was identified in both primitive endoderm and trophoblast cells and greater ($P<0.05$) levels of *GATA6* were detected in primitive endoderm than trophoblast cells. By contrast, *SOX17,* a

primitive endoderm marker in mice was not detected in primitive endoderm. Rather, they were observed in trophoblast cells.

Markers of pluripotency (*NANOG, OCT4*) and trophoblast (*CDX2*, *IFNT*) also were examined to describe how expression of these genes differs in bovine primitive endoderm and trophoblast cells (Fig.4-2). None of the primitive endoderm and trophoblast outgrowths contained detectable amounts of *NANOG* (data not shown) whereas all primitive endoderm and trophoblast cells contained similar amounts of *OCT4*. Both *CDX2* and *IFNT* were very abundant in trophoblast cells. *CDX2* was not detected in any primitive endoderm outgrowths but small amounts of *IFNT* could be detected in some colonies. This most likely reflects a low level trophoblast cells contamination in some primitive endoderm outgrowths at day 20.

A subsequent study was completed to verify that *GATA4* and *CDX2* protein existed in primitive endoderm and trophoblast outgrowths (Fig. 4-3). Antibodies against human *GATA4* and mouse *CDX2* protein were cross-reacted with their bovine counterparts. *GATA4* was detected solely within the nuclei of primitive endoderm cells whereas *CDX2* was detected only within nuclei of trophoblast cells.

The ontogeny of selective pluripotency, primitive endoderm and trophectoderm markers were determined in IVP bovine embryos (Fig. 4-4). The relative abundance of *NANOG*, *CDX2* and *GATA4* transcripts were examined between the morula and hatched blastocyst stage in cultured embryos (day 6 to 8). Transcripts for *NANOG* and *CDX2* were detected at the morula stage, and the relative abundance of both transcripts increased ($P<0.05$) at the blastocyst stage. The abundance of both transcripts did not change thereafter. The expression profile of *GATA4* differed from the other transcripts.

GATA4 was detected throughout all stages examined but the abundance of this transcript increased (P<0.05) coincident with blastocyst expansion and hatching.

Changes in the localization of *CDX2* and *GATA4* protein also were observed in expanded and hatched bovine blastocysts (Fig. 4-5). *CDX2* proteins localized within nuclei of trophectoderm whereas *GATA4* protein localized to some but not all ICM cells. DAPI was used as a counterstain to identify all nuclei in this study. Human and rodent *NANOG* antibody exhibited high non-specific staining and therefore was not included.

Expression of FGFRs in Primitive Endoderm and Trophoblast Cultures

Differences in the relative expression of FGFR isotypes were determined as a first step in describing how FGF2 differentially regulates bovine primitive endoderm and trophoblast development. Each of the four major tyrosine kinase *FGFRs* (*R1-4*) were examined (Fig. 4-6A). Transcripts for *FGFR1* and *FGFR2* were detected in primitive endoderm and trophoblast cells, and the relative abundance of *FGFR1* was greater (P<0.001) in primitive endoderm than TE whereas *FGFR2* was greater (P<0.001) in trophoblast than primitive endoderm cells. *FGFR3* was detected in trophoblast cells but not primitive endoderm. Trace amounts of *FGFR4* could be detected in both primitive endoderm and trophoblast, and no differences in abundance were detected between cell types.

To better define FGFR isotype profiles in primitive endoderm and trophoblast cells, a subsequent study was completed to examine the expression profile for the predominant splice variant forms of *FGFR1* and *FGFR2* (IIIb and IIIc variants; referred to herein as (FGF*R1b*, *FGFR1c*, *FGFR2b* and *FGFR2c*) (Fig. 4-6B). Transcripts for *FGFR1b* and *FGFR1c* were greater (P<0.01) in primitive endoderm than trophoblast cells whereas *FGFR2b* was more abundant (P<0.001) in trophoblast cells than primitive

endoderm. The *FGFR2c* was detected in low abundance in both cells and its relative abundance was not difference between cell types.

Possible Modes of FGF2 Action on Primitive Endoderm

One way that FGF2 may promote primitive endoderm outgrowth formation is through stimulation of primitive endoderm proliferation. To determine whether FGF2 treatment differentially regulates primitive endoderm and trophoblast cell proliferation during outgrowth culture, changes in the mitotic index of trophoblast and primitive endoderm outgrowths was determined by examining the proportion of cell incorporating EdU after treatment with 5 or 50 ng/ml FGF2 (Fig. 4-7). Neither concentration of FGF2 affected the percentage of EdU positive trophoblast cells (Fig. 4-7A). By contrast, both concentrations of FGF2 increased ($P<0.05$) the percentage of EdU-positive primitive endoderm cells when compared with the control (Fig. 4-7B).

To determine how FGF signals influence PE formation and/or proliferation in bovine blastocysts, embryos were treated for 24 h with 50 ng/ml FGF2 or with a pharmacological inhibitor of FGFR kinase activity (1 μM PD173074) to limit the action of any endogenously produced FGFs. The concentration of inhibitor was chosen because it was effective at blocking FGF2-induced effect in bovine trophoblast cultures (Ozawa & Ealy, Unpublished observations). Blastocysts were incubated in treatments prior to primitive endoderm lineage specification (*i.e.* day 6 to 7 post-fertilization) or as primitive endoderm lineage specification began (*i.e.* day 7-8 post-IVF). An immunofluorescence-based approach could not be used to determine numbers of *GATA4*-positive ICM cells in this study because it was too difficult to reliably count *GATA4*-positive and total ICM cells. Therefore, changes in *GATA4* and *NANOG* abundance were used to predict changes in gene expression and/or cell number after treatment with FGF2 or the FGFR

inhibitor (Fig.4-8A). FGF-dependent changes in *GATA4* abundance were evident at both stages of embryo development (Fig. 4-8A). Specifically, exposure to FGF2 did not affect *GATA4* abundance embryos were treated between day 6 and 7 but increased ($P<0.05$) *GATA4* abundance when embryos were treated between day 7 and 8 of development. Also, exposure to the FGFR inhibitor did not affect *GATA4* abundance when embryos were treated between day 6 and 7 but decreased ($P<0.05$) *GATA4* abundance when embryos were treated between day 7 and 8. Changes in the relative abundance of *NANOG* mRNA also were observed in this study (Fig. 4-8B). Treating embryos with FGF2 from day 6 to 7 decreased ($P<0.05$) *NANOG* abundance but had no effect on *NANOG* abundance in embryos treated between day 7 and 8. Treatment with the FGFR inhibitor did not affect *NANOG* abundance when provided between day 6 and 7 but increased ($P<0.05$) *NANOG* abundance when provided between day 7 and 8.

Discussion

Previous work has demonstrated that bovine blastocysts contain transcripts of multiple FGFs including FGF2 and FGF4 [30, 229]. Several FGFs such as FGF2 and FGF4 demonstrate similar receptor binding preference and potentially trigger similar intracellular events [305, 306]. In the present study, bovine recombinant FGF2 was used to activate FGF downstream signal to examine its role in cell fate decisions during bovine embryogenesis using a blastocyst outgrowth system.

Trophoblast outgrowth formation is the default pathway when a single bovine blastocyst was placed in an extended culture. Several bovine trophoblast cell lines such as BT1, CT1 and Vivot were derived from in *vivo* or *in vitro* produced blastocysts and some of those lines show the ability to form binucleate trophoblast cells [150, 307, 308]. Exogenous FGFs or other growth factors were not required for the self-renewal of those

trophoblast cell lines; strikingly, FGF supplementation stimulates *IFNT* production from several trophoblast lines and blastocyst [28, 31, 309]. *IFNT* is exclusively produced by trophoblast cells and acts as a signal for the maternal recognition of pregnancy in ruminants [15, 16]. In this study, FGF2 did not increase the ratio of proliferative trophoblast cells and did not change *CDX2* expression in expanded blastocysts, indicating FGF2 did not acts as a mitogenic factor to bovine trophoblast cells. This observation is different from mouse because FGF4 supplementation induces trophoblast stem cell proliferation from single blastocyst and blocking FGF4 signal leads to trophoblast giant cell formation *in vitro* [310]. In human, FGF2 works with activin to maintain a proliferative state of ES cell in the absence of feed-cell layers, however, withdraw FGF2 does not cause human ES cell to trophoblast lineage commitment[311, 312]. The mechanisms mediating this difference between species remain to be elucidated.

Lineage marker expression in bovine peri-implantation conceptus shows distinct pattern compare to other species. To address the question whether those primitive endoderm like colonies were truly bovine primitive endoderm lineage, firstly we used a well-defined marker transferrin [150]. As expected, those non-trophoblast-like cells produced transferrin along with other primitive endoderm markers such as *GATA4* and *GATA6* which were tested using at the message level. Interestingly, trophoblast cells also contained high amount of *GATA6* mRNA, although this level was lower than that in primitive endoderm colonies. *GATA4* mRNA was exclusively present and its protein was localized in the nuclei of primitive endoderm cells thus were selected as a marker for this lineage. As expected, *CDX2* and *IFNT* were highly abundant in trophoblast

outgrowths and only a trace amount of mRNA was detected in primitive endoderm colonies, probably because of trophoblast cell contamination. Previous work has provided evidences that *OCT4* is not restricted in ICM of blastocyst or epiblast in bovine elongating conceptus. For example, trophoblast cells were stained positive for *OCT4* both at mRNA and protein level [62, 313]. In this study, we found that *OCT4* transcripts were remained high in both trophoblast and primitive endoderm colonies. Therefore this factor was not used as a pluripotent marker in our oncoming studies. In contrast to *OCT4*, *NANOG* mRNA was high in blastocyst, but was missing from both of our cell lines.

FGF receptor profiling is distinct between different cell lineages. We hypothesized that trophoblast and primitive endoderm cells have distinctive FGF receptor expression signatures and thus respond differently to same FGF signal. 4 functional receptors and their 7 splicing variants have been identified for more than 20 FGF members in a variety of tissues in vertebrates [24, 305] . Notably, *FGFR2* was found highly expressed in trophectoderm of mouse blastocyst and its deletion leaded to embryo lethal, because of defects during gastrulation [140, 314]. *FGFR1* was also shown produced by mouse blastocyst and abruption of this gene caused severe growth retardation and defects in primitive steak formation [315, 316]. Here we showed that *FGFR2* was highly expressed in bovine trophoblast outgrowth while *FGFR1* was the primary FGF receptor in primitive endoderm lineage, although their expression was not exclusive. Surprisingly, *FGFR3* transcripts were only detectable in trophoblast colonies. We further examined the specific isoforms of *FGFR1* and *FGFR2*; two receptors were present in primitive endoderm cells. Again, those two lines showed different patterns in relative mRNA

concentration of b and c isoforms. In general, *FGFR1c* was the most abundant receptor in primitive endoderm cells while *FGFR2b* was the highest in trophoblast outgrowths. It is quite interesting because *FGFR1c* has been considered as a receptor mediating mitogenic response in various cellular system studies [317, 318]. All together, we showed firstly that bovine trophoblast and primitive endoderm lineage contained distinguished FGFR subtypes.

FGF signal is required for the derivation of primitive endoderm from bovine blastocyst but not necessary for its continued culture *in vitro*. Previous study has shown that bovine blastocyst could form visceral endoderm when providing with a feed cell layer and this cell line proliferated for multiple passages. In the present study, we used a feed layer free system to examine the effect of exogenous FGF on embryo outgrowth formation. Although the Matrigel used in this study contains low amount of growth factors, only one embryo formed primitive endoderm colony in the absence of FGF2 supplementation. In the presence of 5 ng/ml or 50 ng/ml FGF2, more than 20% of embryos gave rise to primitive endoderm and the proliferation rates of these cells increased dramatically. However, when the isolated cells were cultured *in vitro*, extra FGF2 was not required since the cell number doubled in 48 h and continued to growth on a Matrigel surface for at least 15 passages without any signs of senescence. It is therefore reasonable to conclude that the role of FGF signal was not only providing the cues to induce over proliferation of this specific lineage, it was possible to induce differentiation as well.

To address whether FGF signal induces epiblast differentiation in blastocyst, we tested the possibility that FGF supplementation repressed *NANOG* expression and

promoted primitive endoderm markers in bovine blastocyst. *NANOG* is considered to be the key factor governing the self-renewal and pluripotency of ES cell and induced pluripotent stem cells [129, 130, 310]. *NANOG* and *GATA6* were present in a “salt and pepper” manner in blastocyst and a heterogeneous expression pattern for *NANOG* was identified in mice ES cells [138, 142, 294]. In this model, a subpopulation of ES cells was stained positive for some pluripotent markers such as *OCT4* and SSEA1, but not for *NANOG*, further evidences suggested that these cells also expressed *GATA6*, a primitive endoderm marker in mice. During early development, the primary function of *NANOG* is to the repression differentiate to primitive endoderm and FGFR was the key to mediate this effect in murine stem cells [138, 142, 143, 319]. In the present study, we found that blastocyst receiving FGF2 on day 7 showed lower level of *NANOG* mRNA on day 8, 24 h later, compared to the control, *NANOG* expression was no longer repressed by FGF2; however, the level of *GATA4* mRNA was elevated by 50ng/ml of FGF2. These observations support the idea that FGF signal changes the ground state of pluripotency of ICM and allows them differentiate to primitive endoderm. It appeared that endogenous FGFs also important to maintain the level of differentiation. For example, on day 8 no differences were found in *NANOG* and *GATA4* mRNA level in blastocyst treated with FGFR inhibitor PD173074, however, on day 9, the same inhibitor increased the level of *NANOG* mRNA abundance and repressed the relative level of *GATA4* mRNA.

The appearance of bovine primitive endoderm plays a critical role in conceptus elongation and placentation. An excellent study done by Maddox-Hyttel et al described that a layer of hypoblast emerged underneath the ICM on day 8 when embryo escaped

from zona pellucida and continued to develop and cover the trophoblast layer on day 14 [14]. Proper development of primitive endoderm is critical for embryonic axis formation and subsequent implantation [59, 63]. It is therefore not surprising compared to in vivo derived embryos, *in vitro* produced embryos especially embryos generated from somatic nuclear transfer showed abnormal development of hypoblast and yolk sac formation [320]. However, no transcription factors potentially controlling this lineage commitment has been described. In the present study, bovine embryos started to transcribe *NANOG* mRNA when blastocyst formation occurs and *GATA4* mRNA level began to increase when blastocyst expansion took place. We could conclude that in bovine first lineage segregation occurs between trophectoderm and inner mass on day 6 when embryo starts to form blastocoel and the second lineage differentiation between epiblast and primitive endoderm happens on day 8 as blastocyst expands despite the fact that no immunostaining work has been done in this study because of lack of co-staining antibodies in bovine.

Together, the present study provide evidence that the segregation of primitive endoderm in bovine embryos started on day 8 and *GATA4* could be used as a marker for this lineage. Further we showed that FGF2 signal was required for the derivation of primitive endoderm cells in *vitro* and activation or inhibition of this signal pathway modulates *NANOG* or *GATA4* expression in bovine blastocyst. Further study should be done to find the exogenous or intrinsic cues that regulate bovine embryo development.

Table 4-1. Primers used for quantitative Real Time RT-PCR

Gene	GenBank No.	Primers
NANOG	NM_001025344.1	F 5'GACACCCTCGACACGGACAC-3'
		R 5'-CTTGACCGGGACCGTCTCTT-3'
GATA4	XM_616466.4	F 5'-ATGAAGCTCCATGGCGTCCC-3'
		R 5'-CGCTGCTGGAGCTGCTGGAA-3
GATA6	XM_001253596.2	F 5'-ATACTTCCCCCACCACACAA-3'
		R 5'-AGCCCGTCTTGACCTGAGTA-3'
CDX2	XM_871005	F 5'-CCTGTGCGAGTGGATGCGGAA-3'
		R 5'-CCTTTGCTCTGCGGTTCT-3'
OCT4	NM_174580.1	F 5'-GGTGGAGGAAGCTGACAACAAC-3'
		R 5'-GGCGATGTGGCTAATTTGCTGC-3'
SOX17	XM_868600.3	F 5'-CAGAACCCAGATCTGCACAA-3'
		R 5'-TAGTTGGGATGGTCCTGCAT-3'
FGFR1	NM_001110207.1	F 5'-TGGTCACAGCCACGCTCTGC-3'
		R 5'-GAACATCGTCCCGCAGCCGA-3'
FGFR2	XM_001789706.1	F 5'-GACCTGGTGTCGTGTACCTACCA-3'
		R 5'-CTGGCAGCTAAATCTCGATGAA-3'
FGFR3	AB059430.1	F 5'-GGGGACACCGTGGAGCTGAG-3'
		R 5'-GAACATCGTCCCGCAGCCGA-3'
FGFR4	XM_602166.4	F 5'-GCAGACGCTCCTCACCCGAC-3'
		R 5'-CGAGACTCACGAGGCCAGCG-'3

Table 4-2. Blastocyst outgrowth formation on days 13 and 15 post *in vitro* fertilization

No. of Embryo Cultured	FGF2 (ng/ml)	Floating Embryo %	Attached Embryo %	Outgrowth %	Degenerated %
Day 13					
79	0	11.65±4.57	20.22±5.45	61.56±2.97	6.58±2.28
76	0.5	14.33±7.69	17.67±3.11	62.65±4.63	7.08±3.43
76	5	12.25±7.43	13.92±4.20	71.98±5.18	1.67±1.67
76	50	9.17±3.69	20.37±3.45	63.52±6.17	8.97±4.17
Day 15					
79	0	4.08±0.19	10.82±3.48	78.52±6.06	6.58±2.28
76	0.5	1.12±1.11	16.32±2.49	73.27±4.70	9.32±3.24
76	5	5.42±3.56	10.73±3.22	80.91±4.66	2.95±1.89
76	50	4.45±3.29	12.02±1.96	74.63±3.74	9.82±3.68

Table 4-3. Primitive endoderm formation on days 13 and 15 post in vitro fertilization

No. of outgrowths	FGF2 (ng/ml)	Trophoblast cell Colony (%)	Primitive Endoderm Colony (%)
Day13			
63	0	98.48±3.71 [a]	1.51±1.51 [a]
55	0.5	90.24±6.43 [a]	9.76±6.44 [a]
61	5	76.85±3.52 [b]	23.15±3.52 [b]
56	50	66.36±3.65 [c]	32.12±3.74 [c]
Day15			
63	0	98.81±1.19 [a]	1.19±1.19 [a]
55	0.5	88.51±7.50 [b]	11.49±7.50 [b]
61	5	76.40±2.82 [c]	23.60±2.82 [c]
56	50	69.68±4.33 [c]	30.32±4.33 [c]

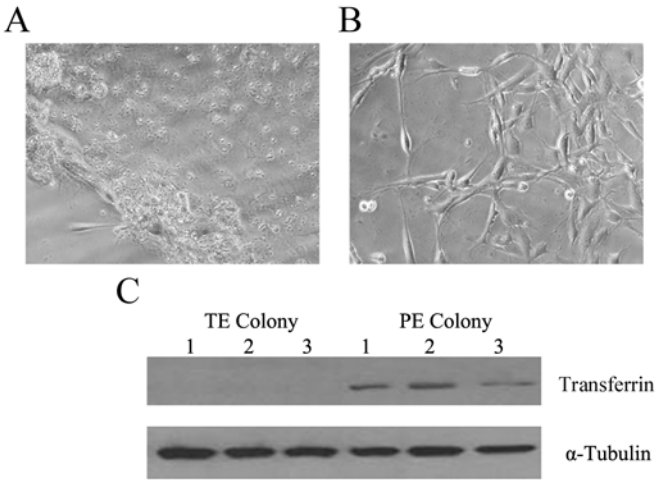

Figure 4-1. FGF2 dependent derivation of primitive endoderm. Bovine day 8 blastocysts were individually cultured in 48-well plate coated with Matrigel in growth medium containing 50ng/ml bovine recombinant FGF2. After 5 days, the morphology of blastocyst outgrowths were examined using a light microscope and the representative pictures of a trophoblast colony (*Panel A*) and putative primitive endoderm colony (*Panel B*) were recorded. To validate the lineage of this new cell type, cells were cultured for extra 5 days and cell lysates were electrophoresed, blotted onto PVDF membrane and immunoblotted with antibodies recognizing transferrin. Membrane were stripped and re-probed with an alpha-tubulin to serve as a loading control.

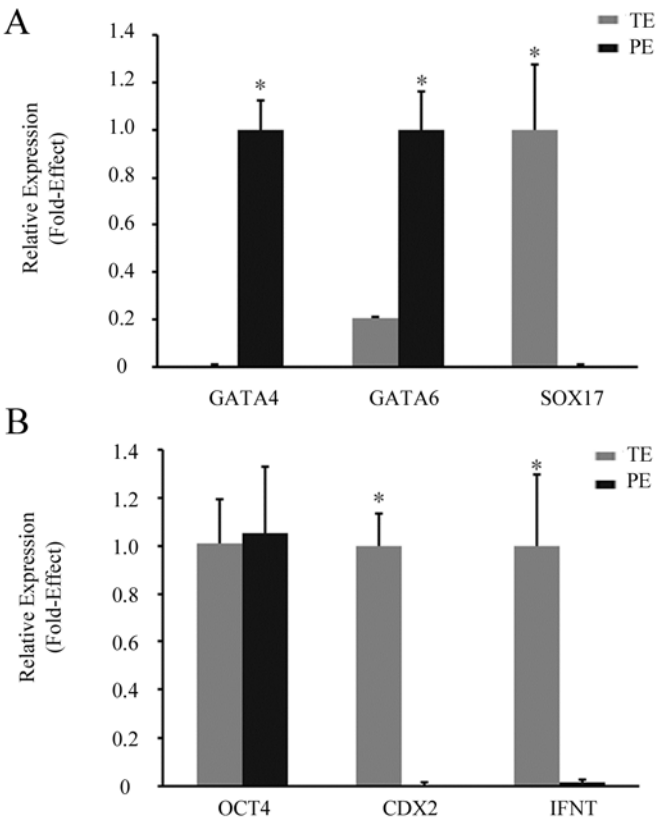

Figure 4-2. Lineage marker expression in primitive endoderm and trophoblast cells. Bovine day 8 blastocysts were individually cultured in 48-well plate coated with Matrigel in growth medium containing 50 ng/ml bovine recombinant FGF2. On day 20 post fertilization, single trophoblast and putative primitive endoderm colony (n=2 each) were collected for lineage marker expression analyses using real time RT-PCR. TcRNA were isolated and qRT-PCR was used to determine the relative abundance of *GATA4*, *GATA6*, *SOX17*, *OCT4*, *CDX2*, *IFNT* and *NANOG*. *GAPDH* RNA was used as the internal control. *NANOG* was under detectable in both lines, relative mRNA abundance of other genes was compared between trophoblast and primitive endoderm colonies. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). The *asterisk* (*) denotes a difference ($P < 0.01$).

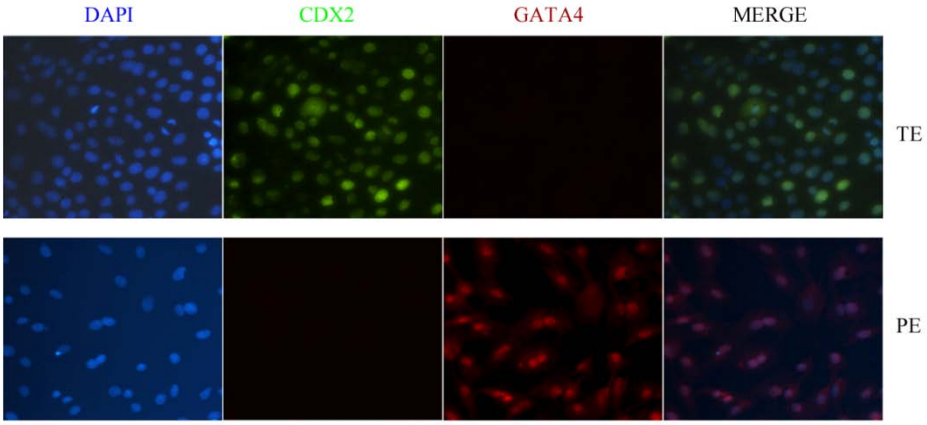

Figure 4-3. Immunostaining of *CDX2* and *GATA4* protein in trophoblast and primitive endoderm cells. Bovine trophoblast or primitive endoderm cells were fixed and co-stained with antibody against *CDX2* and *GATA4*. *CDX2* was only localized in the nucleus of trophoblast (TE) while *GATA4* was only positive in primitive endoderm cells (PE).

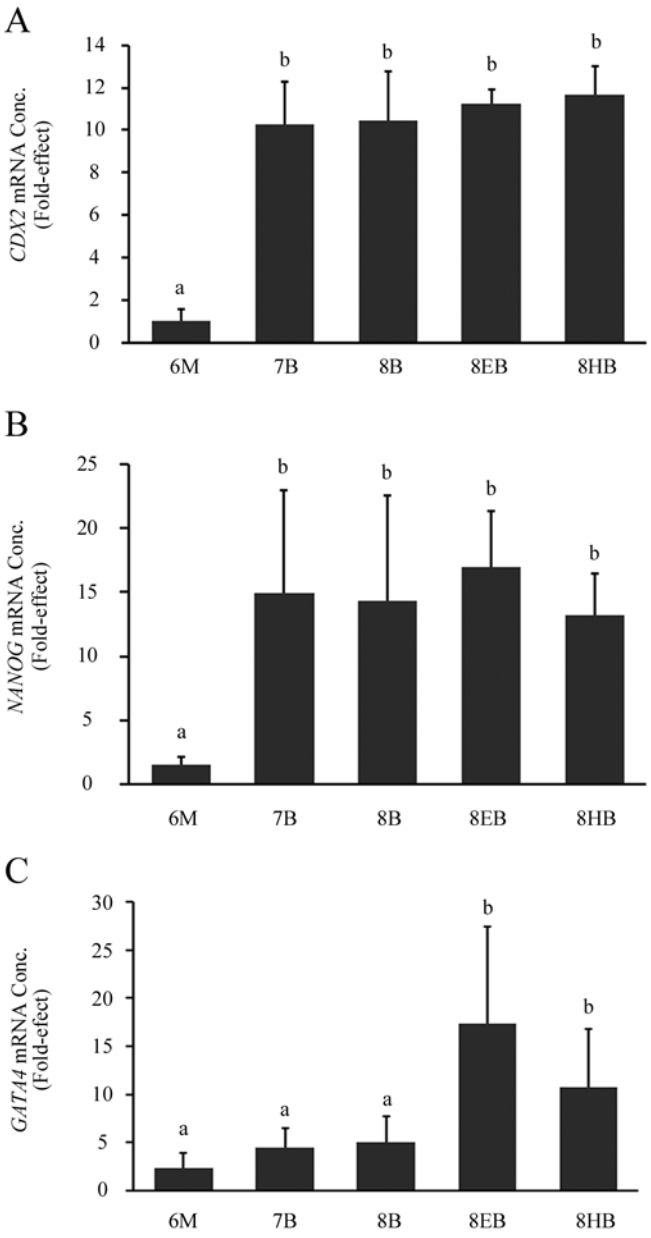

Figure 4-4. Lineage marker expression profiling during early development. Bovine *in vitro* produced morula, early blastocyst, blastocyst, expanded blastocyst and hatched blastocyst were collected between day 6 to day 8 (12-18 per group). TcRNA were isolated and qRT-PCR was used to determine the relative abundance of *CDX2, GATA4 and NANOG*. *GAPDH* RNA was used as the internal control. Relative mRNA abundance was compared between trophoblast and primitive endoderm colonies. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

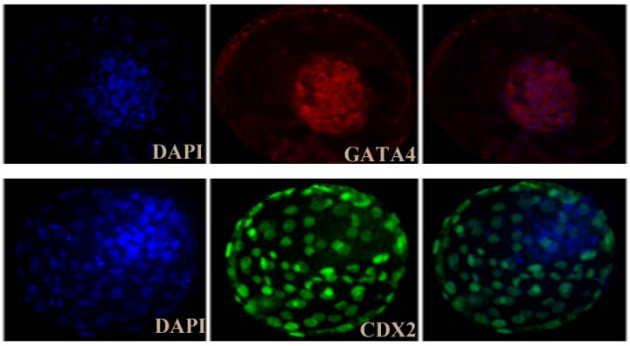

Figure 4-5. Immunostaining of *CDX2* and *GATA4* protein in blastocyst. Immunostaining of *CDX2* and *GATA4* in trophoblast and Primitive endoderm cells. bovine trophoblast or primitive endoderm cells were fixed and co-stained with antibody against *CDX2* and *GATA4*. *CDX2* was only localized in the nucleus of trophoblast (TE) while *GATA4* was only positive in a subpopulation cells within the inner cell mass.

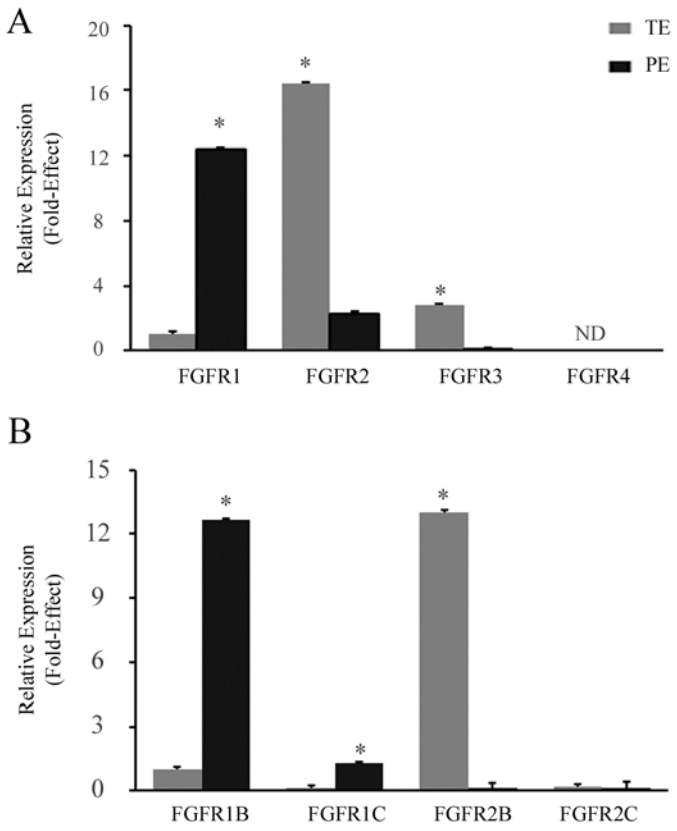

Figure 4-6. FGFR expression profiling is different between primitive endoderm and trophectoderm lineage. Bovine day 8 blastocysts were individually cultured in 48-well plate coated with Matrigel in growth medium containing 50ng/ml bovine recombinant FGF2. On day 20 post fertilization, single trophoblast and primitive endoderm colony (n=2 each) were collected for lineage marker expression analyses using real time RT-PCR. TcRNA were isolated and qRT-PCR was used to determine the relative abundance of *FGFR1, FGFR2, FGFR3, FGFR1b, FGFR1c, FGFR2b* and *FGFR2c*. *FGFR4* is non-detectable (ND). *GAPDH* RNA was used as the internal control. Relative mRNA abundance was compared between trophoblast and primitive endoderm colonies. Data are represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). The *asterisk* (*) denotes a difference ($P < 0.01$).

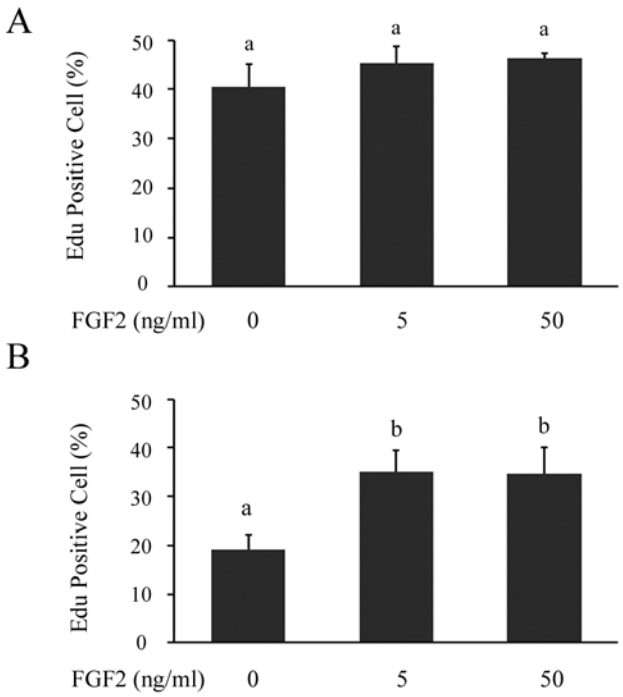

Figure 4-7. FGF2 promotes primitive endoderm proliferation *in vitro*. Trophoblast or primitive endoderm cells were serum starved for 24 h followed by 50 ng/ml FGF2 treatment for 2 h. At the end of FGF2 treatment, EdU reagent (20 µM) was added into each wells for 50 min. Cells were than fixed in 4% paraformaldehyde and proliferative index was examined using a fluorescence microscope. The ratio of EdU positive cells to total cell number (stained with 10 µM DAPI) are represented as mean± SEM (n=3 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

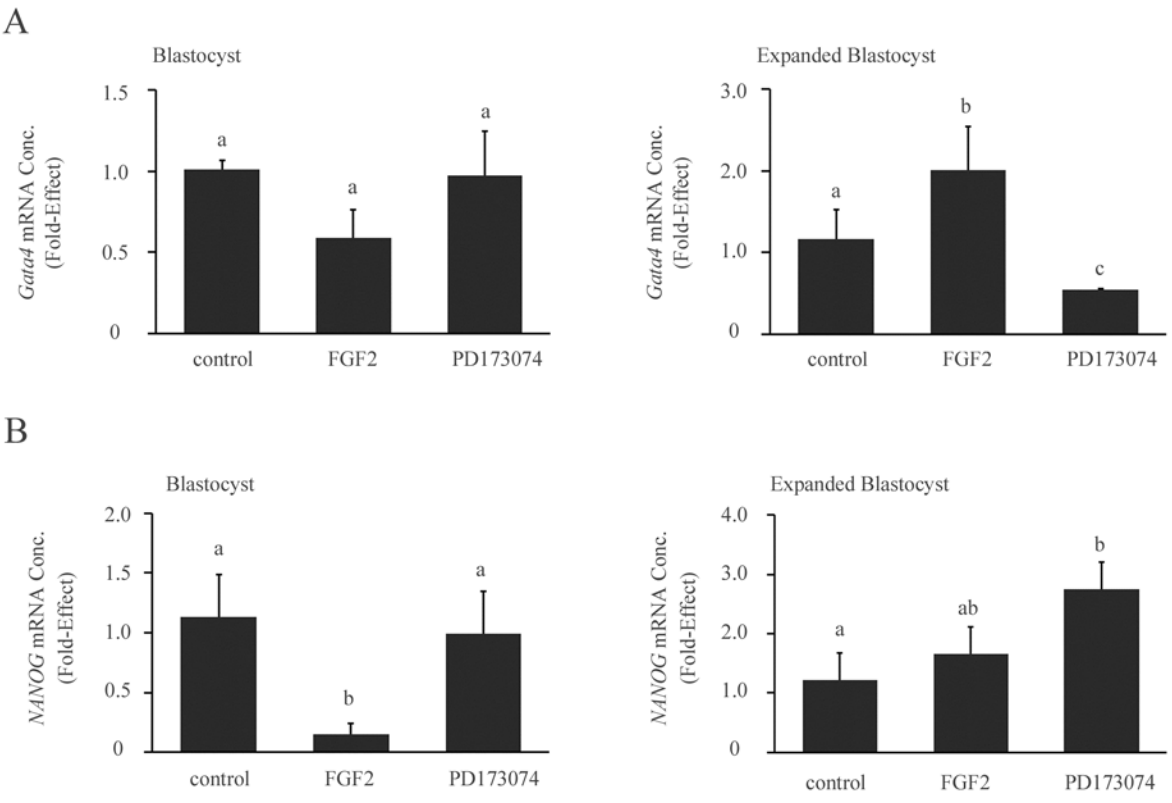

Figure 4-8. Activation or inhibition of FGF signal changes mRNA abundance of early lineage marker in blastocyst. Bovine day 8 blastocysts (*Panel A*) or hatched blastocysts (*Panel B*) (12 per group) were treated with 50 ng/ml FGF2, 1 μM PD173043 for 24 h. TcRNA were isolated and qRT-PCR was used to determine the relative abundance of *GATA4* and *NANOG*. *GAPDH* RNA was used as the internal control. Relative mRNA abundance of each transcript was represented as mean fold-differences ± SEM from the control value (n=3 replicate studies within each panel). Differences ($P<0.05$) are denoted within each panel with different superscripts.

CHAPTER 5
FIBROBLAST GROWTH FACTORS ACTIVATES MITOGEN ACTIVATED PROTEIN KINASE PATHWAYS TO PROMOTE MIGRATION OF OVINE TROPHOBLAST CELLS

Substantial conceptus development occurs in cattle, sheep and other ruminants prior to uterine adhesion and implantation. Unlike rodents and primates, where conceptuses begin implanting soon after hatching from the zona pellucida, ruminant conceptuses remain free-floating for an extended period. In cattle, trophoblast adhesion to the uterine lining is not evident until day 19-21 of pregnancy [321]. Extensive morphological changes in the conceptus occur prior to uterine attachment. Gastrulation and germ layer formation occur within the inner cell during this period. Notable changes also occur within the trophectoderm. Around day 12-13 in sheep and day 14-16 in cattle, a combination of rapid trophoblast cell proliferation and changes in trophoblast cell morphology cause the transformation of a spherical conceptus into an elongated and eventually a filamentous structure that occupies the majority of one uterine horn prior to implantation [247, 321, 322]. The rapid expansion in trophectoderm greatly increases surface area contact with the uterine epithelium and increases the overall production of interferon-tau (*IFNT*), the maternal recognition of pregnancy factor in these species [18, 247]. The timely achievement of both these events is required to maintain pregnancy in cattle and sheep [242, 243].

Uterine gland secretions, also known as histotroph, are required for ovine conceptus elongation [60, 206], and several uterine-derived cytokines and growth factors play critical roles in regulating pre- and peri-implantation conceptus development in cattle and sheep. Several members of the fibroblast growth factor (FGF) family have been implicated in controlling peri-implantation development. FGF2 is detected in luminal and glandular epithelial endometrium throughout the estrous cycle and early

pregnancy in cattle and sheep [28, 29]. FGF10 likely also affects peri-implantation development in ruminants. It is produced by stromal endometrium and functions as a paracrine mediator of epithelial cell function in the uterus [32]. Receptors for FGF2, FGF10 and many other FGFs are evident in bovine and ovine blastocysts and peri-implantation bovine and ovine conceptuses [29, 30, 32, 323]. One known activity of FGF2 and FGF10 in bovine trophoblast cell lines and blastocysts is the stimulation of *IFNT* mRNA and protein production [28, 30, 31, 324]. Additional activities of these paracrine factors likely exist, and one activity that was pursued in work described herein is the ability of FGFs to regulate migratory abilities of bovine and ovine trophoblast cells.

Trophoblast cell migration is one component to trophoblast cell reorganization and morphogenesis that plays an important role in mediating conceptus elongation [67, 321, 325]. Several uterine- and conceptus-derived factors induce trophoblast cell migration. These include epidermal growth factor (EGF), insulin-like growth factor 2 (IGF2), galectin-15 (SGAL15), Wnt5a and periostin (POSTN) [23, 176, 218, 223, 326, 327]. Several signaling molecules have been linked with this activity, and several of the aforementioned factors utilize mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), Rho-kinase or a combination of these pathways to control migration rates [176, 223, 327]. The potential involvement of FGFs in trophoblast cell migration has not been examined in ruminant species, but specific FGFs regulate cell migration in other systems [24, 267, 328-330]. Also, an embryonic-lethal phenotype exists in mice lacking FGF receptor 1 (FGFR1) because of failures in extraembryonic and mesoderm cell migration during gastrulation [331, 332]. A series experiments were conducted to establish that FGF2 and FGF10 stimulates ovine and

bovine trophoblast cell migration and determine whether ERK1/2, p38MAPK and JNK pathways are required for this activity.

Materials and Methods

Reagents

Unless indicated otherwise, cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA). Bovine recombinant (br) FGF2 was purchased from R&D Systems (Minneapolis, MN) and human recombinant (hr) FGF10 was purchased from Invitrogen Corp. (Carlsbad, CA). Matrigel^{TM} was purchased from BD Biosciences (San Jose, California). Transwell inserts were purchased from Corning Inc. (Lowell, MA). Prolong antifade reagent and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Corp. (Carlsbad, CA). Pharmacological inhibitors for MAPK kinases (MEK1/2; PD98059; upstream mediators of extracellular signal-related kinases [ERK1/2]), p38 MAPK (SB203580) and stress-activated protein kinase and Jun kinase (SAPK/JNK; JNK inhibitor I, cell permeable) were purchased from EMD Chemicals (Gibbstown, NJ). Antibodies recognizing phosphorylated or total ERK1/2, p38 MAPK and SAPK/JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). The proteinase and phosphatase inhibitors cocktails, BCA Protein Assay and BioMax film were purchased from ThermoFisher Scientific (Pittsburgh, PA). Polyvinylidene Difluoride (PVDF) membrane (Immobilon-P) was purchased from Millipore Co. (Bedford, MA). Enhanced chemiluminescence (ECL) western blot detection system was purchased from GE Healthcare (Piscataway, NJ). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Trophoblast Cell Culture

The ovine trophoblast cell line (oTr) was kindly provided by Dr. Thomas E. Spencer (Texas A&M University). Cells were cultured on plastic (non-Matrigel-coated) in DMEM/F12 medium containing 10% [v/v] fetal bovine serum (FBS), 700 nM insulin, 100 µM NEAA , 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphotericin B. Cells were passaged by enzymatic disruption (0.25% [w/v] trypsin) as described previously [218]. Cells were serum-starved by replacing medium with DMEM/F12 lacking FBS but containing all other supplements.

Bovine CT1 cells were propagated on Matrigel-coated plates in DMEM (with high glucose) containing 10% FBS, 100 µM non-essential amino acids (NEAA), 55 µM β-mercaptoethanol and antibiotic-antimycotic (100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B) at 38.5°C with 5% CO2 in air as described previously [28, 150]. CT1 cells were passaged manually by separating them from plates with a cell scraper and dissociating them into small clumps with repeated dissociation through a 20-ga needle. Cells were serum-starved by replacing medium with DMEM lacking FBS but containing all other supplements and a serum-substitute mix (10 µg/ml insulin, 5.5 µg/ml transferrin and 6.7 ng/ml sodium selenium).

Migration Assay

Trophoblast cell migration assays were completed as described previously [223] with minor modifications. Cells were serum-starved for 24 h before harvesting from plates. Cells (30,000 oTr or 50,000 CT1 in 100 µL serum-free medium) were seeded onto Transwell inserts (8-µm pores; Costar #3422). Treatments were added to each well (0, 0.5, 5 or 50 ng/ml FGF2 or FGF10) (n=3 wells/treatment). After 8 h for oTr1 cells and 12 h for CT1 cells, medium in the top chamber was removed and cells remaining on

the top chamber were removed with a cotton swab. Cells migrating through to the bottom side of the insert evaluated by fixation in 4% [w/v] paraformaldehyde for 15 min at room temperature and staining with 10 µM Hoechst 33342. After staining, membranes were removed from inserts and mounted on a glass slides with the migrating surface facing up, were overlaid with Prolong antifade reagent and then with a coverslip. Migrated cells were counted in seven no overlapping locations in each membrane using a Nikon TE2000 inverted phase epifluorescence microscope and CoolPix CCD camera and NIS-Elements Software (Nikon Corp., Melville, NY). Each study was repeated on at least three separate occasions with different batches of cells.

For studies examining the effects of pharmacological inhibitors on basal and FGF-induced cell migration, oTr cells were serum-starved for 24 h and treated with 50µM PD08059, 25 µM SB203580, 2 µM SAPK/JNK inhibitor or carrier only (0.1% DMSO) for the final 2 hours of serum-starvation. Cells then were harvested and seeded onto Transwell inserts containing 0, 50 ng/ml FGF2 or FGF10. The migration assay was terminated after 8 h as described previously.

Western Blots

Ovine cells (oTr) were serum-starved for 24 h, and then either 0 or 50ng/ml FGF2 or FGF10 was added to culture medium. Cells were harvested either immediately before adding FGFs (time 0) or 5, 15, 30, 60 or 120 min after treatment. Cells were rinsed with Dulbecco's PBS (DPBS) and dissolved in NP40 buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 20 mM EDTA, 1% [v/v] NP40) supplemented with protease and phosphatase inhibitor cocktail. Cell lysates were sonicated and supernatant protein concentrations were determined using a BCA Protein Assay. Protein samples (20 µg) were loaded and separated on 10% [w/v] SDS-PAGE gels and transferred onto 0.45 µm

PVDF. Membranes were blocked with 5% [w/v] nonfat dry milk in TBST (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% [v/v] Tween-20), then incubated overnight at 4°C with antibodies against phosphorylated-ERK1/2 (1:2000), phosphorylated-p38 MAPK (1:2000) or phosphorylated SAPK/JNK (1:1000). Horseradish peroxidase-conjugated anti-rabbit IgG were used in combination with ECL to visualize reactive bands after exposure to BioMax film. After detection, membranes were washed with TBST, stripped according to manufacturer's instructions and used to detect total-ERK1/2 (1:2000), total-p38MAPK (1:2000) and total-SAPK/JNK (1:2000). Three independent western blots generated from different batches of oTr cells were completed, and band intensities were quantified after scanning using ImageJ software (NIH, USA). Representative immunoblots were photographed and presented in figures.

Statistical Analyses

All analyses were performed by least-squares ANOVA using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between individual means were compared using pairwise comparisons [PDIFF (probability of difference) analysis in SAS]. Results were presented as the mean ± SEM. A p-value < 0.05 was considered to be significant.

Results

FGF2 and FGF10 Stimulate Migration of Ovine Trophoblast Cells

Several FGFs, including FGF2 and 10 stimulate chemotaxis and migration in several cell types [24, 267, 328-330]. Since these FGFs are present in the uterus during peri-implantation development in cattle and sheep, we set out to determine if FGF2 and 10 regulate trophoblast cell migratory activity by examining how these factors impact oTr cell migration *in vitro*. This cell line was developed from trophoblast cells

derived from an elongating ovine conceptus (day 15 of gestation) [218] and has been used extensively to examine how uterine factors affect various aspects of trophoblast activity, including cell migration [23, 176, 218, 326, 333]. Studies were completed using a previously described assay examining the movement of oTr cells through Corning Transwell inserts containing 8-μm pores [218, 223]. Supplementation with 0.5 ng/ml FGF2 or 10 did not affect oTr migration but providing 5 or 50 ng/ml FGF2 or 10 increased ($P<0.05$) the percentage of migrated cells after 8 h when compared with non-treated controls (Fig. 5-1A&B).

FGF2 and 10 Stimulate ERK1/2, p38 MAPK and SAPK/JNK Activity in oTr Cells

Several MAPK pathways have been linked with trophoblast cell migration [325, 327]. Since FGFs utilize MAPK signals for various purposes in many cell types [334], studies were completed to identify specific MAPKs involved with basal- and FGF-induced migration of oTr cells.

The first MAPKs examined were ERK1/2. Western blot analyses (Fig. 5-2A&C) and subsequent analysis of densitometry scanning of ERK1/2 band intensities (Fig. 2B&D) determined that both FGF2 and FGF10 increased ($P<0.05$) ERK1/2 phosphorylation status within 5 min of treatment. This stimulation in phosphorylation remained greater ($P<0.05$) than controls for 60 min for ERK2 and at least 120 min for ERK1. Changes in ERK1/2 phosphorylation status were not assessed after 120 min.

A separate set of studies determined that FGF2 and 10 also stimulated p38 MAPK activation in oTr cells (Fig.5-3). Increases ($P<0.05$) in p38 MAPK phosphorylation were evident within 5 min after initiating FGF2 and 10 treatments. Effects were short-lived, and phosphorylation status of p38 MAPK returned to pre-treatment levels after 15 min

for FGF2-treated samples (Fig. 3 A&B) and after 30 min for FGF10-treated samples (Fig. 3C&D).

Another set of studies determined that FGF2 and 10 also regulate SAPK/JNK in oTr cells (Fig. 5-4). FGF2 induced a rapid increase ($P<0.05$) in SAPK/JNK phosphorylation within 5 min after treatment (Fig. 5-4A&B). This effect was short-lived, and SAPK/JNK phosphorylation status returned to basal levels 15 min after FGF2 treatment. FGF10 treatment also caused a rapid phosphorylation in SAPK/JNK within 5 min (Fig. 5-4C&D). However, SAPK activation remained greater ($P<0.05$) than the pre-treatment level at 15 min and returned to basal levels after 30 min whereas JNK phosphorylation remained elevated at 15 and 30 min and returned to pre-treatment levels after 60 min.

ERK1/2, p38 MAPK and SAPK/JNK Mediate FGF2/10 Effects on oTr Cell Migration

Specific pharmacological inhibitors against ERK1/2 (PD98059), p38MAPK (SD203580) and SAPK/JNK (JNK inhibitor I) were utilized to examine whether these kinases are utilized by FGF2 and/or 10 to mediate oTr cell migration. Each inhibitor was added to cultures 2 h before FGF supplementation. Similar concentrations of each inhibitor were used in previous studies on oTr cells [223, 335]. Western blot analyses verified that treating oTr cells with each inhibitor for 2 h prevented phosphorylation of each respective MAPK after FGF2 or FGF10 treatment (data not shown). None of the pharmacological inhibitors increased the incidence of apoptosis in oTr cells (data not shown).

Specific MAPKs that transduced FGF2 and FGF10 effects in oTr cells were described by pre-treating oTr cells with specific MAPK inhibitors 2 h before FGF supplementation and initiation of the migration assay (Fig. 5-5). In the absence of

inhibitors, FGF2 and FGF10 increased (P<0.05) oTr migration. Pre-treatment with PD98059 (ERK1/2 inhibitor) did not affect oTr migration in non-FGF-treated samples but prevented FGF2- and FGF10-induced increases in cell migration. Similarly, pre-treatment with SB203580 (p38 MAPK inhibitor) did not affect migration in non-FGF-treated cells but prevented FGF2 and FGF10-dependent increases in cell migration. The SAPK/JNK inhibitor (JNK inhibitor I) caused a slight numerical reduction in oTr migration in non-FGF-treated cells that did not reach statistical significance (P=0.1). The SAPK/JNK inhibitor blocked FGF2- and FGF10-dependent increases in cell migration.

FGF2 and FGF10 Stimulate Migration of Bovine Trophoblast Cells

A final study was completed to determine if the migratory activity of a bovine trophoblast cell line could be influenced by FGF2 and 10. The CT1 cells were used [28, 256]. These cells migrated across 8-µm pores at a somewhat delayed rate when compared with oTr cells, and the assay time was extended from 8 to 12 h to compensate for this difference in basal migratory activity (data not shown).

FGF2 or FGF10 influenced the migratory activity of CT` cells (Fig. 5-6). Migration of CT1 cells was increased (P<0.05) by supplementation with 0.5 ng/ml FGF2 and greater concentrations of FGF2 induced further increases in CT1 migration rates (Fig. 5-6A). Specifically, CT1 migration rate was increased further (P<0.05) in cells supplemented with 50 ng/ml FGF2 (2.6 ± 0.6 fold increase over non-treated controls). In FGF10-treated CT1 cells, supplementation at 0.5 ng/ml did not affect cell migration but supplementation with 5 or 50 ng/ml FGF10 increased (P<0.05) migration indices over non-treated controls (2.0 ± 0.2 fold increase in cells treated with 50 ng/ml FGF10 versus non-treated controls) (Fig. 5-6B).

Discussion

The purpose of this work was to describe new biological activities for FGF2 and 10 during early conceptus development in ruminants. The timing of uterine and conceptus FGF2 and 10 production suggest they may be important regulators of early conceptus development in ruminants. FGF2 is produced primarily in luminal and glandular epithelium throughout the estrous cycle and early pregnancy in cattle and sheep [29, 30]. In the ewe, uterine luminal FGF2 protein concentrations increase at days 12-13 post-estrus coincident with conceptus elongation in this species [29]. It is unknown if cattle also experience an increase in uterine FGF2 during conceptus elongation. FGF2 mRNA also is detected in bovine conceptuses throughout pre- and peri-implantation periods but the predominant conceptus-derived FGF transcript identified in elongating and filamentous bovine conceptuses is FGF10 [30]. The uterine stroma also produces FGF10 throughout the estrous cycle and early pregnancy and it is likely that some of this reaches the uterine lumen before implantation [32, 33].

It also is evident that conceptuses are responsive to FGFs during early pregnancy. Bovine and ovine pre- and peri-implantation conceptus expresses multiple FGF receptor isoforms, including those that interact with FGF2 and 10 [29, 30, 32]. One recently identified activity of FGFs in bovine trophoblast cells is the stimulation of *IFNT* production [28-31]. FGF2 and likely other FGFs increase *IFNT* mRNA and protein abundance in trophoblast cell lines and blastocysts by regulating the activity of a novel protein kinase C, termed PKC-delta (Chapter 3). Also, FGF2 supplementation to bovine blastocysts increased the incidence of primitive endoderm outgrowth formation in extended cultures (Chapter 4). This activity was controlled by FGF2 stimulating

endoderm proliferation and potentially by promoting endoderm lineage commitment in IVF-derived blastocysts.

This project was completed to determine if FGF2 and 10 participate in conceptus elongation in ruminants. The mechanisms controlling conceptus elongation are not well understood in ruminants, but there is ample evidence that uterine histotroph is required for elongation to proceed normally. In the ewe, conceptuses generated in ewes lacking uterine glands, the primary source of histotroph, fail to elongate properly [204, 225]. Also, ovine conceptuses collected at day 12 post-mating failed to elongate after their removal from the uterus, but reacquired the ability to elongate after their transfer back into uteri of surrogates [336]. In cattle, blastocysts cultured for extended periods in agarose tubes with medium containing large amounts of serum acquire an elongated-like phenotype, but the rate of this elongation is much slower than observed *in utero* [337, 338]. Trophoblast migratory activity is one of several cell activities that likely contribute to conceptus development and elongation. It has been postulated that the differentiation, morphogenesis and reorganization events controlling conceptus development and elongation in ruminants may be examined in vitro by studying how uterine factors regulate cell migration, proliferation, adhesion and motility [67, 339].

This work demonstrated that supplementation with FGF2 or FGF10 increased ovine and bovine trophoblast cell migration *in vitro*. The oTr cell has been used previously to discover several uterine-derived migratory factors [218, 327]. Several other uterine factors are implicated in controlling conceptus development. Two uterine factors identified in the sheep are IGFBP1 and LGALS15. The expression of both factors increases in luminal and glandular epithelium around the time of conceptus

elongation, and supplementing these factors increased oTr cell migration and adhesion [23, 218, 340]. In cattle, IGFBP1 expression also increases around the time of elongation [23], suggesting it plays similar roles during peri-implantation development in both species. The same may not be said for LGALS15. The bovine homolog is not produced in the bovine uterus during diestrus and early pregnancy [340], and currently it is unclear whether a paralog for LGALS15 contains a uterine-dependent expression profile or if this factor or a related molecule is not required during early pregnancy in cattle.

Another interesting facet of previous work is that these migratory factors have little influence of trophoblast proliferation. IGFBP1 and LGALS15 have little to no effect on oTr cell proliferation [23, 218, 340]. FGF2 and 10 also have little to no effect on bovine trophoblast or blastomere proliferation [28, 30, 31] (Yang et al., endoderm Chapter 4). These observations indicate a divergence in trophoblast control of these activities.

The MAPK signaling modules were examined to describe those utilized by FGF2 and 10 in regulating oTr migration. FGFs are well-known activators of MAPK pathways in several cells [334], and examining the Ras/MEK/ERK pathway was of special interest given the importance of this pathway, and ERK2 in particular, in promoting trophoblast lineage specification and placental cell differentiation in mouse embryos [116, 341]. FGF2 and 10 increased ERK1/2 phosphorylation status in oTr cells. The ERKs exhibited differential responses to FGF activation, with ERK1 phosphorylation lasting longer lasting than for ERK2 (>120 versus 60 min). The importance of this outcome remains unclear. Exposure to the MEK1/2 inhibitor prevented FGF1 and 10 from activating ERK1/2, and exposure to this inhibitor prevented both FGFs from stimulating

oTr migration. The results implicate ERK1 or 2, or perhaps both, as mediators of FGF2- and 10-dependent migration in these cells. Other factors also utilize the Ras/MEK/ERK pathway to control ovine and bovine trophoblast migration. Kim *et al* (2008) determined that IGF2 activated ERK1/2 in oTr cells and stimulated oTr migration. The essentiality of ERK1/2 for this activity was not examined in that work. EGF also activated ERK1/2 in F3 cells, a bovine trophoblast line derived from a mid-gestation bovine placenta, and inhibiting these kinases interfered with EGF-induced cell migration and motility [327, 342].

The p38 MAPK and SAPK/JUNK pathways also are activated by FGF2 and 10 in oTr cells, and inhibiting these kinases prevented FGF-mediated migration in these cells. FGF2 also induces p38 MAPK in CT1 cells (Yang, PKC chapter), and several uterine factors regulate p38 and SAPK/JNK phosphorylation in oTr cells [176, 218, 223]. These observations indicate that several MAPK systems must be activated to promote trophoblast migration. It also remains possible that other signaling systems also are required for FGF2 and 10 to control cell migration. PI3K, FRAP1 and Rho-kinase pathways are associated with uterine-dependent activation of trophoblast cell migration in other studies [176, 218, 223, 327]. The need for such multiplicity of signaling for controlling migratory activity in trophoblast cells is not clear. Perhaps migratory activity is highly coordinated in these cells to ensure that conceptus development only progresses when several uterine factors exist in concentrations needed to induce the correct combination of signaling molecules.

Although our understanding of conceptus development and elongation in ruminants is far from complete, this work describes a new activity for uterine- and

conceptus-derived FGFs that may be of importance in regulating conceptus development during the second and third weeks of pregnancy in cattle and sheep. Insight also was made describing cellular mechanisms that respond to FGFs and control trophoblast cell migration.

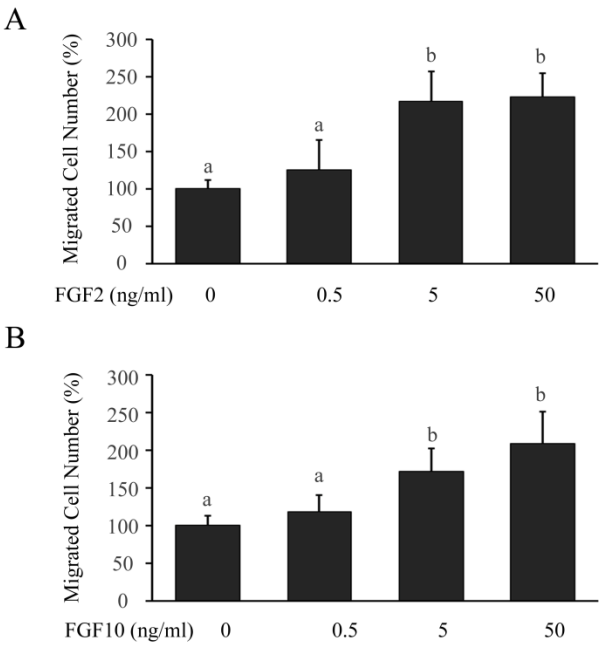

Figure 5-1. Supplementation with FGF2 and FGF10 promotes ovine trophoblast cell (oTr) migration. Cells were serum-starved for 24h, then were trypsinized and seeded on Transwell inserts containing 8-µm pores (30,000 cells/insert) in serum-free medium containing 0, 0.5, 5 or 50 ng/ml brFGF2 (*Panel A*) or hrFGF10 (*Panel B*). Cells migrating to the lower chamber of the insert were counted 8 h later. Results represent means and SEM of fold-differences relative to control values (n=5 replicate studies in Panel A and 4 replicate studies in Panel B). Different superscripts denote differences ($P<0.05$) between FGF treatments within each panel.

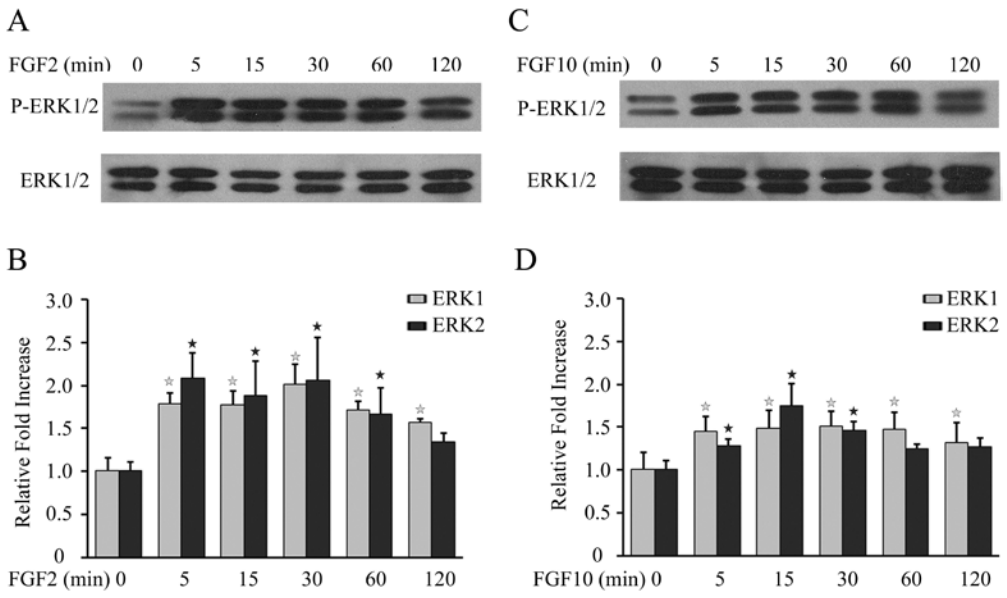

Figure 5-2. Erk1/2 phosphorylation status before and after oTr supplementation with FGF2 or FGF10. Western blotting was used to examine changes in ERK1/2 phosphorylation status. oTr cell lysates were collected either immediately before (time 0) or at specific periods after treatment with 50 ng/ml FGF2 or FGF10. Lysates were electrophoresed, blotted onto PVDF membrane and immunoblotted with antibodies recognizing phosphorylated ERK1/2 (P-ERK1/2) or total ERK1/2. Four independent studies were completed and a representative blot for outcomes from supplementing FGF2 (*Panel A*) and FGF10 (*Panel C*) is provided. As anticipated, two immunoreactive bands of the correct molecular mass were observed in all blots. The higher molecular mass band represents ERK1 and the lower band represents ERK2. The relative intensities of ERK1 (gray) and ERK2 (black) bands over time were quantified and analyzed for cells supplemented with FGF2 (*Panel B*) or FGF10 (*Panel D*). Data are presented as mean fold-differences ± SEM from the control value. An asterisk (*) denotes an effect of FGF2 or 10 treatment that differed from the control value ($P < 0.01$).

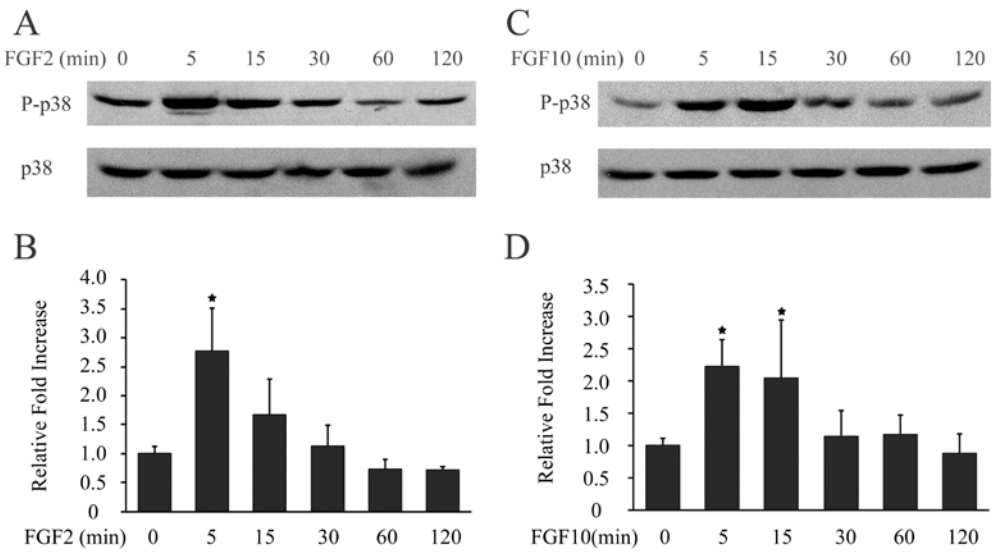

Figure 5-3. p38 MAPK phosphorylation status is enhanced by FGF2 and FGF10 in oTr cells. Western blotting was used to examine changes in p38 MAPK phosphorylation status. oTr cell lysates were collected either immediately before (time 0) or at specific periods after treatment with 50 ng/ml FGF2 or FGF10. Lysates were electrophoresed, blotted onto PVDF membrane and immunoblotted with antibodies recognizing phosphorylated (P-p38) or total (p38) p38 MAPK. Three independent studies were completed, and a representative blot for outcomes from supplementing FGF2 (*Panel A*) and FGF10 (*Panel C*) is provided. A single immunoreactive band of the correct molecular mass was observed in all blots. The relative intensities of bands over time were quantified and analyzed to determine the effects of treatment with FGF2 (*Panel B*) or FGF10 (*Panel D*). Data are presented as mean fold-differences ± SEM from the control value. An asterisk (*) denotes an effect of FGF2 or 10 treatment that differed from the control value ($P < 0.01$).

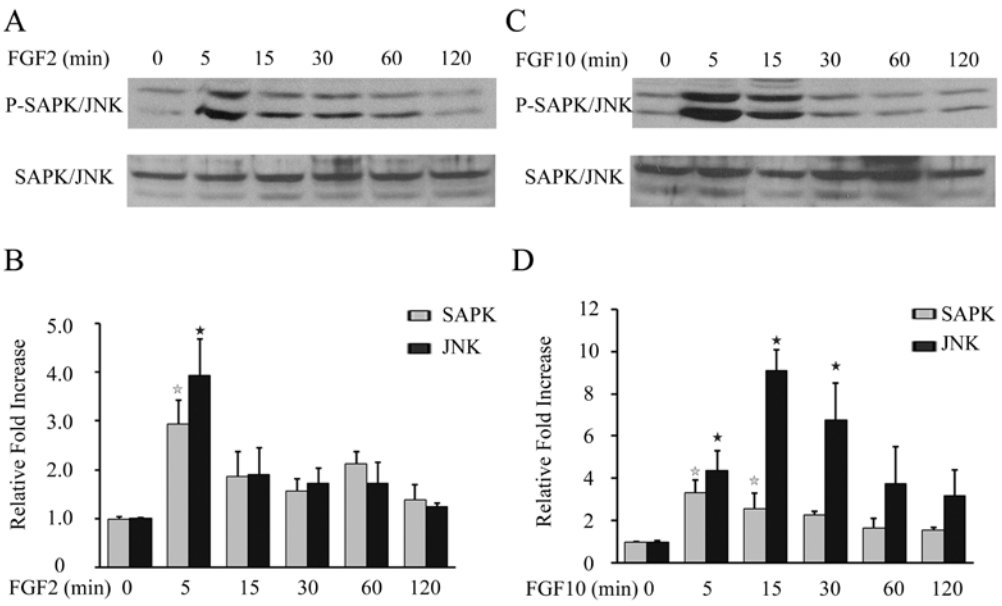

Figure 5-4. SAPK/JNK phosphorylation status is increased in oTr cells after supplementation with FGF2 or FGF10. Western blotting was used to examine changes in SAPK/JNK phosphorylation status. oTr cell lysates were collected either immediately before (time 0) or at specific periods after treatment with 50 ng/ml FGF2 or FGF10. Lysates were immunoblotted with antibodies recognizing phosphorylated (P-SAPK/JNK) or total (SAPK/JNK) MAPK. Four independent studies were completed, and a representative blot for outcomes from supplementing FGF2 (*Panel A*) and FGF10 (*Panel C*) is provided. Two immunoreactive bands of the correct molecular mass were detected in all blots. The higher molecular mass band represents SAPK and the lower band represents JNK. The relative intensities of SAPK (gray) and JNK (black) bands over time were quantified and analyzed to determine the effects of treatment with FGF2 (*Panel B*) or FGF10 (*Panel D*). Data are presented as mean fold-differences ± SEM from the control value. An asterisk (*) denotes an effect of FGF2 or 10 treatment that differed from the control value ($P < 0.01$).

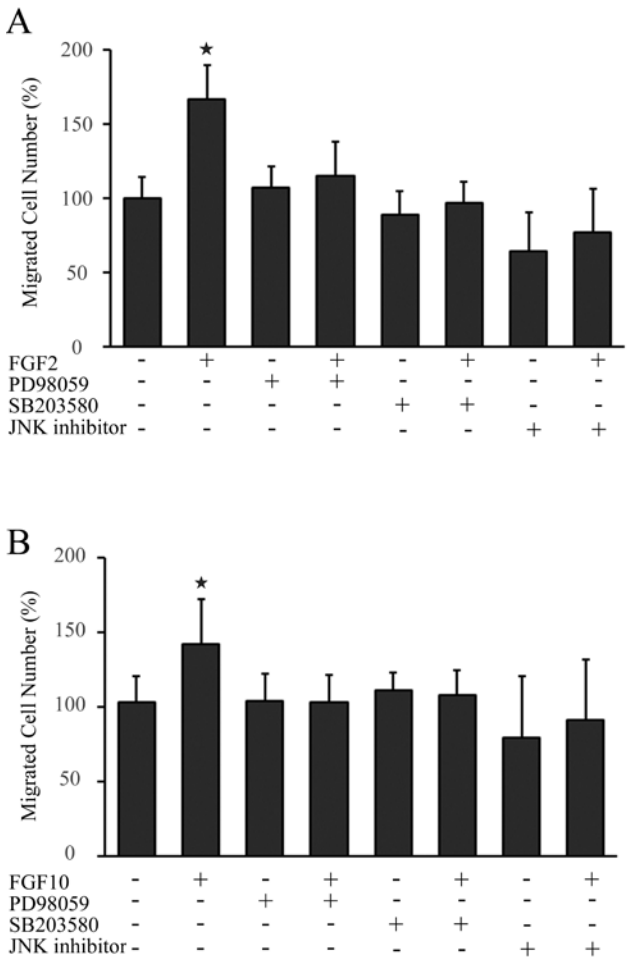

Figure 5-5. Delineation of MAPK molecules involved with FGF2- and FGF10-dependent increases in oTr cell migration. Cells were serum-starved for 24 h. Either 50µM PD98059 (ERK1/2 inhibitor), 25 µM SB203580 (p38 MAPK inhibitor), 2 µM JNK inhibitor (SAPK/JNK inhibitor) or DMSO (vehicle) was added to cultures for the last 2 h of serum-starvation. Cells were trypsinized and seeded on Transwell inserts containing 8-µm pores (30,000 cells/insert) in serum-free medium containing 0 or 50 ng/ml FGF2 (*Panel A*) or 0 or 50 ng/ml FGF10 (*Panel B*). Cells migrating to the lower chamber of the insert were counted 8 h later. Results represent means and SEM of fold-differences relative to control values (n=4 replicate studies in each panel). An asterisk (*) denotes treatment effects that differ significantly from the control value ($P<0.05$).

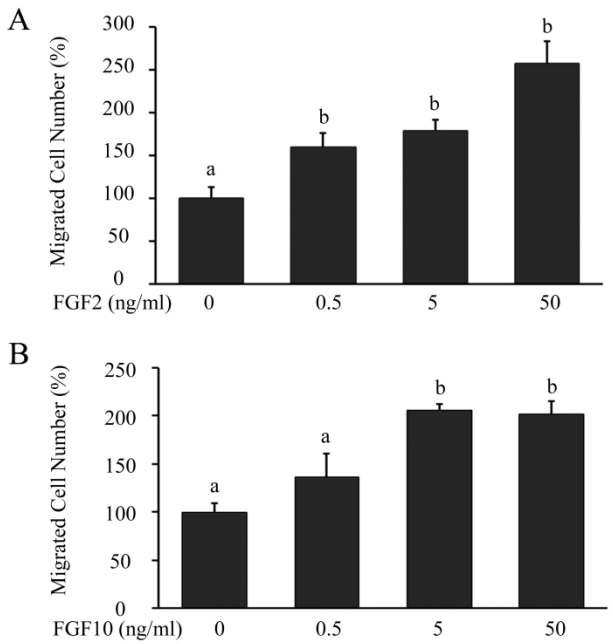

Figure 5-6. FGF2 and FGF10 treatment promotes the migration of bovine trophoblast cells (CT1). Cells were serum-starved for 24 h prior to their collection. Cells were seeded onto Transwell inserts containing 8-µm pores (50,000 cells/insert) in serum-free medium containing 0, 0.5, 5 or 50 ng/ml rbFGF2 (*Panel A*) or rhFGF10 (*Panel B*). Cells migrating to the lower chamber of the insert were counted 12 h later. Results represent means and SEM of fold-differences relative to control values (n=5 replicate studies in Panel A and 4 replicate studies in Panel B). Different superscripts denote differences ($P<0.05$) between FGF treatments within each panel.

CHAPTER 6
SUMMARY AND DISCUSSION

Multiple lines of evidences suggest that defects in conceptus elongation and miscues in *IFNT* signaling are prominent causes of early pregnancy loss in dairy cattle [1, 2, 6]. This lab previously described that uterine-derived FGFs regulate *IFNT* production [28]. The focus of the present work was to elucidate the underlying mechanisms by which FGF signaling regulates *IFNT* production in bovine trophoblast cells. At the same time, an attempt was made to discover the new roles for FGF in regulating conceptus growth and development in bovine.

Firstly, using two established cell lines (CT1 and Vivot) and primary trophoblast outgrowths, the novel protein kinase C member, PKC delta was identified as the predominant mediator of FGF2-induced *IFNT* production in trophoblast cells. The working hypothesis in this lab is that uterine-derived FGFs stimulate the maximal *IFNT* production and therefore play an important role in establishing the two-way communication between the embryo and uterus during the establishment of a pregnant state in ruminants. Because of the multiplicity of FGFRs found in trophoblast cells, it is unlikely to expect that a single FGFR subtype is targeted by FGFs. FGFR1, FGFR2, FGFR3 all were detectable in trophoblast cell and it is well established that these receptors can play compensatory role during development. By contrast, PKC delta is a conserved intracellular molecule potentially used by multiple FGFR subtypes, and this factor likely serves as bridge for several FGFs to stimulate *IFNT* production. For this reason, PKC-delta would be ideal target for testing some of the FGFR-mediated effects in peri-implantation conceptuses. It is unclear if PKC-delta serves other critical roles

during conceptus elongation, and addressing this question should be given high priority in future work.

A second project was conducted to uncover the role of FGF2 and potentially other FGFs in mediating early bovine embryogenesis. Bovine trophoblast stem cell-like cell lines have been established in several labs using *in vitro and* in *vivo* derived blastocysts as well as blastocysts generated from somatic cell nuclear transfer. Different from mouse trophoblast stem cells, the derivation and maintenance of these cells were not dependent on FGF4 supplementation. The generation of endoderm outgrowths required FGF4 and its signaling effects in mice and until completion of this work it was unknown if FGFs also were needed to mediate primitive endoderm lineage specification and proliferation in ruminants. The FGF2-dependent signaling modulates the expression of lineage markers in blastocysts, suggesting that they likely affect lineage specification in addition to promoting endoderm proliferation. Future lineage trace experiments should be completed to further examine how this signal changes other gene expression. One gene of particular interest is *NANOG*. Current findings suggest that FGF signaling plays a role in controlling the expression level of *NANOG*. This is important because no convincing stem cell lines have been reported in ruminant animals. Future work should address if blocking FGF downstream signaling could maintain the pluripotency of epiblast.

Conceptus elongation is one of the development puzzles that have attracted the attention of several animal scientists over the last a couple of decades. Conceptus elongation does not occur in culture systems spontaneously or even in the uterus with impaired uterine glands [206]. Based on an early observation that cell activities such as

migration and invasion are associated with phenotypes that trophoblast cells behavior during blastocyst elongation [343], endeavors have focused on discovering the role of uterine derived factors on these activities in trophoblast cells [218, 223, 344, 345]. Trophoblast migration was sued as the endpoint for examining if FGF2 and 10 potentially are involved with events associated with elongation. As a potent factor that stimulates migration of other cell types, FGF2 and 10 are discovered as migration inducers in ruminant trophoblast cells. Using two different cell lines from bovine and ovine, this study concluded that FGF2 and FGF10 activates multiple MAPK signaling transduction cascades to regulate trophoblast cell migration. The cellular pathways utilized by FGF2 to control cell migration are different from the one used to regulate *IFNT* production. Further experiments are necessary to elucidate how this factor triggers different cellular events through different intracellular mechanisms.

In summary (as shown in figure 6-1), uterine derived FGF2, mesenchymal-secreted FGF10 and potentially other FGFs produced by uterine endometrium or embryo participates several events including primitive endoderm development, *IFNT* production and trophoblast migration to regulate conceptus development and influence uterine response to pregnancy.

Understanding the cellular and molecular mechanisms that regulate early embryogenesis and conceptus elongation is crucially important not only because this kind of knowledge will fulfill our scientific curiosity but also because it is the inevitable step toward recovering a serious problem in mordent dairy industry: early pregnancy loss.

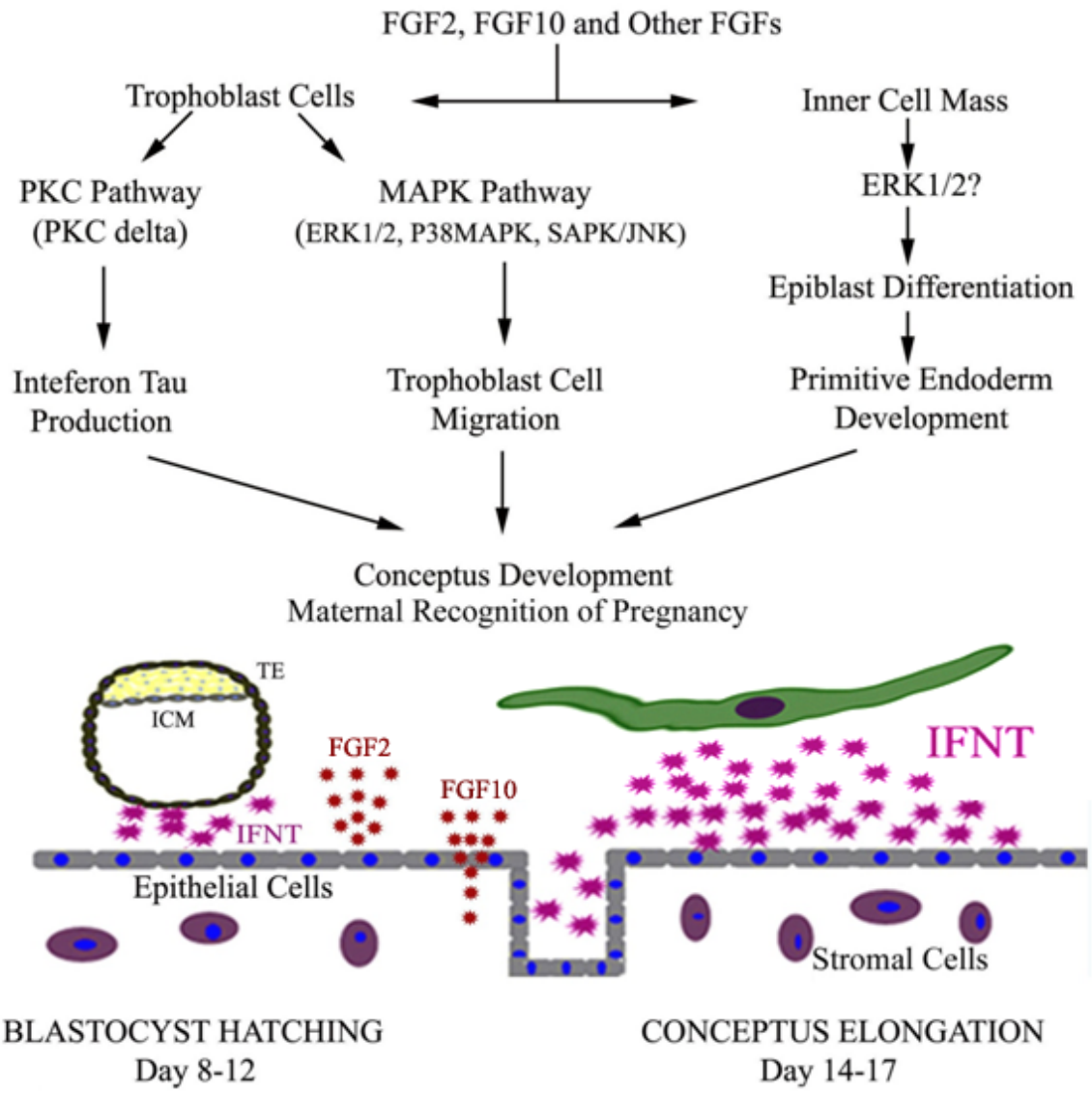

Figure 6-1. The actions of FGF signaling during peri-implantation development in bovine: a suggested model. Embryogenesis and peri-implantation development in ruminant is a unique process featured by conceptus elongation which occurs between days 14 to 17 post fertilization. During this period of development, trophoblast cells undergo proliferation, migration and differentiation. Meanwhile, mononucleate trophoblast cells secret interferon tau (*IFNT*) to initiate the maternal recognition of pregnancy. Uterine epithelial derived fibroblast growth factor 2 (FGF2) and stromal cell secreted FGF10 regulate trophoblast functions such as *IFNT* production and trophoblast migration. In expanded blastocysts, although FGF signaling also stimulates *IFNT* secretion by cells from trophectoderm lineage (TE), more importantly, FGF plays a critical role in promoting primitive endoderm development, thus contributing early lineage segregation in this species.

APPENDIX A
FIBROBLAST GROWTH FACTOR 2 AND 10 MEDIATE TRANSCRIPT ABUNDANCE OF SELECTIVE INTEGRINS AND METALLOPROTEASE 2 IN BOVINE BLASTOCYSTS AND TROPHOBLAST CELLS

This laboratory recently completed a preliminary examination of how FGF2 and FGF10 affects the bovine blastocyst transcriptome by using a bovine microarray (Zhang, Yang and Ealy; Unpublished observations), and several molecules involved with cell attachment, migration and invasion were induced with FGF2 and/or FGF10 treatment. One of these was metalloprotease 2 (*MMP2*), a gelatinase that is well studied for its involvement with controlling trophoblast cell attachment, migration, invasion and other aspects of implantation [325, 346]. This and other MMPs, notably *MMP9*, and their inhibitors are expressed throughout gestation in the bovine endometrium and placenta [347]. Increases in *MMP2* mRNA abundance are detected in mouse trophoblast stem cells treated with FGF4, and increases the enzymatic activity of *MMP9* occur in human cytotrophoblasts treated with FGF4 or FGF10 but not FGF2 [285, 348]. Roles for FGF mediation of *MMP* expression and biological activity has not been described in ruminant placentae.

Several transcripts that encode integrins also were found in greater abundance in FGF2 and FGF10-treated blastocysts in the preliminary microarray analysis. These transmembrane proteins mediate various aspects of cell-to-cell and cell-to-extracellular matrix interactions during implantation [349]. Integrins are essential components of trophoblast cell attachment, adhesion and migration in various species [333, 350-352]. Potential roles for FGFs in regulating integrin expression and activity in trophoblast cells have not been described. We hypothesize that FGF2 and 10 control trophoblast cell migration and other aspects of early placental development and implantation by

controlling the expression of selective integrins and *MMP2*. An expression profile study was completed to determine whether FGF2 and 10 affects the relative abundance of integrin and *MMP2* transcript abundance in bovine blastocysts and trophoblast cells.

Materials and Methods

Reagents

Unless indicated otherwise, cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA). Matrigel™ was purchased from BD Biosciences (San Jose, California). Bovine recombinant (br) FGF2 was purchased from R&D Systems (Minneapolis, MN) and human recombinant (hr) FGF10 was purchased from Invitrogen Corp. (Carlsbad, CA). The PicoPure™ RNA isolation kit was purchased from MDS Analytical Technologies (Sunnyvale, CA). Trizol™ reagent and the PureLink™ RNA Mini Kit were purchased from Invitrogen. RNase-free DNase was purchased from New England Biolabs (Ipswich, MA). PCR primers were synthesized by Invitrogen. The High Capacity cDNA Reverse Transcription kit, SYBR® Green Detector System and consumables for qRT-PCR were purchased from Applied Biosystems Inc. (Foster City, CA).

In vitro Production of Bovine Blastocysts

In vitro production of bovine embryos was completed as described previously [30, 353]. In brief, bovine ovaries collected from a local slaughterhouse (Center Hill, FL) were transported to the laboratory and sliced to liberate cumulus oocyte complexes (COCs). COCs with compact cumulus were matured in a 50-µl drop of oocyte maturation medium (~30 COCs/drop) overlaid with mineral oil for 20–22 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in air. Matured oocytes were fertilized with Percoll-purified spermatozoa from frozen-thawed semen from at least three bulls.

Putative zygotes were denuded of cumulus cells and incubated in 50-µl drops of modified synthetic oviduct fluid (mSOF) [30] at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 (~30 presumptive zygotes/drop). On day 8 post-fertilization, blastocysts were collected and placed in Dulbecco's modified essential medium (DMEM) containing high glucose (25 mM) and 1% [w/v] BSA and 0, 5 or 100 ng/ml brFGF2 or hrFGF10 (n=12-15 blastocysts/well). Both FGFs were prepared in DMEM containing 1% BSA. After 8 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air, blastocysts were collected, snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Trophoblast Cell Cultures

Bovine CT1 cells were propagated on Matrigel-coated plates in DMEM (with high glucose) containing 10% [v/v] fetal bovine serum (FBS), 100 µM non-essential amino acids (NEAA), 55µM β-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphotericin B at 38.5°C with 5% CO2 in air as described previously [28, 150]. CT1 cells were passaged manually by separating them from plates with a cell scraper and dissociating them into small clumps with repeated dissociation through a 20-ga needle. Cells were serum-starved by replacing medium with DMEM lacking FBS but containing all other supplements and a serum-substitute mix (10µg/ml insulin, 5.5µg/ml transferrin and 6.7ng/ml sodium).

RNA Isolation and Quantitative, Real Time RT-PCR

Total cellular RNA (tcRNA) was isolated from blastocysts using the PicoPure RNA isolation kit and from CT1 cells using Trizol reagent and the PureLink RNA Mini Kit. TcRNA quality and concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific). Samples with A_{260}/A_{280} ratios ≥1.8 were

incubated with RNase-free DNase for 30 min at 37°C. After heat-inactivating the DNase (75°C for 10 min), RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit and random hexamers. Primers (200 nM) for *ITGs*, *MMP2* and the internal loading control (*GAPDH*) (Table A-1) were used in combination with a SYBR® Green Detector System and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to quantify transcript abundance in embryos and CT1 cells as described previously [30]. A dissociation curve analysis (60-95°C) was used to verify the amplification of a single product. Each sample was completed in triplicate reactions. A forth reaction lacking reverse transcriptase was included to control for genomic DNA contamination. The comparative threshold cycle (C_T) method was used to quantify mRNA abundance relative to the reference control (*GAPDH*).

Statistical Analyses

All analyses were performed by least-squares ANOVA using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between individual means were compared using pairwise comparisons [PDIFF (probability of difference) analysis in SAS]. qRT-PCR data (fold-differences between treatments) were analyzed after log-transformation. Results were presented as the mean ± SEM. A *p*-value < 0.05 was considered statistically significant.

Results

FGF2- and 10-dependent Changes in Selective *ITGs* and *MMP2* in Bovine Blastocysts

Several *ITGs* and *MMP2* were identified as candidates for FGF2 or FGF10 regulation in bovine blastocysts after a preliminary microarray analysis (Zhang, Yang & Ealy; Unpublished observations). Quantitative RT-PCR was used to examine whether

the abundance of selective transcripts were indeed regulated by FGF2 or FGF10 in bovine blastocysts. Each of the six *ITGs* examined (See Fig. A-1 and A-2, panels A-F) were readily detected by qRT-PCR (dCT values using *GAPDH* as the reference control). According the delta CT value, integrin alpha 6 (*ITGA6*) is highly expressed by bovine blastocyst (3.56±0.12 vs 7.04±0.34 (*ITGA3*), 7.98±0.18 (*ITGA7*), 8.31±0.55 (*IGTA2B*), 12.27±0.36 (*ITGB4*), 10.58±0.49 (*ITGB6*)). 5ng/ml FGF10 increased *ITGA7* ($P<0.05$). 5ng/ml FGF2 tended to increase I*TGB4* ($P=0.07$), 50ng/ml FGF2 significantly increased *ITGB4* ($P<0.05$). *ITGA3, ITGA6, ITGB6, and ITGA2B* was not affected by FGF treatments. Interestingly, FGF2 increased the *MMP2* level ($P<0.05$)

FGF2- and 10-dependent Changes in Selective *ITGs* and *MMP2* in CT1 Cells

Bovine blastocysts contain multiple cell types (trophoblast, epiblast and primitive endoderm) [14], therefore a follow-up study was completed using CT1 cells to examine how FGF2 and 10 influence trophoblast-specific expression of selective *ITG*s and *MMP2*. To evaluate whether FGF2 or FGF10 changes the integrin expression in trophoblast cells, a bovine trophoblast cell line (CT1) was treated with FGF2 or FGF10 for 8 h. According to the blastocyst data, 5ng/ml FGF2 or FGF10 could induce changes in integrin expression so lower doses of FGF2 or FGF10 (5 or 50ng/ml) was used in this study. In CT1 cells, *ITGA6* was the most abundant integrin as indicated by the C_T value (3.42±0.32 vs. 12.87±1.03 (*ITGA3*), 16.55±0.31 (*ITGA7*), 12.28±0.55 (*IGTA2B*), 6.33±1.11 (*ITGB4*), 6.13±0.71 (*ITGB6*)). Similar to blastocyst, 3 alpha subunits of selected integrin were not responsive to FGF2 or FGF10 treatment. *ITGA2B* was stimulated by 5ng/ml and 50ng/ml FGF2 ($P<0.01$). *ITGB4* was consistent among different treatments. *ITGB6* was also stimulated by 5ng/ml and 50ng/ml FGF2

treatments (P<0.05). 5ng/ml or 50ng/ml FGF2 treatment significantly increased the mRNA level of *MMP2* in CT1 cells (P<0.01).

Discussion

Conceptus elongation is a complex and highly regulated process that features cell differentiation, proliferation and migration. Trophoblast cell migration has been noticed during this period of development and several factors have been identified directly controlling the migration of ovine or bovine trophoblast cells *in vitro*. In the present study, we found that FGF2 and FGF10, two growth factors previously described having a role in ruminant uterus changes the abundance of several ITGs and increases *MMP2* mRNA concentration in both blastocyst and trophoblast cells.

The switch in integrin expression is related with trophoblast cell migration. Previous work in mouse demonstrated that the dynamic changes in integrin expression during the period of embryo implantation play a critical role in trophoblast differentiation, migration and attachment [354, 355]. Several integrins were selected in the present study not only because a previous microarray study suggested that several integrin can be regulated by FGFs in bovine blastocysts (data not shown), but also because those integrins play a crucially important role in trophoblast migration and development in other species. For example, *ITGA2B* was expressed by trophoblast cells and it paired with ITGB3 to mediate the trophoblast attachment and migration during peri-implantation development in mice [355, 356]. *ITGA7* was specifically expressed by trophoblast cells and highly unregulated in trophoblast giant cells, which migrate and invade into uterine lining during embryo implantation in the mouse [354, 357]. In elongating bovine conceptus, *ITGA3* and *ITGA6* were constitutively detectable in mononucleated trophoblast cells and a subpopulation of binucleated trophoblast cells

while *ITGA5* was only stained in endoderm cells [222, 358]. Here we showed that *ITGA3* and *ITGA6* were abundantly present in bovine blastocyst and trophoblast cells. *ITGB4* and *ITGB6* were also transcribed by bovine trophoblast cells.

The interaction integrin and extracellular matrix mediates trophoblast cell migration in ruminant. It has been proposed that changes in ITG expression and its extracellular molecules govern the migration of binucleated cells in bovine placenta [220]. It is not surprising because one of the well-known characteristics of bovine binucleated cells is migration. However, several experiments have established the role of integrin signaling in mediating mononucleated trophoblast cell migration and suggested that this type of cell migration is important for conceptus elongation in ruminant species [219, 349]. Here we showed that FGF2 and FGF10 supplementation elevated mRNA levels of *ITGA2B* and *ITGB6*, indicating that changes in integrin expression may be related with FGF induced cell migration. FGF2-induced changes in integrin expression have been found in many cell types and it is directly linked to cell migration and adhesion [359, 360]. In the present study, the FGF response is moderate, however, changes in integrin expression does not need to be robust to induce a physiological event [361].

MMP activity is critical for cell migration. *MMPs* are a group of enzymes that responsible for the turnover of structural protein in extracellular matrix [345]. *MMP2* and *MMP9* have been considered as key enzymes for trophoblast invasion [362] . For example, In human placenta, FGFR1-4 were identified in trophoblast cells and supplementation FGF10 to cultured trophoblast cell increases outgrowth formation and cell migration through a Matrigel coated membrane by modulating *MM9* activity [363]. Bovine trophoblast cells have restricted invasive ability; therefore *MMP* seems play a

role in promoting trophoblast migration. Indeed, a recent study showed that EGF stimulates bovine trophoblast migration by *MMP9* and *TIMP1* activities [344]. Here we demonstrated that *MMP2* was induced by FGF2 in both blastocyst and trophoblast cells and potentially plays a role in bovine trophoblast migration. The reason that FGF10 showed less effect on *MMP2* expression in bovine trophoblast is unclear.

Table A-1. Primers used for quantitative Real Time RT-PCR

Gene	GenBank No.	Primers
ITGA3	BC149926.1	F 5'-GGACATGTGGCTCGGCGTGA -3' R 5'-GCCAGTCATCCCTGGCGTCC -3'
ITGA6	XM_616466.4	F 5'-TGCGAGGGCTGGACAGCAAG-3' R 5'-ACCTGAGTGCCTGCGTTGGG -3
ITGA7	NM_001191305.1	F 5'-TGGAGGAGTACTCAGCTGTG -3' R 5'-AGCAAGTTCTTGATGGAGGATT-3'
ITGA2B	NM_001014929.1	F 5'-GGGCAAGGACTCGGAGCGTC -3' R 5'-CTGTGCGGTCGCCGTTTGG -3'
ITGB4	NM_001193257.1	F 5'-GCTACGAGGGTCAGTTCTGC-3' R 5'- TCCGTGTAGAGCGACTGTTG-3'
ITGB6	NM_174698.2H	F 5'-CTCGTGCAGTGGGAGAGGCG-3' R 5'-CGCAGAGCAGCCCCTTGTGT-3'
MMP2	NM_174745.2	F 5'-GGGCCTGAGCACCAGGGAAG -3' R 5'-ATGGAGGGGGAGGGACACCC -3'
GAPDH	NM_001034034.1	F 5'-ACCCAGAAGACTGTGGATGG-3' R 5'-CAACAGACACGTTGGGAGTG-3'

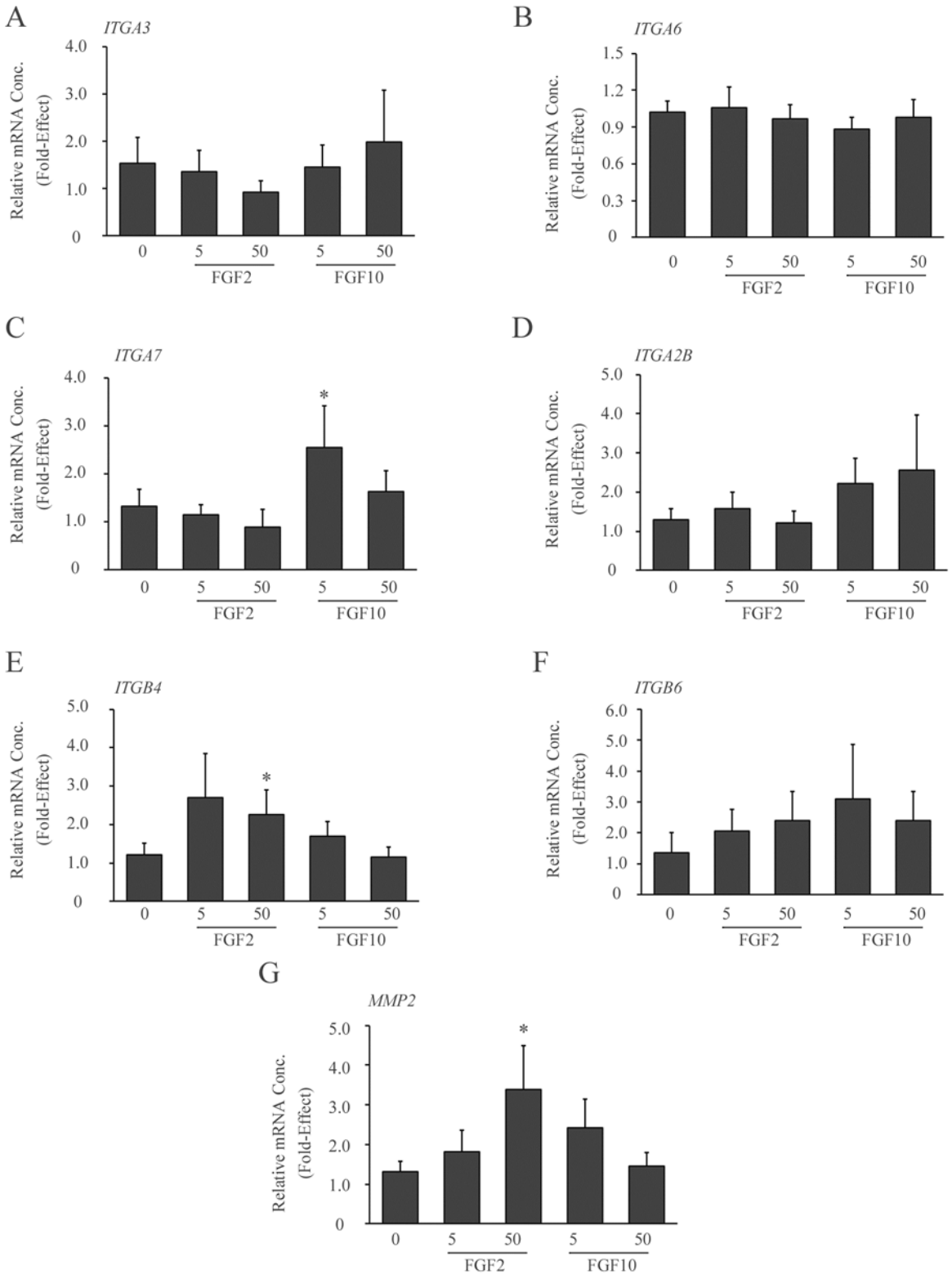

Figure A-1. FGF2 and FGF10 modulate selected gene expression in bovine blastocysts. Results represent means and SEM of fold-differences relative to control values (n=5). The asterisk (*) denotes a difference ($P < 0.05$).

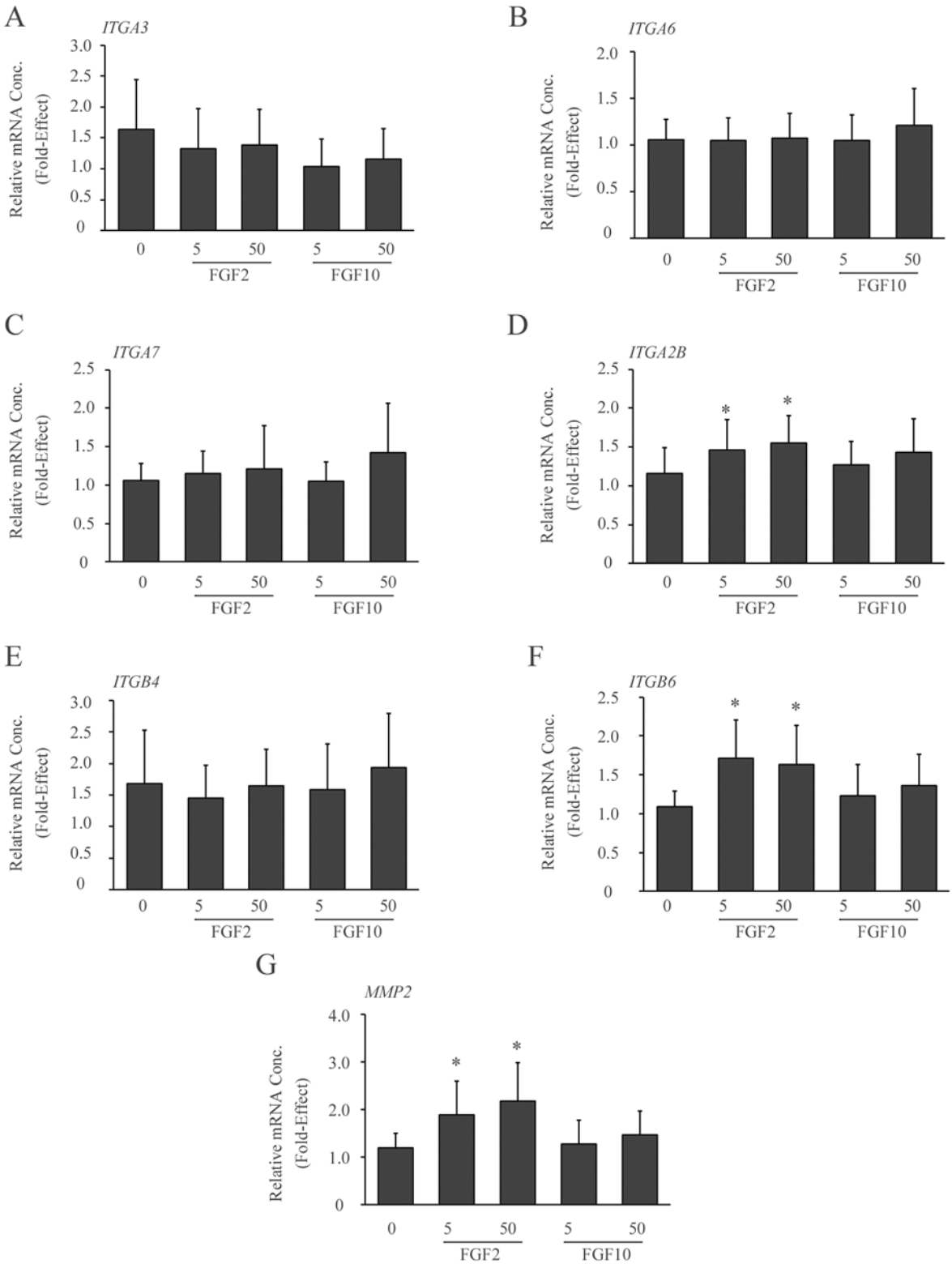

Figure A-2. FGF2 modulates selected gene expression in CT1 cells. Results represent means and SEM of fold-differences relative to control values (n=5). The asterisk (*) denotes a difference ($P < 0.05$).

APPENDIX B
DELIVERY OF SIRNA OLIGOS INTO BOVINE TROPHOBLAST CELLS

This protocol has provided successful knockdown results when tested three PKC delta (PRKCD) siRNAs in a bovine trophoblast cell line, Vivot cells. The reasons of using this cell line are Vivot cells produce high amount of *IFNT* mRNA, which makes it a good model to study *IFNT* gene expression (Figure B-1), and the individual cell has different membrane structure when compare with CT-1 cells by a light microscopy. This protocol describes the key points of using Hiperfect transfection reagent (Cat.No. 301705, Qiagen) in Vivot cells. In this protocol, cell plating and siRNA transfection are conducted on the same day (reverse transfection).

Trophoblast Cell Culture

- Vivot cells will be cultured in T-75 flask for 4 or 5 days with a confluence of 50%. At this time, the cells are at rapid proliferation stage. On the day of transfection (Day 0), cells will be washed with PBS 3 times and suspended in 3 ml of OPI-MEM medium using a scraper.
- Transfer cell suspension to a 15 ml conical tube and split cells physically by breaking the cell clump using a 22G syringe needle. Dependent upon the size of the colonies, 2 or 3 times will be enough to dissociate all cell clumps.
- 100 µl of cell suspension will be added to each well in a 24-well plate. The plate must be coated with Matrigel 4 hours earlier. To distribute cells equally, shake the tube well each time. Cell confluence determines the efficiency of siRNA. Incubate cells under normal conditions or this step can be done after step 2 of this protocol.

Preparation of siRNA Complex

- siRNA**:** dilute siRNA in 100 µl of OPI-MEM with proper concentration. This should be 2 times higher than the final siRNA concentration in the well. For example, if working concentration is 50 nM, prepare 100nM siRNA at this step. Mix by vortexing.

- Transfection complex: HiPerFect from Qiagen will be used in this protocol. Based on the preliminary results, the optimal dose of this reagent in each transfection is 4.5 µl. Add 4.5 µl of HiPerFect to the diluted siRNA. Mix by vortexing for 10s.Incubate the solution for 10 min in the hood to allow formation of transfection complexes.

- siRNA delivery: Add siRNA complexes in the cells in a drop-wise manner. Gently shake the plate to distribute the complexes equally. Incubate cells under normal condition. 18 hours later, add 400 µl serum containing medium and continue to culture the cells.

Cell Maintenance

- Day 2: Check cell viability 48 hours after transfection, if needed, add 200 serum containing medium in each well.

- Day 3: Check cell viability, 72 hours later, dependent upon experimental setup, the effect of siRNA could be checked using real time PCR or western blot analysis.

- Day 3 (Optional): If the purpose is to study the effect of siRNA on *IFNT*, 60 hours after siRNA delivery, the medium containing siRNA complexes will be replaced by serum free medium (ITS medium) and the cells will be starved for 12 hours. 72 hours post transfection; cells will be treated with FGF2 for 20 hours.

- Day 4 (Optional): Normally the cell confluence will be around 30 to 40%. Isolate tcRNA with appropriate approach and dilute into 30 to 40 µl RNase free H2O.qRT-PCR will be used to If the confluence is lower than 15%, no endpoint should be conducted because tcRNA quality will be a concern. If the cells are over confluent, endpoint should not be performed since the effect of siRNA will be limited.

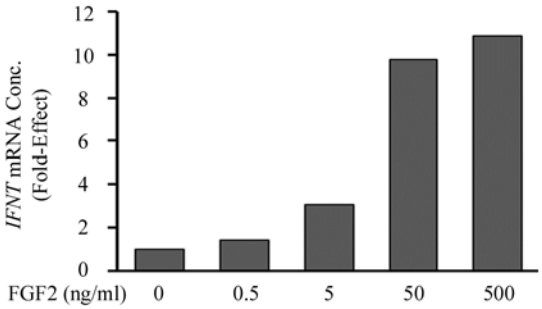

Figure B-1. FGF2 increases *IFNT* mRNA abundance in Vivot cells. Cells were serum starved from 24h then treated with 0.5, 5, 50, 500 ng/ml FGF2 or vehicle (1% BSA in ITS medium) for 24 h. tcRNA was isolated at the end of the incubation period and qRT-PCR was used to determine the relative abundance of *IFNT* mRNA. 18S RNA was used as the internal control.

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BIOGRAPHICAL SKETCH

Qien Yang grew up in Qinghai province northwest of China. He attended the China Agricultural University in Beijing and received his bachelor's degree in animal science in July 2004. Following graduation, Qien began his master's program in Dr. Zhu Shien's group in the key laboratory for embryonic biotechnology at the same university. He continued his undergraduate research on mouse embryo development and embryo cryopreservation and received a Master of Science in animal reproductive biology. 2006, Qien came to the University of Florida and started his PhD in animal molecular and cellular biology graduate program under Dr. Alan Ealy's guidance. His research project has been on early embryonic development and maternal recognition of pregnancy in cattle, with particular focus on molecular regulation of early lineage segregation and trophoblast function in cattle. Following his PhD, Qien plans on pursue a postdoctoral position to gain more training in developmental biology and finally start his own lab in the field of reproductive biology. He is a trainee member of the Society for the Study of Reproduction.