Development of an Interferon Bioassay and Primitive Endoderm Cell Lines to Study Lineage Specification During Early Bovine Embryogenesis

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

Master of Science
In
Animal and Poultry Science

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November 7, 2014
Blacksburg, VA

Key words: Interferon-tau, Bioassay, Primitive Endoderm, Cell Lines

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Abstract

Embryonic wastage is rampant in cattle during early stages of pregnancy, particularly the first few weeks of gestation, a time recognized for significant remodeling of the embryo. Of particular interest to this laboratory are the first two lineage specification events, trophectoderm (TE) and primitive endoderm (PrE) specification, occurring between days 6 and 8 of gestation. The TE is responsible for uterine attachment and production of interferon-tau (IFNT), the factor of maternal recognition of pregnancy in ruminants. The PrE forms the yolk sac, which provides nutrients to the developing embryo. It is probable that developmental miscues during these differentiation events are responsible for the high rate of pregnancy loss, however, information on these early lineage processes is lacking in ruminants. The objective of the first study was to improve the current methods for detecting IFNT in biological samples. A novel interferon stimulatory response element (ISRE)-reporter assay was created, and provides adequate quantification to measure IFNT. Additionally, it has a shorter completion time than previous bioassays, and does not require the use of a live virus. The second study describes the development of a PrE cell line derived from bovine embryos. The PrE outgrowths can be produced at high rate, and can be maintained in a continuous culture system for about 6 weeks. As a true bovine PrE cell line does not currently exist, these lines hold great potential for the study of early

development. Collectively, these studies improve knowledge of bovine embryogenesis, and provide insights that may be used to limit the pregnancy failures occurring in this species.

Acknowledgements

I would first like to thank my parents for always supporting me, and for teaching me that hard work pays off. I would also like to thank Will Stetson for always letting me know how proud he is of me, and for keeping me level-headed. To Laura Nelson, Meghan MacGhee, and Erin Mabry, thank you for helping me find a happy medium between work and play. I would like to thank all the members of the Johnson-Ealy lab, especially Jen Bradley, for always lending a hand when needed. And finally to Dr. Ealy, thank you for teaching me how to “think like a scientist”, and for encouraging me to find research that I find exciting. You’re all awesome!

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List of Abbreviations

AI	Artificial insemination
aPKC	Atypical protein kinase C
AVE	Anterior visceral endoderm
BMP	Bone morphogenic protein
BNIP1	BCL2/Adenovirus E1B 19kDa interacting protein 1
BSL2	Biosafety level 2
CDX2	Caudal-related homeobox 2
CL	Corpus luteum
COC	Cumulus-oocyte complex
CXCR4	Chemokine (C-X-C motif) receptor 4
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme-linked immunosorbant assay
EMK	ELKL motif kinase
emVE	Embryonic visceral endoderm
EPI	Epiblast
ET	Embryo transfer
ESC	Embryonic stem cell
ExE	Extraembryonic endoderm
exVE	Extraembryonic visceral endoderm
FGF2	Fibroblast growth factor 2
FGFR1	Fibroblast growth factor receptor 1
FBS	Fetal bovine serum
FST	Follistatin
GAS	Gamma activating sequence
GATA4	GATA binding protein 4
GGATA6	GATA binding protein 6
HHEX	Hematopoietically expressed homeobox
ICAM1	Intracellular adhesion molecule 1
ICM	Inner cell mass
IFN	Interferon
IFNA	Interferon-alpha
IFNAR1/2	Type 1/2 IFN receptor
IFNG	Interferon-gamma
IFNT	Interferon-tau
IL6	Interleukin 6
IRF9	Interferon regulatory factor 9
ISRE	Interferon stimulatory response element
ISG	Interferon stimulatory gene
ISGF3	Interferon stimulatory gene family 3
IVF	*In vitro* fertilization

IVP	*In vitro* produced
Jak2	Janus kinase-2
Luc	Luciferase
NK	Natural killer
OCT4	Octamer-4
OT	Oxytocin
OTR	Oxytocin receptor
PAR	Partitioning-defective
PARD6b	PAR-6 family cell polarity regulator beta
PDGFRA	Platelet-derived growth factor receptor alpha
PE	Parietal endoderm
PGF2α	Prostaglandin-F2α
PrE	Primitive endoderm
PTHrP	Parathyroid hormone related peptide
RIA	Radio immunoassay
RPS9	Ribosomal protein S9
SOF	Synthetic oviduct fluid
SOX7	SRY-box 7
SPARC	Secreted protein, acidic, cysteine-rich
STAT	Signal transducer and activator of transcription
STRA6	Stimulated by retinoic acid 6
TE	Trophectoderm
TEAD4	TEA domain family member 4
THBD	Thrombomodulin
TGF-β	Transforming growth factor-beta
Tyk1	Tyrosine kinase-1
VE	Visceral endoderm
VEGFA	Vascular endothelial growth factor A
VSV	Vesicular stomatitis virus
Yap	Yes-associated protein

Chapter 1

Literature Review

Reproductive efficiency in dairy cattle has been steadily decreasing in recent years. One of the major problems associated with infertility in cattle is pregnancy failure, and substantial pregnancy loss occurs within the first few weeks of gestation. The events responsible for pregnancy loss can be assessed based on the specific developmental events occurring throughout early pregnancy. Two such events are trophectoderm (TE) development and primitive endoderm (PrE) lineage specification, each of which occurs between days 6 to 8 of gestation in cattle [1]. The TE is responsible for uterine attachment, while the PrE will eventually give rise to the yolk sac of the developing embryo. Inadequate development of either of these cell lineages could result in pregnancy failures. The TE is also responsible for secreting interferon-tau (IFNT), the maternal recognition factor in ruminant species. IFNT is required for the maintenance of pregnancy in cattle, and insufficient levels result in pregnancy loss. It is important that we gain a better understanding of these early embryological events in order to alleviate the drop in reproductive efficiency that is currently plaguing the dairy industry. The following studies were completed to improve the present knowledge of early bovine embryogenesis.

Pregnancy Loss In Cattle

Over the past several decades, the dairy industry has experienced a boom in milk production with the help of genetic selection practices and various reproductive technologies, including artificial insemination (AI), and *in vitro* fertilization (IVF). These improvements in milk yield, however, have coincided with a period of steady decline in animal reproductive efficiency. The relationship between these two events is not by chance, but rather they are inversely correlated to each other [2, 3]. Studies have shown that approximately 60% of all pregnancies fail in lactating dairy cattle, with between 40-50% of all pregnancies failing to progress past the first 3 months of gestation [4, 5]. Studies utilizing embryo transfer (ET) as well as early pregnancy detection have shown an even more drastic effect, with fewer than 50% of viable embryos failing to maintain a pregnancy past days 27-30 of gestation [6].

Economic loss and pregnancy failures are directly related in the dairy industry. The cost of pregnancy loss in dairy cattle is about $555/pregnancy based on the costs of re-breeding animals, and loss due to decreased milk production over the animal's lifetime [7]. Though less severe, the beef industry has not escaped the economic loss of pregnancy failure, which costs the United States an estimated $1.2 billion dollars each year [8]. Reports indicate rates of early embryonic loss in beef cattle ranging from 20-44% [9].

Etiology of Pregnancy Loss

In order to standardize bovine reproductive terms, the Committee on Bovine Reproductive Nomenclature recommended specific terms be used when referring to the

times of a lost pregnancy. Pregnancy failures occurring within the normal range of an estrous cycle, or before day 24 of gestation, are referred to as “early embryonic losses”. By contrast, losses occurring after a normal estrous cycle length, but before a fetus has developed (i.e. between days 24-42) are classified as “late embryonic losses”. Pregnancy loss detected after day 42 of gestation is characterized as fetal loss. In a review of several published studies, the average incidence of late embryonic loss in lactating dairy cows is 12.8%, while the incidence of fetal loss averages 2.5%. Pregnancy failures occur most frequently in the time of early embryonic development. A summation of studies aimed at determining the embryo viability of multiparous lactating, multiparous nonlactating, and nulliparous heifers found embryo viability rates to be 50, 57.9, and 71.9%, respectively, by day 5-6 post-insemination [4]. The low viability rates point to disruptions during early embryonic development as the cause of most pregnancy loss in cattle. For this reason, it is imperative that we investigate this window of development, a time of drastic change in the embryo.

Prominent Periods of Early Embryonic Losses

Although embryonic losses may occur at any time during early development, several key periods seem especially vulnerable to pregnancy failure. Dairy cows experience significant pregnancy loss during the first 7-8 days of gestation, during which time the embryo undergoes extensive cellular divisions, as well as several vital developmental events (Fig. 1-1). For example, during the earliest days of pregnancy, and specifically at day 3 to 4 post-fertilization in cattle, the embryonic genome becomes activated [10]. Any perturbations in this intricate process, which will be discussed later,

may result in halted embryonic growth and pregnancy failure. Soon after genome activation, embryonic lineage specification events occur. The first of these events is specification of the TE, which takes place on embryonic days 7-8 in cattle. TE cells are responsible for the production of *IFNT*, the main factor accountable for the maintenance of early pregnancy in ruminants, as well as the conceptus' eventual implantation into the uterus. This and other lineage specification events, and the role of *IFNT* in early pregnancy will be explained in greater detail in subsequent sections.

Of those pregnancies surviving the initial week of gestation, ~40% are lost between days 8-17 of development [11]. This window of time spans several crucial developmental events. Two prominent events include conceptus elongation, and the production of massive amounts of *IFNT*. Beginning on day 12 of gestation, the previously spherical embryo evolves into a tubular structure. The bovine embryo gains a filamentous shape, and becomes completely elongated by day 16 of pregnancy [12]. Day 16 is also an important time point for *IFNT* production. Sufficient levels of *IFNT* must be produced by this stage in development, or the pregnancy will be lost [13]. The high level of pregnancy loss at this stage of gestation suggests a role of inadequate *IFNT* production and/or inadequate conceptus growth. As will be discussed later, these two events are not mutually exclusive, but rather are highly interrelated with one another.

To summarize, pregnancy losses are rampant during early pregnancy in cattle, and this is especially evident in lactating dairy cows. Not surprisingly, these losses undoubtedly occur because of mishaps in several early developmental events that are critical to embryonic growth and the maintenance of a pregnancy. More insight into

these developmental events is needed before we can devise schemes to limit pregnancy losses in cattle. The following sections will discuss many of these events, including TE and PrE development, conceptus elongation, and maternal recognition of pregnancy.

Early Embryogenesis

Initial Embryonic Cell Divisions

In cattle, embryos do not progress to the first cell cleavage until 23-31 hours after fertilization [14, 15]. Cleavage to the 4-cell stage occurs at approximately 36-50 hours after fertilization, and the 8-cell stage is reached at 56-64 hours. By 80 hours post-fertilization, a healthy embryo reaches the 16-cell stage [16]. By day 5 of pregnancy, the maternal genome becomes inactivated and the embryo begins transcribing its own genome, termed embryonic genome activation (EGA)[17]. Prior to EGA, the maternal genome controls all aspects of embryonic development, from directing early cellular patterning, to coordinating the first mitotic divisions [18]. This transition from maternal to embryonic control is important to survival, as it allows the embryo to respond to environmental stresses, such as nutritional imbalances, that may otherwise harm its development.

Soon after EGA has occurred, the embryo begins a process referred to as compaction. Embryonic compaction occurs when blastomeres increase contact with nearby cells, forming a compact sphere of cells. This change in cell behavior is often referred to as the beginning of the morula stage. Initially, the morula does not contain an internal cavity, however, a cavity is formed by a process known as cavitation [19].

Cavitation and the formation an internal cavity, termed the blastocoel cavity, marks the progression of the embryo from the morula to blastocyst stage. This process generally occurs around day 7 post-fertilization in cattle.

The first cell fate decision in the developing embryo is the differentiation of the TE from the inner cell mass (ICM). This process has been well studied in mice, and presumably the same or similar progression exists in other mammalian embryos, including cattle. The process begins during compaction, when the *PAR* (partitioning-defective) proteins, *EMK* (ELKL motif kinase), and *aPKC* (atypical protein kinase C), each known for their roles in asymmetric cellular divisions, are disproportionately distributed within the embryo [20, 21]. A study conducted by Louvet-Vallée et. al. revealed that a member of the *PAR* protein family, *PARD6b* (par-6 family cell polarity regulator beta), and *EMK1* are initially co-localized in the nuclei in all blastomeres prior to compaction. However, following compaction, *PARD6b* travels to the apical pole of blastomeres, while *EMK1* re-distributes along the basolateral domain of each blastomere. By the 16-cell stage, *aPKC* co-localizes with *PARD6b*, forming a PAR/aPKC complex. [22]. The importance of this complex in the allocation of cell polarity was observed when blastomeres from 4-cell mouse embryos were subjected to a down-regulation of *aPKC*. Subsequent daughter cells localized to the inner embryo rather than to their “normal” distribution along the apical pole [21].

During the succeeding cellular divisions, cells divide in one of two manners; symmetric or asymmetric. Symmetric divisions result in two polar daughter cells that both maintain an outer position within the embryos, while asymmetric divisions produce

one polar outer cell and one apolar, or inner cell [23]. At the conclusion of these consecutive cell divisions, two distinct cell populations are formed; outer TE cells and inner ICM cells. Though the determination of cell fate begins at the 8-cell stage, a level of plasticity is preserved in mouse embryos. This phenomenon can be observed in studies where the ICMs are removed from early blastocysts, and maintain their ability to form blastocyst-like structures containing both ICM and TE cells [24]. This suggests that in early development, exposure of ICM cells to an outer position within the blastocyst is sufficient to cause cells to polarize and form the TE lineage. However, this plasticity is lost by the expanded blastocyst stage, at which point cells are fated to the ICM or TE [25, 26].

Molecular Control of Trophectoderm Differentiation

The molecular control of TE specification has been well described in mice. In rodents, TE commitment requires the expression of caudal-related homeobox 2 (*CDX2*) and concurrent repression of octamer-4 (*OCT4*) expression, a factor responsible for maintaining the pluripotent state of the ICM [27]. The role of *CDX2* can be observed through the use of CDX2-null embryos. These mutants form seemingly normal blastocysts, however, they fail to implant as a result of inadequate TE production [28]. Niwa and colleagues observed the importance of this heightened ratio of *CDX2* to *OCT4* expression by decreasing *CDX2* expression in embryonic stem cells (ESCs). This condition promoted the maintenance of a pluripotent ICM, while the opposite state forced cells toward a TE fate [29]. This suggests that *CDX2* and *OCT4* interact in a manner to control each other's expression. In mice, Cdx2 and Oct4 begin to be

restricted to the TE and ICM around the morula stage, and achieve complete restriction at the blastocyst stage [30].

TEAD4 (TEA domain family member 4) has also emerged as a factor with a role in TE segregation in the mouse model, acting upstream of *CDX2*. *TEAD4* was first implicated as a potential promoter of TE formation as it plays a role in the development of the blastocoel cavity. A deficiency in *TEAD4* results in a failure to express TE-specific genes, and halted TE formation [31, 32]. Not lineage-restricted, *TEAD4* is expressed in both the ICM and TE lineages, though it is only required for TE differentiation, not for the maintenance of the ICM [31, 33]. *TEAD4* functions as regulator of the Hippo signaling pathway, which is the mechanism behind contact inhibition in proliferating cells [34]. At low confluency, cells actively proliferate, however, at a high density the process stops. Hippo signaling is differentially expressed within the cells of the developing embryo; strong expression is observed in the inner cells, which contain a high level of cell contact, and weak in the outer cells [33]. This differential activation of the Hippo pathway results in variations in Yes-associated protein (Yap) expression as well. Yap proteins are present in the nuclei of the outside cells, and absent from inner cells. Studies by Nishioka et al. show that when Hippo signaling is suppressed, inner cells express Yap, as well as Cdx2 [33]. Thus, the outer cells of the embryo, those with limited cell-to-cell contact, experience weak Hippo signaling, and increased Yap accumulation. This in turn activates *TEAD4*, which induces *CDX2* expression, and thereafter fates cells to the TE lineage.

Many of these TE specification processes have not been explored in bovine embryos. However, several key components of the processes delineated in mice appear to also exist in cattle. TE-specific expression of *CDX2* and *TEAD4* exists in bovine embryos. Interestingly, *OCT4* expression differs in cattle, and presumably other ruminants. In the mouse model, Oct4 only maintains its expression profile in TE-fated cells to the blastocyst stage. In bovine embryos, *OCT4* is produced in both ICM and TE cells well after blastocyst formation. *OCT4* expression exists in bovine TE until day 11 post-fertilization [1]. Thereafter, *OCT4* is localized solely within the ICM. The importance of this disparity between species is not clear, but the distinction in *CDX2/OCT4* profiles suggests that the relative importance of *OCT4* in lineage specification in cattle may not be as important as believed for rodent embryos.

Conceptus Elongation

In several species, including humans and mice, embryonic implantation into the uterus occurs almost immediately after the blastocyst stage, days 7-9 and 4 respectively [35]. The same does not hold true for cattle. Following the blastocyst stage, bovine TE cells undergo expansive proliferation, resulting in the elongation of the conceptus. By day 16 of pregnancy, the point at which maternal recognition of pregnancy must occur, the bovine conceptus reaches a length of 10-30 cm. This elongation process continues, and by day 21, the time of uterine attachment, the conceptus reaches a length of 50-200 cm. At this time, the conceptus can span throughout one entire uterine horn, and into part of the second horn [36]. The process of elongation is important for creating more

surface area for conceptus attachment. It also increases the number of IFNT-producing trophoblast cells.

Primitive Endoderm

The second fate-determining step of embryogenesis is the allocation of a population of ICM cells to form the PrE [37]. Shortly after TE formation, the ICM forms two distinct cellular populations; the PrE and the epiblast (EPI). Once segregated, PrE cells migrate along the inner blastocoel border, and continue traveling down the TE layer until they completely line the blastocyst cavity. The PrE will differentiate into visceral endoderm (VE) and parietal endoderm (PE), which give rise to distal and proximal portions of the yolk sac, respectively. These endoderm sub-types act as structures providing gas, nutrient, and waste exchange between the embryo and the maternal environment before the chorio-allantoic placenta has been established [38-40]. The yolk sac is present during both conceptus elongation and maternal recognition of pregnancy, making PrE a particular interest of this lab. By day 20 of pregnancy in cattle, the yolk sac reaches peak development, and then rapidly regresses as its nutritive function is taken over by the emerging allantois [41, 42].

Model for Primitive Endoderm Cell Sorting

In order to establish the timing of PrE/EPI differentiation, early research focused on isolating cells of the ICM from mouse blastocysts at different stages of development. These studies revealed that by the expanded blastocyst stage, cells of the ICM could no longer differentiate into the TE lineage; however, they were able to form both PrE and EPI cells [25, 43]. When the ICMs of early blastocysts were isolated, cells were able to

form each of the primary lineages [44, 45]. This established a clear window of when PrE and EPI fate-determining events occur, though the allocation of the lineage precursors was still unknown.

By embryonic day 4.5 in the mouse, the PrE and EPI cell lines exhibit morphologically distinct fates [46]. This observation led to the initial model for PrE-EPI segregation that cells of the ICM are identical and will differentiate depending on their position; cells lining the outside of the ICM will differentiate into PrE, while cells localized deeper in the structure will adopt an EPI fate [47]. This early model was later challenged when it was observed that the cells of the ICM expressed transcription factors at varying levels, thus creating a “salt and pepper” model. Several studies showed *NANOG* and *GATA6*, markers of EPI and PrE respectively, were differentially expressed before the emergence of a definitive PrE layer within the blastocyst [48]. This model is also supported by studies utilizing lineage-tracing experiments. A study by Chazaud and colleagues studied the patterns of individually labeled ICM cells from early blastocysts, and noted that in most cases, individual cells contributed to only one cell lineage, either the PrE or EPI, and the location of these cells within the ICM did not dictate the resulting cell fate [49]. These data suggest cell fate is determined prior to the appearance of a distinct PrE layer, and have lead to the proposition of a new paradigm referred to as the three-phase model. The proposed first step in lineage allocation is the co-expression of *GATA6* and *NANOG* at the early morula stage of development. This is followed by the restricted expression of the PrE- and EPI-precursors, and their “salt and pepper”

distribution within the ICM. The final step is the physical segregation of the two cell types to form the two distinct lineages [50].

Through the use of live imaging technology, it has become apparent that cell sorting is not as simple as expression or repression of *GATA6* and *NANOG*. One such study utilized the PrE-specific single-cell resolution fluorescent reporter, $Pdgfra^{H2B\text{-}GFP}$. The study revealed several cell behaviors involved in the process of sorting the PrE from the ICM. Cells that were already lining the blastocoel cavity rarely changed their position, and were shown to upregulate GFP expression. Those cells located deeper within the ICM showed more complex behaviors, where some GFP-positive cells were shown to migrate to the blastocoel border, and give rise to the PrE. Interestingly, other GFP-positive cells also deep within the ICM were seen to either downregulate GFP expression or undergo apoptosis [50]. This study revealed the complexity of the cell sorting process. It is not simply a matter of gene expression or inhibition, but also that cells failing to assimilate to the PrE lineage undergo apoptosis, or actively revert to an EPI fate.

Transcriptional Interactions of PrE and EPI Precursors

In mice, the allocation of cells to the PrE lineage over the EPI lineage is controlled by interactions between and the expression of two lineage-specific transcription factors, Gata4/6 and Nanog. EPI progenitors are shown to express markers of pluripotency, *SOX2* and *OCT4*, as well as *NANOG*; however *NANOG* is the only factor to be solely expressed by EPI cells. This finding sparked the idea that *NANOG* is the influential factor forcing cells toward an EPI fate. Similarly, PrE

progenitors express *SOX2* and *OCT4* until implantation, though the defining characteristic of these cells is their upregulation of *GATA4/6*. However, this upregulation in lineage-specific transcription factors does not become apparent until the early blastocyst stage. Up to this point, the ICM seems to be a homogenous structure, with similar gene patterns among cells; most notable is the co-expression of *GATA6* and *NANOG* [49, 50].

After reaching the early blastocyst stage, cells within the mouse ICM begin to exclusively express lineage-specific transcription factors. One subset of cells will downregulate *NANOG*, and simultaneously increase *GATA6*, resulting in an upregulation of *SOX17* [51]. This expression pattern primes cells to follow the PrE lineage. The remaining cells will downregulate *GATA6*, and increase *NANOG* levels, resulting in an EPI fate.

While *GATA4/6* and *NANOG* are differentially expressed within the two cell lines, recent studies suggest a level of cross-talk between the two transcription factors. Messerschmidt and Kemler used *NANOG* mutant mouse embryos to bring light to this phenomenon. Their work showed that *NANOG*-null embryos failed to produce the PrE marker *GATA4* [52]. This finding contradicts the previous belief that *NANOG*-null embryos would show rampant expression of PrE factors because of the lack of inhibition from *NANOG*. Instead, these findings suggest the *NANOG* may be essential for proper PrE development, and the interactions between *NANOG* and *GATA6* are essential for proper embryonic development.

Role of Fibroblast Growth Factors in Primitive Endoderm Development

Several studies implicate fibroblast growth factors (*FGF*s) as a driving force behind PrE development. Embryos lacking *FGF4*, its receptor *FGFR2*, or the receptor adaptor protein GRB2 fail to form PrE [53-56]. In addition, embryos lacking GRB2 fail to express *GATA6*, a transcription factor essential for proper PrE development [49]. Similar work showed embryoid bodies derived from $Fgfr1^{-/-}$ ESCs expressed a decreased level of the PrE marker α-fetoprotein, and failed to produce viable visceral endoderm (VE), a product of PrE [57]. Work conducted by Wells and Melton further supported the role of *FGF* in PrE development in their study that found recombinant *FGF4* affects the patterning of endoderm in a dose-dependant manner [58].

The Parietal and Visceral Endoderm

Upon its complete formation, the PrE begins to differentiate into two major subtypes of extra-embryonic endoderm (exVE); the parietal and visceral endoderm, PE and VE respectively. The PE and VE will ultimately act as a structure of maternal-fetal nutrient and waste exchange [38-40]. Together, the PE and VE provide vital support for the continued development of the embryo.

In addition to the several tissue subtypes that arise from VE, it also imparts patterning cues on the EPI, a role not shared by PE. As with other cell types of the embryo, VE cell location affects function, as differing cell-to-cell contacts result in differing signaling environments. An example of this can be observed in the VE that is in direct contact with the epiblast, referred to as embryonic VE (emVE) [59]. The emVE, later becoming anterior VE (AVE), is important in the formation of the anterioposterior

axis of the embryo, as well as stimulating neural development [60-62]. Still, a population of emVE cells remains associated with the epiblast, and will eventually contribute to the early gut tube [63].

Inducing Visceral and Parietal Endoderm Differentiation

The ability to artificially differentiate PrE into PE and VE has proven to be a valuable technique to study early embryonic development in rodents and humans. Members of the transforming growth factor (*TGF-β*) family play a role in the VE specification. An example is the necessity of bone morphogenic proteins (BMPs), specifically BMP2 and BMP4, in VE differentiation, as shown by Coucouvanis and Martin [64]. Furthermore, studies show that embryos lacking BMP2/4 receptors have a more constricted and disorganized VE layer [65]. Smad4 has also been implicated in VE differentiation, as its absence results in an even more chaotic VE expression [66].

Similar signaling processes are required for PE formation, though less is known about the initial segregation. Parathyroid hormone related peptide (PTHrP), a factor produced by TE cells is believed to be the main inducer of PE formation during embryonic development [67, 68]. Once activated, PE cells undergo an epithelial-to-mesenchymal transition, show little cell-to-cell contact, and begin to express mesenchymal markers such as Snail and Vimentin [69-71]. Studies show this PTHrP-induced PE differentiation is initiated by the type 1 PTH/PTHrP receptor, however, type 1 (-/-) embryos do not show any apparent abnormalities in PE development, though they are smaller than controls [72]. These findings suggest that though PTHrP and its type 1

receptor are not necessary for the initial PE development, it may play a role in PE maintenance.

Primitive Endoderm Formation in Cattle

In cattle, PrE markers show diverse expression profiles compared to those of other species. Studies from this lab indicate that *GATA4/6* mRNA levels increase when blastocysts undergo hatching and expansion, soon after initiation of *CDX2* expression in TE cells [73]. These levels are shown to increase in day 8 blastocysts; however, unlike in mice, *GATA4* can be detected in both TE and ICM cells, whereas *GATA6* is restricted to the ICM. These findings suggest that although the bovine embryo is similar to the mouse in that lineage-specific transcripts are expressed in a salt-and-pepper model within the ICM, expression may be less restricted in the bovine [74].

A potential role of fibroblast growth factors (FGFs) in bovine PrE development also has been brought to light. Work from this laboratory showed that FGF2 is produced by the endometrium in early pregnancy, and FGF2, FGF4 and FGF10 are produced within the peri-implantation embryo [75-77]. Also, various FGF receptors, including the FGFR2 receptor implicated in PrE specification in mouse embryos, is produced in peri-implantation stage bovine embryos [77, 78] Moreover, a study by Yang and colleagues determined that PrE outgrowths could be formed by supplementing FGF2 to bovine blastocysts [73]. In fact, the frequency of PrE outgrowth formation was great enough that it now appears that this strategy could be used to produce PrE cell types in cattle. Cell lines for bovine endoderm are noticeably absent.

Epiblast Differentiation

A small population of undifferentiated cells is maintained within the blastocyst throughout early development, and is referred to as the EPI. The outermost layer of the EPI, the ectoderm, differentiates by forming an elongated ridge along the germ-disc. This ridge, called neural ectoderm, will eventually give rise to the brain, spinal cord, and various other derivatives of the nervous system [79].

By day 10 of pregnancy, the inner surface of the TE is surrounded by endoderm cells [36]. At day 14, the next layer of cells, the extraembryonic mesoderm (ExM), begins to form between the endoderm and the TE [80]. The mesoderm will give rise to connective tissues, vascular systems, bones, and muscle [79] whereas the ExM will juxtapose with TE and form the chorion. As the embryo continues to develop, the chorion begins to grow upward, forming “wing-like” structures, referred to as amnionic folds. These folds will eventually fuse to surround the entire conceptus, creating the amnion which directly surrounds the embryo/fetus [81]. Initially, a large cavity separates the chorion and the amnion, however, as the pregnancy progresses, the amnionic membrane fuses with the chorion to form the amnio-chorionic membrane [79].

Coinciding with amnion development is the formation of the allantois from the primitive gut, occurring around day 20 in cattle [80]. During this time, the embryonic yolk sac begins to regress [79]. The allantois serves as a collection chamber for embryonic waste, but also supports blood vessels that will provide blood flow to the chorion and amnion. Finally, the chorion and allantois fuse, bringing the fetal blood supply physically closer to the maternal tissue, and become the chorio-allantoic placenta [82].

Maternal Recognition of Pregnancy

During early gestation, it is pertinent that the maternal system recognizes that a pregnancy exists so that estrous cycles can be interrupted and the uterus can continue to support the pregnancy. IFNT is the factor responsible for this in ruminants. IFNT is produced by the trophoblast cells, and is a member of the Type 1 interferon family. Type 1 interferons (IFNs) play a role in immune response after viral infection, and also possess anti-proliferative properties without showing signs of cytotoxic behaviors on the exposed cells [83]. Unlike other Type 1 IFNs, however, IFNT has a major function in maternal recognition of pregnancy in ruminant species.

IFNT Expression Profiles

IFNT expression occurs in a very distinct window of conceptus development. *IFNT* mRNA and proteins can first be detected at the morula to early blastocyst stage, days 6-7 of gestation [84, 85]. Comparatively low at the blastocyst stage, *IFNT* levels steadily increase until around day 14 for pregnancy, when a surge in mRNA is observed in cattle [85, 86]. This dramatic increase in *IFNT* mRNA levels is also observed in sheep at day 12 of pregnancy [87, 88]. The sharp increase in *IFNT* levels is coincident with conceptus elongation in both the bovine and ovine model.

The expression of *IFNT* by trophoblast cells is short-lived. *IFNT* mRNA levels drop dramatically after day 19 to 21 in cattle and day 16 in sheep, corresponding with uterine attachment [89, 90]. Though the mechanisms responsible for this reduced expression are unknown, a change in gene expression resulting from TE-uterine interactions is probable.

Mechanism of IFNT Action

The current theory for maternal recognition of pregnancy in ruminants is that IFNT is solely responsible for conferring this signal in early pregnancy. This contention is based on work where estrous cycle lengths were extended when IFNT was infused into the uterus of non-pregnant ewes [91, 92]. Similar work in the bovine model found that intrauterine injections of IFNT extended the lifespan of the CL as well as the interestrous interval, and eliminated the increase in prostaglandin-F2α (PGF2α) that initiates luteolysis [93]. Though considered the main factor responsible for maternal recognition of pregnancy, IFNT is only present until implantation, day 21 of gestation in cattle, which suggests the presence of other recognition factors in later pregnancy. IFNT must then only be the first in a series of pregnancy recognition factors.

Several activities for IFNT have been identified. The primary action of IFNT is to act as an anti-luteolytic factor. In non-pregnant animals, uterine pulses of PGF2α cause corpus luteum (CL) regression, and the resulting loss of progesterone causes animals to return to estrus for another breeding. These PGF2α pulses are oxytocin (OT)-dependent, wherein OT acts through its receptors (OTR), whose development is stimulated by estrogens, to stimulate PGF2α production. A feed-forward system then ensues, where CL-derived OT is released in response to PGF2α so it may act to produce more endometrial PGF2α [89]. In pregnant ruminants, IFNT prevents CL regression by directly blocking the expression of OT-receptors, so OT cannot induce PGF2α synthesis [81]. It is also possible, however, that IFNT acts indirectly by inhibiting

estrogen receptor expression, and thus preventing the development of OT receptors in the uterine epithelium [94].

Several additional actions for IFNT have been identified, and these activities are presumed to aid in maintaining a pregnant state in cattle and other ruminants. IFNT acts on the endometrium to induce the expression of several IFN-stimulated genes (ISGs). ISGs are known to play a role in uterine receptivity to conceptus elongation and implantation [90]. Several ISGs, such as *ISG15*, are expressed in the uterine epithelium during pregnancy. Studies show these genes increase in expression during conceptus elongation, and then decline in expression in a manner paralleling the decline in IFNT concentrations [95, 96]. IFNT may also play a role in modulating the maternal immune response in order to promote maternal recognition of pregnancy, though immune function during the time of elongation has not been well defined [97].

Schemes For Detecting and Quantifying IFNT

A few selective laboratories have developed IFNT antisera, and generated either radio or enzyme-linked immunoassays for quantifying IFNT [98-100]. However, none of these assays have been commercialized, and this lack of commercially available reagents has encouraged the continued use of cytopathic-based antiviral assays to study the ontogeny and regulation of IFNT production [77, 86, 101-103]. The term "cytopathic" refers to the necessary use of a virus to perform the assay. Although cytopathic antiviral assays provide equal, if not greater, sensitivity for detecting IFNT than immunoassays, their use has diminished because of the heightened regulations and restrictions for using many of the viruses traditionally employed for these assays

[104]. The most commonly used virus has been vesicular stomatitis virus (VSV), and although the strains used in today's assays are lab attenuated, they remain infectious, and must be handled under biosafety level 2 (BSL2) conditions [104]. Other disadvantages of these assays are that they are time consuming, usually requiring 2 to 3 days to complete, and a high assay-to-assay variation is commonly observed. Radioimmunoassays (RIAs) and enzyme linked immunosorbent assay (ELISA) are available as an alternative to cytopathic assays, however, these options do not allow for the quantification of IFNT biological activity. Because of these shortcomings, a new cell-based biological assay that does not require virus challenge has been developed to examine IFNT and other IFNs in biological samples.

Summary and Implications of Previous Findings

The dairy industry is currently experiencing an alarming decline in the reproductive efficiency. Pregnancy loss most commonly occurs during early embryonic development, and specifically throughout the first 2-3 weeks of gestation, a time recognized for significant structural remodeling of the embryo and maternal recognition of pregnancy. Some of the more pronounced developmental events are summarized in Figure 1-1. The first cell fate decision is the differentiation of the TE from the ICM, with the TE beginning to form around day 6 post-fertilization. The TE is responsible for the production of IFNT, a factor necessary for early maternal recognition of pregnancy in ruminants. The overlap in the timing of IFNT secretion and heightened pregnancy loss suggest that the embryo's failure to produce adequate levels of IFNT is to blame for pregnancy failures. Traditionally, cytopathic antiviral assays serve as the conventional

method for studying IFNT action. Though these assays are effective, they have several shortcomings including required handling under BSL2 conditions, assay-to-assay variability, and a completion time of up to 3 days. These inadequacies lead us to develop a new bioassay to study the actions of IFNT.

The second lineage specification event occurring in the developing embryo is the segregation of the PrE from the EPI. The establishment of FGF2 as a factor required for PrE production in vitro was a key discovery in early lineage specification in mice, primates and ruminants. However, current knowledge of PrE formation and differentiation is limited in cattle and other ruminants. Notably, very few endoderm cell lines exist for cattle, and the lack of these reagents has hindered progress in our understanding of endoderm-trophectoderm interactions in cattle and other ruminants.

These past observations have prompted the need to better understand the events of early embryogenesis so that solutions to the increasing rates of pregnancy losses can be discovered and tested. The overall aim of the following work was to provide new resources for studying crucial embryonic events in cattle. Specifically, the work completed for this thesis research were aimed at

1) Developing a new quantitative assay for the detection of IFNT in biological samples that may reduce the shortcomings of the currently available assays, and
2) Examining ways to induce PrE lineage specification, and develop a bovine PrE cell line as a preface to studying PrE development and PrE-TE interactions during early embryogenesis.

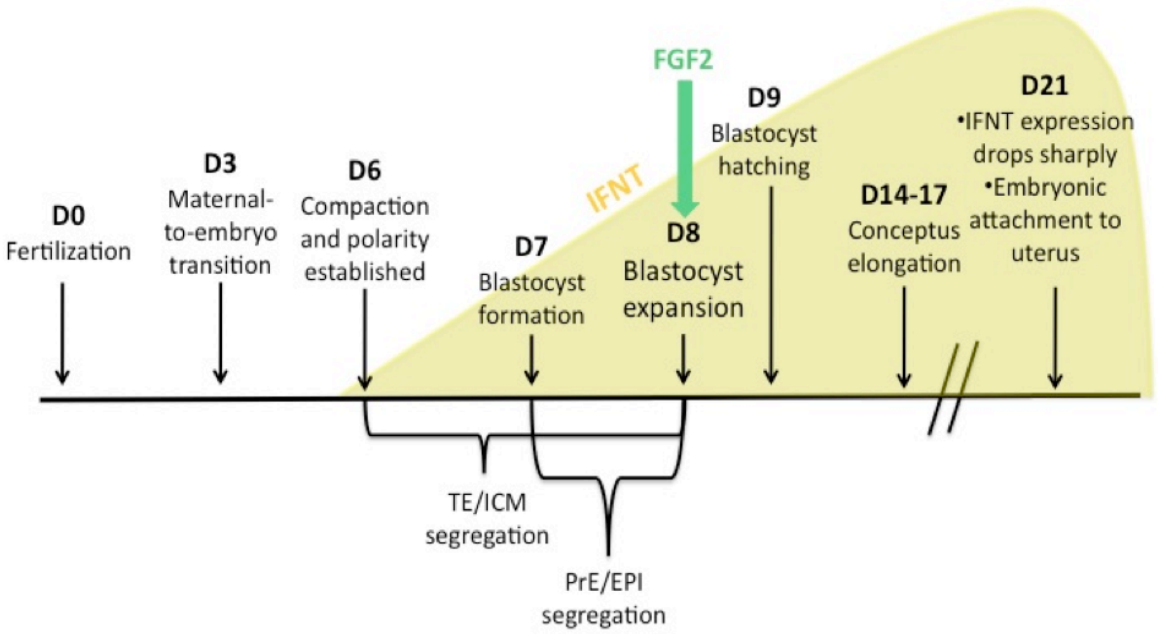

Figure 1-1 A time-line of the major developmental events occurring during early embryogenesis in cattle. The shaded area represents the production of IFNT during early embryogenesis. Epiblast (EPI), fibroblast growth factor 2 (FGF2), inner cell mass (ICM), interferon-tau (IFNT), primitive endoderm (PrE), trophectoderm (TE).

Chapter 2

Development of a Luciferase-Based Reporter Assay for Detecting Interferon-Tau in Biological Samples

Introduction

IFNs are cytokines that have long been recognized for their ability to respond to viral infections. Two main types of IFNs exist; type 1 and type 2 [105]. IFNs are induced by various cell types in response to viruses, and contain antiviral and/or inflammatory activity. An IFN of particular interest to scientists studying early pregnancy in ruminants is IFNT. This protein is structurally similar to other Type I IFNs, and contains the same antiviral, antiproliferative, and immunomodulatory activities distinct to this group of cytokines [106]. A defining characteristic of *IFNT* is that its expression is not viral-dependent like other Type I IFNs, but rather is expressed exclusively by the TE. During early pregnancy, prior to implantation, IFNT binds to Type I IFN receptors (IFNAR1/2), and acts to signal to the mother that she is pregnant, and thus maintains the pregnant state [89, 107]. Several laboratories have utilized immunoassays for quantifying IFNT [98-100], but the lack of commercially available reagents has encouraged the continued use of cytopathic antiviral assays to study the mechanism behind and regulation of IFNT production [77, 86, 101-103].

The anti-viral behaviors of IFNs have been utilized in the development of biological assays to study IFN activity in cell-conditioned medium, plasma, and lymph fluid. These cytopathic antiviral assays are commonly used as a substitute for commercially available assays. RIAs and ELISAs have shown to be successful

alternatives to antiviral assays because of their ability to distinguish between various IFNs, and their detection sensitivity. However, these current assays do not have the ability to quantify the biological activity of IFNs, making the antiviral assay a necessity for this information.

The antiviral assay, though effective, has many shortcomings as well. The most obvious is use of a live virus, commonly the vesicular stomatitis virus (VSV). Although the virus is attenuated, it still has the ability to cause infection, and handling must occur under BSL2 conditions. Also, while the antiviral assay is sensitive, it has a high assay-to-assay variability, which can result in data inaccuracies. Lastly, most antiviral assays require 2-3 days for completion to allow for proper viral challenge. These shortcomings make a new biological assay desirable.

The premise behind the following work was to develop a new cell-based biological assay to quantify IFN activity, specifically that of IFNT, in biological samples. The new assay was developed in an attempt to overcome the many pitfalls of the current cytopathic assays, while achieving a similar level of sensitivity.

Methods

Cell Transduction

Madin-Darby bovine kidney cells (MDBK; ATCC#CCL-22) were propagated and passaged as described previously [75] in Dulbecco's modified eagle medium (DMEM, 25 mM glucose; Life Technologies, Grand Island, NY) containing 10% [v/v] fetal bovine serum (FBS), and antibiotics (50 IU Penicillin G and 50 μg/ml Streptomycin sulfate) at 37°C in 5% CO_2 in humidified air. For transduction, cells at 50% confluence were

incubated in DMEM containing 3 μg/ml Polybrene (Santa Cruz Biotechnology Dallas, TX). Lentiviral particles containing the ISRE-Luc reporter (Cignal™ Lenti ISRE; SA Biosciences Valencia, CA) were added to cells for 4 h (multiplicity of infection = 2). The VSV-g pseudotyped lentivirus particle expresses Luc under the control of a minimal mammalian CMV promoter and tandem repeats of ISRE. Cells were allowed to recover in DMEM containing FBS and antibiotics for 3 days before selection with 10 μg/ml Puromycin (Life Technologies) for 14 days. After selection, cells were propagated and passaged in DMEM with FBS and antibiotics.

ISRE-Luc Assay

Cells were trypsinized and plated into 96-well polystyrene plates with opaque walls and optically clear bottoms (Corning Inc., Corning, NY) at a density of 5-10 x 10^5 cells/well. After 4 h incubation at 37°C in 5% CO_2, medium was replaced with 50 μl of medium containing FBS, antibiotics, and either the sample or standard. Recombinant human IFNA was used as the assay standard (3.87 x 10^8 IU/mg; EMD Biosciences, Billerica, MA). A 1:3 serial dilution of this protein was completed to generate the standard curve. Samples were prepared by mixing DMEM containing 10% FBS and antibiotic with reconstituted recombinant proteins, conditioned media, or flush solution (no more than one-half the final volume of medium added to each well). Cells were incubated at 37°C overnight (16-24 h). Luciferase activity was determined by adding 50 μl of One-Glo Luciferase Assay Substrate (Promega Corp., Madison, WI) to each well. After 10 min of agitation, the plate was read using an Infinite M200 PRO Plate Reader

(TECAN Systems Inc., San Jose, CA). Steady levels of Luciferase activity could be measured for up to 6 h after the addition of the One-Glo Substrate.

Recombinant Proteins

Ovine recombinant IFNT ($1x10^7$ IU/mg; ProSpec, East Brunswick, NJ), human recombinant IFN gamma (IFNG) ($2x10^7$ IU/ml; EMD Biosciences, Billerica, MA), and human recombinant interleukin 6 (IL6) (Kingfisher Biotech Inc., St. Paul, MN) were reconstituted according to manufacturers' instructions.

Bovine Trophoblast Conditioned Media and Uterine Flushes

One set of conditioned media samples was collected from a bovine trophoblast cell line that produces IFNT (CT1) [75, 108]. Several flasks of cells were cultured for 48-72 h. Media was then harvested, centrifuged to remove debris (10,000 x g for 2 min), and stored at -80°C until use.

Another set of samples was obtained from previous work that collected uterine flushes from pregnant cows, and conditioned media in elongated bovine conceptuses [109]. In brief, *in vitro* produced bovine embryos were transferred at day 7 post-estrus to crossbred beef heifer recipients (predominantly Charolais and Limousin). Cows were slaughtered at day 16 post-estrus, and excised reproductive tracts were flushed with phosphate-buffered saline (PBS, 0.01 M, pH 7.2). Each conceptus was retrieved and cultured individually in 500 µl synthetic oviduct fluid (SOF; Caisson Labs, Logan, UT) containing 5% FBS. After 24 h, the conditioned medium was removed, centrifuged, and the supernatant was stored at -80°C. The uterine flush was also centrifuged (3,000 x g for 15 min), and the supernatant was stored at -80°C.

Ovine Uterine Flushes

Work was completed at Washington State University using previously described procedures [110]. Animal work was completed in accordance with and following the approval of the Institutional Animal Care and Use Committee. Ewes (n=9) were mated to rams of proven fertility, and were sacrificed at day 14 post-mating. The reproductive tract was excised, and the uterine lumen was flushed with 10 mM Tris [pH7.2]. Conceptuses were removed, the flush was centrifuged (3,000 x g for 15 min), and the supernatant was stored at -80°C.

Bovine Blastocyst Culture

In vitro production of bovine embryos was completed as described previously, with minor modifications [111, 112]. Ovaries from beef and dairy cattle were obtained from Brown Packing Co. (Gaffney, SC). Cumulus-oocyte-complexes (COCs) were obtained by slashing follicles with a scalpel blade, and then by searching with a Nikon SMZ745 stereomicroscope (Nikon Instruments Inc., Melville, NY). Groups of 10-15 COCs were cultured in 50 µl drops of oocyte maturation medium (TCM199 containing Earle's salts [Life Technologies], 10% FBS, 25 µg/ml bovine FSH [Bioniche Life Sciences, Bellvill, ON, CA], 2 µg/ml estradiol [Sigma-Aldrich, St. Louis, MO], 22 µg/ml sodium pyruvate, 1 mM glutamine, and 25 µg/ml gentamicin sulfate [Life Technologies]) at 38.5°C in 5% CO_2 in humidified air. After 21-24 h, COCs were transferred to fertilization medium, and exposed to BoviPure™ gradient-purified (Nidacon, Spectrum Technologies, Healdsburg, CA) bovine spermatozoa derived from a pool of frozen semen from 3 Holstein bulls. After 18 h at 38.5°C in 5% CO_2 in humidified air, cumulus

was removed by vortexing, and groups of 20-30 presumptive zygotes were placed in 50 µl drops of SOF containing 20 µg/ml essential amino acids (Sigma-Aldrich), 10 µg/ml nonessential amino acids (Life Technologies), 4 mg/ml fatty acid free bovine serum albumin (Sigma-Aldrich), and 25 µg/ml gentamicin sulfate (Life Technologies) [113], and cultured at 38.5°C in 5% O_2, 5% CO_2, 90% N_2. At day 8 post-fertilization, blastocysts were collected and placed individually (expanded blastocysts), or in groups of 3-5 (regular and expanded blastocysts) in 50 µl drops of DMEM containing 5% FBS, and antibiotics (50 IU Penicillin G and 50 µg/ml Streptomycin sulfate) at 38.5°C in 5% CO_2 in humidified air [114]. After 48 h, medium was harvested and stored at -80°C.

Antiviral Assay

A cytopathic antiviral assay was used as described previously [75, 115]. Samples were examined in duplicate by serial dilution (1:3) in a 96-well plate. MDBK cells were added, and plates were incubated for 20-24 h at 37°C in 5% CO_2 in humidified air with DMEM containing 10% FBS and antibiotics. Cells were challenged with VSV in DMEM lacking FBS and antibiotics for 1 h, and were then washed and incubated in DMEM containing 10% FBS and antibiotics for 18-20 h. Viable cells were detected by fixation in 70% ethanol, and staining with 0.5% [w/v] Gentian Violet. The concentration at which samples prevented cell lysis by 50% was then compared to a hIFNA standard (3.8×10^8 IU/mg; EMD Biosciences).

Statistical Analyses

Correlation analyses (Pearson Correlation) were completed using GraphPad Prism Software (Version 5; San Diego, CA).

Results

Preliminary studies determined that maximal ISRE-Luc responses occurred when cells were permitted to attach to plates before being exposed to treatments (data not shown). Also, 16 h exposure to IFNs produced a much more expansive and more sensitive dose-response curve than shorter incubations, whereas incubation for > 16 h did not generate any further improvements in assay sensitivity (data not shown).

A dose-dependent increase in luciferase activity was detected after incubation with human IFNA (Fig. 2-1A). The resulting sigmoidal curves contained minimum and maximum values when exposed to 0.5 and 1000 IU/ml IFNA, respectively (1.3 pg/ml and 2.5 ng/ml, respectively). The linear portion of this response (20% above the nadir and 20 below the peak) contained a linear equation with a high coefficient of determination after log transformation (R^2 range = 0.89 to 0.97).

The specificity of the ISRE-Luc reporter for Type I IFNs was examined by determining the sensitivity of this system to IFNT, IFNG, and IL6 (Fig. 2-1B). A dose-dependent increase in Lucerifase activity was detected after exposure to IFNT. More IFNT was needed to induce a response than IFNA because the IFNT preparation contained a lower biological activity than IFNA (1×10^7 IU/mg vs. 3.87×10^8 IU/mg, respectively). Biological responses overlapped when data were presented based on specific activity (data not shown). The ISRE-Luc system reporter did not respond to IFNG or IL6.

One primary interest in developing this assay was to use it for analyzing IFNT content of biological samples from cattle and sheep. A series of studies was completed

to verify assay usage for these purposes. Dose-response studies were completed with biological samples containing IFNT to examine parallelism in responses when compared with the IFNA standard (Fig. 2-2). Media harvested from bovine trophoblast cells (CT1) (Fig. 2-2A) exhibited a parallel response when compared with IFNA (Fig. 2-2A). Parallelism in dose-responses also was observed between IFNA and uterine flushes collected from pregnant ewes at day 14 post-estrus (Fig. 2-2B).

The sensitivity of the ISRE-reporter system for IFNT was determined by examining its ability to detect IFNT in media conditioned by bovine blastocysts, which contain small amounts of IFNT (Fig. 2-3). IFNT activity was detected in 4 of 5 media samples derived from individual blastocyst cultures, and in all media samples derived from group-cultured blastocysts.

A final series of studies examined the relationship between outcomes for various samples assayed with both the ISRE-Luc system and the conventional cytopathic antiviral assay (Fig. 2-4 and 2-5). Two bovine sample sets obtained from previously published work [109] were examined (Fig. 2-4). The samples were comprised of uterine flushes recovered from pregnant heifers at day 16 of pregnancy (Fig. 2-4A), and conditioned medium collected after culture of elongating conceptuses recovered from these flushes (Fig. 2-4B). There was a significant positive association between antiviral and ISRE-Luc activities in both the uterine flushes ($P=0.0001$; $R^2=0.8$) and conditioned medium ($P=0.0004$; $R^2=0.84$) samples. The ISRE-Luc and antiviral activities also were compared in a set of ovine uterine flushes collected from ewes at day 14 of pregnancy

(Fig. 2-5). There was a positive correlation between the ISRE-Luc and antiviral activities for these samples ($P=0.003$; $R^2=0.74$).

Discussion

This work established the use of a reporter-based system for quantifying Type I IFNs in biological samples. The impetus for this work stemmed from the ever-increasing difficulty in using highly regulated viruses such as vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV) and Sindbis virus (SINV) which are necessary for cytopathic assays [104]. Although the creation of the ISRE-Luc system required lentiviral transduction under BSL2 conditions, subsequent usage and propagation of the transduced cells can be completed under biosafety level 1 (BSL1) conditions, thereby avoiding the need for continued use of heightened culture safeguards, monitoring, and record keeping. The new assay also contains several other advantages when compared with the conventional antiviral assay. It can be completed within 20-24 h, whereas the conventional cytopathic assay requires 72 h. The new assay also contains a linear dose-response region that spans two orders of magnitude, while the conventional cytolytic response occurs within a single order of magnitude [115, 116].

Another advantage of the ISRE-Luciferase assay is that it is more selective in the molecules it detects. The conventional antiviral assay responds to various IFNs, and other cytokines that generate a variety of intracellular events that limit viral production and release. The IFN stimulatory genes (ISGs) that respond to IFNs and some other cytokines contain activities that promote viral RNA degradation, inhibit viral transcription, inhibit viral particle assembly, and limit membrane budding and viral release [117].

Several ISGs are ISRE dependent, and respond well to Type I IFNs, but not to IFNG or other cytokines. These IFNs signal their antiviral responses after binding to Type I IFN receptors (termed IFNAR1 and R2) and activating Tyk1 and Jak2 [118]. These Janus kinases then phosphorylate STAT1 and 2, and in combination with IRF9 form IFN stimulatory gene factor 3 (ISGF3), which reacts directly with ISRE to activate transcription [118]. By contrast, IFNG does not act through ISGF3 and ISRE. Instead it utilizes a STAT1 homodimer transduction response to stimulate the gamma activating sequence element (GAS element) present on a subset of ISGs [118].

The entire gambit of Type I IFNs was not tested here. Rather, work focused on establishing if the new assay could quantify IFNT in biological samples. The conventional antiviral assay has been used ever since this factor was identified as a Type I IFN in the 1980's [115]. IFNT contains a dynamic production profile during early pregnancy in ruminants. The conventional antiviral assay has been well-suited for detecting both the small amounts of IFNT produced as trophoblast cells first emerge at the morula and blastocyst stages, and the large amounts of IFNT produced 7 to 10 days later as conceptuses undergo an exponential growth phase before implanting into the uterus [89]. IFNT is the predominant IFN produced by ruminant conceptuses [119, 120]. Therefore, the use of antiviral assays to quantify IFNT is generally accepted as long as pathologic conditions that generate other IFNs are absent (*e.g.* uterine inflammation or infection).

The sensitivity of the ISRE-Luc assay is comparable with a conventional cytopathic assay. Although not tested directly, the sensitivity of both systems to IFNA

appears similar. The ISRE-Luc system detected IFNT in media from individual blastocyst cultures. This also can be achieved by using a cytopathic assay [102, 103]. Interestingly, the ISRE-Luc system detected IFNT in several bovine uterine flushes and conceptus-conditioned media samples that did not contain any antiviral activity when using a cytopathic assay (Fig. 4). This may imply that the ISRE system is more sensitive than the conventional assay. Alternatively, this outcome may simply reflect differences in assay reading. The antiviral assay depends on the visual detection of at least 50% live cells to register a positive reading, and the new ISRE-Luc system does not have this requirement.

Several reporter gene assays have been reported over the past 20 years as alternatives to antiviral assays. These systems have employed various cell lines (WISH, HeLa, MDBK, Vero), and a variety of regulatory sequences (*e.g.* Mx promoter) and reporter genes (luciferase, chloramphenicol acetyltransferase, growth hormone) [121-125]. Levels of sensitivity for detecting IFNs are comparable to those in the ISRE-Luc reporter gene system used herein. One advantage of the ISRE-Luc system is its ease in measuring luciferase activity, especially now that highly robust and stable luciferase reagents exist commercially. Another advantage is the ease of creating the reporter system in cells using the commercially available lentiviral particles (Cignal^{TM} Lenti Reporters). Transduced cells may also be easily shared among laboratories since the pseudovirus is replication defective, and cells can be maintained at BSL1 conditions after they are transduced.

The MDBK cell line is commonly used for cytopathic assays. It is sensitive to various IFNs, including Type 1 IFNs, and it recognizes IFNs from several species, including primates, cattle, sheep and pigs [126-128]. MDBK cells are equally sensitive to ovine and bovine IFNT proteins, which are 77 to 81% identical in primary amino acid sequence [86, 101, 129]. IFNT proteins share substantial sequence similarity among ruminants, especially in receptor binding domains [129], and this permits cross-species use of proteins. For example, recombinant ovine IFNT induces a pseudopregnant state in cattle, goats (*Capra hircus*) and red deer (*Cervus elaphus*) [130-132]. This raises the possibility that IFNT from goats, antelope, bison, musk ox, giraffe and other ruminants could be quantified using this MDBK-based assay.

To conclude, these outcomes provide convincing evidence that an ISRE-based reporter system can substitute for conventional, virus-dependent bioassays when determining IFN bioactivity. The work also established that this new assay contains the sensitivity and specificity needed for quantifying IFNT concentrations in biological samples for cattle, sheep and likely other ruminants.

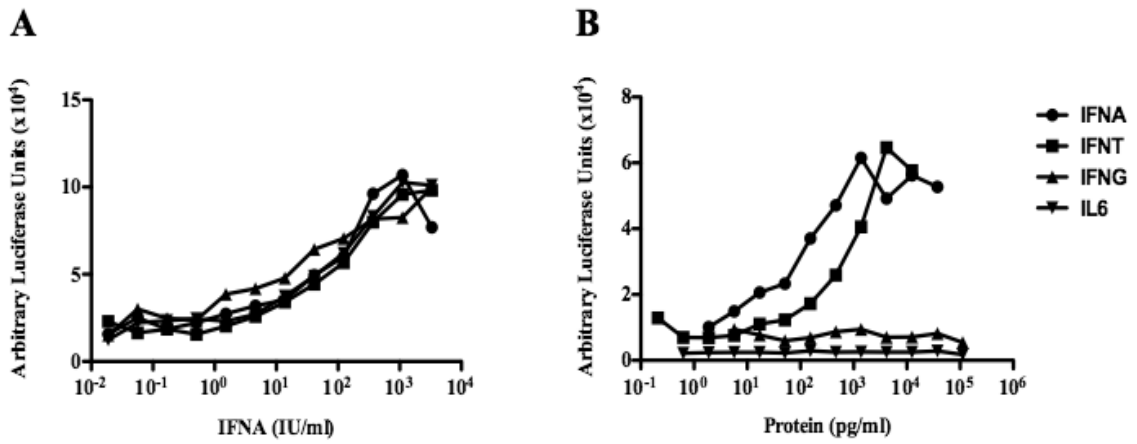

Figure 2- 1 Dose-response curves for recombinant interferons using the ISRE-Luc reporter system in MDBK cells. Panel A: Dose response curves for recombinant human IFNA conducted on 4 separate occasions. Panel B: Dose responses for Type I IFNs (IFNA, IFNT), Type 2 IFN (IFNG) and a non-interferon cytokine that utilizes Jak-STAT signaling (IL6). Both studies represent luciferase activity after 16 h exposure to serial-diluted proteins. Data represent averages of duplicate well readings.

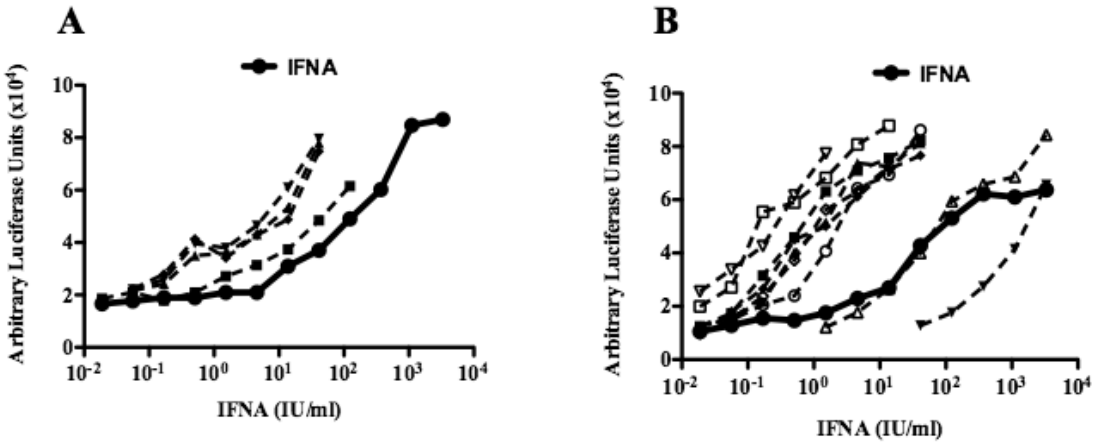

Figure 2-2 Parallelism in luciferase readings between IFNA and biological samples. Panel A: Comparison between IFNA and CT1 cell conditioned media (n=4 samples). Panel B: Comparison between IFNA and uterine flushes collected from ewes at day 14 of pregnancy (n=9). IFNA and each sample set were serial-diluted (1:3). Data represent averages of duplicate well readings.

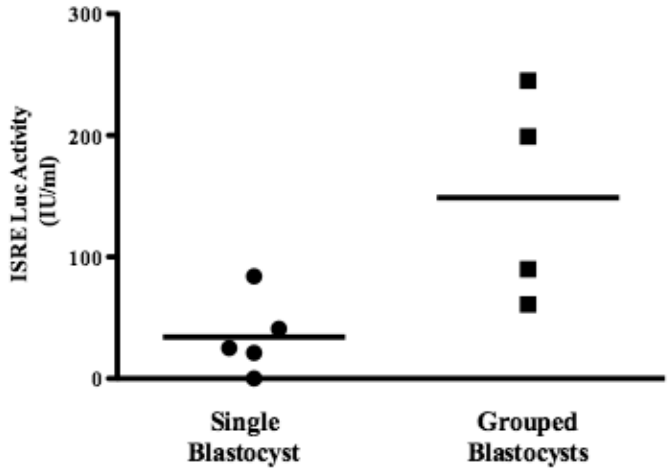

Figure 2-3 Detection of *IFNT* activity in media collected after culture of individual or groups of bovine blastocysts. *In vitro*-derived bovine embryos were examined at day 8 post-fertilization, and individual expanded blastocysts (n=5) or groups of regular and expanded blastocysts (n=4 cultures, each with 3 to 5 embryos) were incubated in 50 µl drops of medium for 48 h. Data represent averages of duplicate well readings.

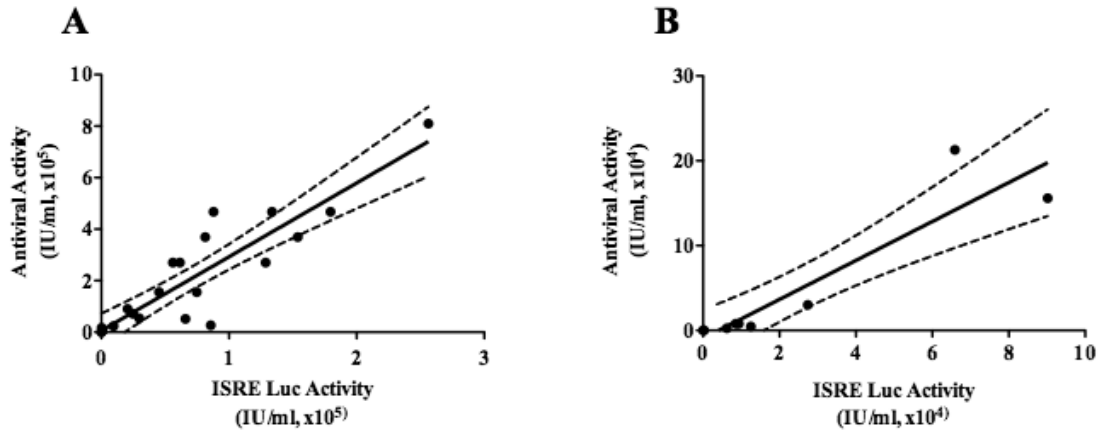

Figure 2-4 Correlation between antiviral and ISRE-Luc activities in biological samples obtained from cattle. Panel A: Conditioned media obtained from conceptuses recovered from heifers at day 16 of pregnancy and cultured individually in 500 µl media for 24 h (n=17). Panel B: Luciferase activity of uterine flush solution from the pregnant heifers (n=7). Data represent averages of duplicate well readings. Positive linear associations were detected between antiviral and ISRE-Luc activities for both the conditioned culture media (P=0.0004; R^2=0.84) and uterine flushes (P=0.0001; R^2=0.8).

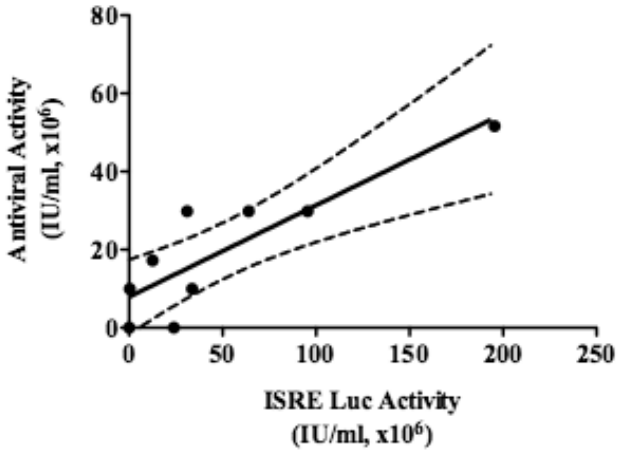

Figure 2-5 Correlation between antiviral and ISRE-Luc activities in uterine flushes from pregnant ewes. Uterine flushes were collected after sacrificing ewes at day 14 of pregnancy (n=9). Data represent averages of duplicate well readings. A positive linear association was detected antiviral and ISRE-Luc activities (P=0.003; R^2=0.74).

Chapter 3

Production of Bovine Primitive Endoderm Outgrowths and Primitive Endoderm Cell Lines for the Study of Early Bovine Embryogenesis

Introduction

The dairy industry has been experiencing an alarming decrease in pregnancy rates over the past several decades. Fewer than 50% of all viable embryos progress past day 27-30 of gestation [4, 6]. At least some, and likely a fair portion of these losses likely occur because of problems associated with early embryogenic processes. One such event is the specification of the PrE from the EPI, occurring between days 7 and 8 of gestation. The PrE evolves to become the yolk sac, which provides nutrition to the developing embryo [40]. The yolk sac continues to develop until day 20 of pregnancy, when it promptly regresses [41, 42]. Yolk sac development coincides with the time of conceptus elongation and immense TE proliferation, though the importance of these overlapping events on embryonic development is lacking.

Past research from this lab has revealed the role of FGF2 in PrE formation in bovine embryos [73]. Namely, FGF2 supplementation increased PrE outgrowth rates in bovine blastocysts. The change in PrE outgrowth development was substantial, increasing outgrowth rates from 1% in non-treated controls to 23% in blastocysts exposed to 10 ng/ml FGF2. We propose that the incidence of PrE formation could be improved further by enhancing the culture conditions employed during blastocyst exposure to FGF2.

Another unclear feature of bovine PrE is their specific endoderm lineage. Previous work on this topic has not been fully described, for example whether PrE is maintained in its primitive state during culture or if it differentiates into other lineages, such as PE and VE. Also, the longevity and usefulness of bovine PrE cell lines as cell culture models is not known. Only two reports exist of bovine PrE cell lines [73, 133], and this laboratory failed to maintain both of these lines for more than 4-6 weeks in culture (McCoski & Ealy, Unpublished observations). Useful PrE, VE, and PE cell lines exist for the human and mouse, and these cells have been studied extensively. The lack of these cell lines in cattle and other ruminants has dampened our ability to examine this important feature of embryogenesis. Our contention is that understanding endoderm development and its interaction with TE will help us to better understand early embryologic processes in cattle, and to determine how these processes may be interrupted in cattle that experience pregnancy loss. If we are correct, then this improved understanding of early bovine embryonic development will help us to limit some of the pregnancy losses ailing the dairy industry. The goals of the following research were to 1) improve the protocol for developing PrE outgrowth cultures from bovine blastocysts, 2) delineate the type(s) of endoderm lineages represented in these cultures, and 3) produce and define bovine PrE cell lines for use in future studies.

Methods

In Vitro Embryo Production

The *in vitro* production of bovine embryos was completed as described previously [111, 113]. The only exception is that in some studies bovine oocytes were purchased

from DeSoto Biosciences (Seymour, TN). These oocytes were derived from Brown Packing (Gaffney, SC), and cumulus-oocyte complexes (COCs) were isolated and shipped overnight in a transport incubator at 38.5 °C. Individual tubes containing COCs were gassed with 5% CO2 in air before sealing. Oocytes were subsequently fertilized, and zygotes were cultured as described earlier in Chapter 2.

Endoderm Outgrowth Cultures

At day 8 post-fertilization, individual blastocyst stage embryos were transferred to 12-well plates (3.8 cm^2; Corning, Tewksbury, MA) coated with Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, California). Embryos were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5.5 mM glucose, 20% fetal bovine serum (FBS), antibiotic/antimycotic mix (Life Technologies, Grand Island, NY) and 10 ng/ml recombinant bovine FGF2. Embryos were then incubated at 38.5 °C in 5% CO_2 in air. At day 12 post-fertilization, half of the culture medium was replaced with fresh medium containing 5 μl of 10ng/ml FGF2. Embryos were visualized under a stereomicroscope during media aspiration to ensure they were not removed. At day 15, outgrowths were assessed to determine the presence or absence of an outgrowth (Fig. 3-3 A and B). Outgrowths were then maintained in DMEM containing 5.5 mM glucose, antibiotics, and 10% FBS at 38.5°C in 5% CO_2 in air. Medium was changed on outgrowths every 2 to 3 days. Efficiency of outgrowth production was noted on day 15 post-fertilization. Outgrowths were determined to be PrE rather than TE based on the differing physical appearances of the two cell types (Fig. 3-1A and B). Outgrowths were maintained thereafter for cell line production, or lineage marker analysis.

Endoderm Cell Line Production

Upon reaching >90% confluency, passage 0 (P0), or initial, outgrowths were passaged for further culture as described previously [73]. Briefly, outgrowths were treated with trypsin in order to dislodge cells from the plate. Cells were then passaged onto either Matrigel-treated or untreated T75 (75cm^2; Corning) flasks. Medium was changed every 2-3 days, and cells were passaged as needed. This usually was needed every 7 days. Initial work determined that these cells did not require Matrigel so most of the studies utilized cells that were passaged onto untreated flasks.

Throughout their continued culture, cells were frozen and stored for future culture. In brief, cells were dislodged from plates using trypsin. The contents of the flask were collected into a 15ml conical tubes, and centrifuged at 500g for 2 minutes. The liquid content of the tubes was aspirated off, and the resulting pellet was re-suspended into a freezing media made of 92% FBS and 8% DMSO (Sigma-Aldrich, St. Louis, MO). Cells were then aliquoted into 1ml samples, and stored in liquid nitrogen. When thawed, cells were warmed in a 37°C water bath. A 10% FBS growth media, described previously, was then added to the cells, and the entire contents of the tube were removed and added to a T75 flask containing 10ml of the 10%FBS growth media. Cells were propagated as described earlier. RNA samples were also collected at several different passages, and the process will be described later.

By passage 6 (P6), cells began undergo quiescence. Their slowed-rate of growth was accompanied by a cell morphology that was less tightly packed than previous passages.

Fluorescent Staining

Upon reaching confluency, PrE cells were passaged onto 35mm glass-bottom dishes with a 20mm bottom well (In Vitro Scientific, Sunnyvale, CA) treated with Purcol, a purified bovine collagen solution (Advanced Biomatrix, San Diego, CA). Cells were passed at varying rates (1:10, 1:20, 1:30, 1:40), and cultured for 3 days in the 10% FBS medium described previously. Cells were then fixed with 4% paraformaldehyde in PBS, and stored at 4˚C until staining. Cells were stained with ActinGreen reagent (Life Technologies) and DAPI (Life Technologies) according to the manufacturer's instructions, and were then observed under fluorescence.

Quantitative RT-PCR Analysis

PrE outgrowths were processed for RNA isolation at days 16, 19, 21, or 23 post-IVF. In most cases, the day of RNA isolation corresponded to when endoderm reached approximately 80-90% confluency (n=3 to 4 for each stage; n=15 total). These outgrowths were referred to as passage 0 (P0) outgrowths. RNA was also isolated from the endoderm lines at P2, P5, and P6. RNA was isolated from both sample sets using TRIzol reagent (Life Technologies) and the PureLink RNA mini kit (Life Technologies), according to the manufacturer's instructions. RNA was stored at -80° C.

All RNA samples were incubated with RNAse-free DNAse for 30 minutes at 37°C (Life Technologies) before completing quantitative reverse transcription polymerase

chain reaction (qRT-PCR). Then samples were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was performed using the SybrGreen detection system (Life Technologies) in combination with primers for endoderm, trophoblast, and epiblast-specific transcripts (Figure 3-3), which were identified by the NCBI website, and then synthesized by Life Technologies. RPS9 was used as the internal reference control. PCR was completed with the use of and Eppendorf Realplex4 Mastercycler (Hamburg, Germany). Samples were heated to 95°C for 10 minutes. Then, 40 cycles of denaturation (95°C, 15 sec), annealing (57°C, 15 sec), and synthesis (68°C, 20 sec) were completed. Thereafter, a melting curve analysis was completed to verify the amplification of a single product. For this, the temperature was once again brought to 95°C for 15 seconds, dropped to 60°C, and then over the course of 20 minutes it was raised back to 95°C. Primer efficiency was determined for each primer set by running a standard curve analysis on pooled samples (efficiencies ranged from 83 to 100%). Each PCR sample was run in triplicate, and a fourth sample lacking the reverse transcriptase was included as a negative control.

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

Statistical Analyses

Changes in the relative abundance of endoderm transcripts were determined by least-squares analysis of variance using the general linear model of the statistical

analysis system (SAS Institute, Cary, NC). Differences were partitioned further by completing pair-wise comparisons (PDIFF analysis in SAS).

Results

Outgrowth Production Efficiency and Lineage Profiling

We proposed that previous attempts to generate endoderm outgrowths from bovine blastocysts were suboptimal. Therefore, improvements in endoderm outgrowth formation were attempted by increasing the amount of serum included in the cultures to 20% [v/v] and by supplementing FGF2 throughout the initial period of outgrowth development. These minor adjustments in culture conditions improved the efficiency of endoderm outgrowth generation to 80.3 ± 5.6% when assessed at day 15 post-fertilization (Fig. 3-2).

A series of lineage-specific transcript markers were examined to verify that endoderm outgrowths harvested at P0 represented endoderm. This assessment was completed various times post-fertilization (day 16-19 and day 21-23) (Fig. 3-3). *GATA4* and *GATA6* mRNA, which are expressed in all types of endoderm, were present in samples at each time point. Several PE-specific transcripts (*CXCR4, HHEX, THBD*) and VE-specific transcripts (*BNIP1, VEGFA*) also were detected in all P0 outgrowths at both time points. Several TE-specific markers (*CDX2* and *IFNT*) were detected in most, but not all P0 samples. Expression for *NANOG*, a marker for pluripotency and the ICM, was non-existent in the day 16/19 P0 samples. However, it could be detected, albeit at a low level, in the day 21/23 samples.

Examination of the Endoderm Lines

During continued culture, actively growing cells had a division rate of about 12-18 hours, and usually required passaging every 7-10 days. Just prior to passaging, cells seemed to undergo contact inhibition, as proliferation was halted upon reaching 90% confluency. The period of active growth continued until around P5 when cell growth began to slow. By P6, cell lines entered a quiescent state. These behavioral characteristics were maintained when cells were frozen, and then thawed again for culture. Cells were also observed to grow in colonies while in culture (Fig. 3-4 A) Colonies contained cells of a fairly consistent shape, though size varied (Fig. 3-4 B).

Lineage marker profiles were examined in two cell lines, termed E1 and E2, to determine potential changes in gene expression as the cell lines move from a highly proliferative (P2) to a quiescent (P6) state (Fig. 3-5 and 3-6). General endoderm markers, *GATA4* and *GATA6*, were expressed in both early and later passages. PE-specific transcripts, *CXCR4, THBD* and *HHEX* were also present in all sample passages. The VE-specific transcript, *VEGFA*, was observed in all samples as well, and increased in expression with continued time passaging ($P<.05$). Interestingly, *BNIP1*, also VE-specific, was present in P2 to P5 samples; however, the transcript could not be detected at P6.

Both TE and EPI markers were likewise examined in these cell lines over time in culture (Fig. 3-6). The two TE-specific transcripts, *CDX2* and *IFNT*, showed to increase expression with consecutive passaging, as did ICM-specific, *NANOG* ($P<.05$).

Discussion

Though the importance of the three main germ layers of the developing embryo has long been recognized, PrE development and function during embryogenesis remains vastly understudied in cattle and other ungulates. One main reason for this lack of study, at least in cattle, results from the lack of cell culture lines that represent endoderm lineages. The work contained herein describes new insights into the developmental tendencies and gene characteristics of endoderm outgrowths derived from IVP bovine embryos.

Although there are several reported studies on the use of a mouse endoderm cell culture, there is limited research involving the continued culture of an endoderm cell line in ruminants, and a true bovine endoderm cell line remains non-existent [133-136]. A bovine endoderm line would prove to be useful in studying early embryo development, particularly yolk sac development, which is different in the bovine model compared to the mouse.

Previous studies from this lab recognized FGF2 as a promoter of PrE development in bovine blastocyst outgrowths [73]. FGFs were first implicated for their role in PrE formation when it was realized that FGF2 is produced in the endometrium, and is secreted both before and after implantation [75, 76]. Previous studies produced outgrowths with a 23.5% success rate [73]. This rate is far lower than that of the current study, which achieved a final outgrowth rate of 80.3%. The current study produced outgrowths under similar conditions, with the only deviation being a change in FBS concentration within the culture media. Previous work used 5%, while the current study

used 20%. With this in mind, the heightened rate of success likely is attributed to the increased concentration of FBS. Another possible explanation for the high success of producing outgrowths is the high concentration of Matrigel used to coat wells prior to plating. Matrigel serves as an artificial basement membrane matrix, allowing for increased growth and attachment of cultured cells. Like FBS, Matrigel is not a well-defined product. Several growth factors, included FGF, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), and transforming growth factor beta (TGFβ) exist in Matrigel [137]. Therefore, it is possible that these growth factors within the Matrigel also played a role in the increase rate of successful outgrowths.

The exact composition of FBS is unknown. It is a naturally occurring substance with marginal variation among distributers and within collection lots [138]. In general, FBS is made up of various supplements including vitamins, fatty acids, and growth factors. One question that remains unresolved is whether the increased concentration of FBS in blastocyst cultures increased the proliferation of all cells, notably TE, or if it acted solely on PrE. Low amounts of *CDX2* and *IFNT*, TE-specific transcripts, existed within the P0 samples, and levels seemed to increase slightly as outgrowths aged. These outcomes suggest that the endoderm cells within the P0 cultures may have overwhelmed and prevented the growth of TE cells, but as outgrowths reached quiescence TE cells were able to reestablish their numbers in the cultures. Alternatively, It is possible the increased concentrations of FBS promoted PrE attachment, and subsequent growth occurred independent of the FBS concentrations. After the cultures

were established, a reduction in FBS from 20% to 10% did not impact the ability of PrE to proliferate.

It also is important to note that the need for FGF2 supplementation occurs only as the endoderm cultures are being initiated. Work by Yang et al determined that FGF2 supplementation promoted endoderm outgrowth formation, but that FGF2 supplementation does not impact endoderm proliferation after cell lines have been established [73]. The same was evident in the present work. A definitive comparison of extended FGF2 supplementation versus non-supplementation was not completed, but removing FGF2 supplementation at day 15 did not impact the ability of endoderm cell lines to continue to proliferate. What remains unknown, however, is whether continuous FGF2 supplementation may impact endoderm transcript expression profiles and/or the onset of cell senescence.

The second study was aimed at establishing the precise type of endoderm represented in the P0. A variety of markers of PrE, VE, and PE exist in these outgrowths. These patterns of expression may indicate that the outgrowths maintain a level of pluripotency, where they may become either the PE or VE subtype. It also is interesting to note that several other endoderm markers were tested, though transcripts could not be detected. These transcripts included those associated with PrE, PE, and VE (*FGFR1, FST, ICAM1, PDGFRA, SOX7, SPARC, STRA6*). The lack of these transcripts could have important implications, but proper primer efficiency testing could not be completed on these primers since we did not have proper control tissues for this work. Therefore, it remains unclear if the absence of these transcripts is real. TE-

specific transcript expression was also observed within P0 samples; however the ICM-specific marker, *NANOG*, was not detected in any day 16/19 samples. This may be because the earlier outgrowths (day 16/19) contain multiple cell types at a more diluted concentration, and as they progress in culture the cells increase in number. Based on these results, these outgrowths seem to be PrE with an apparent ability to differentiate into either PE or VE should they be given the proper signals. Treating the outgrowths with lineage-specific differentiation factors can test this hypothesis. If the hypothesis is correct, and the outgrowths are in fact PrE, they should be able to segregate into either endoderm sub-type. Completing this work is imperative for proper description of these endoderm outgrowths and their multipotent potential.

The final portion of this study examined continuous culture of selective endoderm lines. Cells grew in colonies when in a continuous culture environment, and colonies contain cells of a fairly consistent shape, though size of cells varied throughout the colonies. This difference may be further proof of the outgrowths' multipotent potential, as size variation may suggest different endoderm subtypes. The cell lines that were actively dividing had a cell division rate of about 12-18 hours, and required passaging every 7 days. However, cells did seem to encounter contact inhibition as they became more confluent immediately before each passage. This behavior is reminiscent of what is observed in TE cells when the Hippo signaling pathway is initiated, causing contact inhibition. The 2 cell lines studied each divided until P5, or for about 5 to 6 weeks, and when began to grow more slowly. Growing cells beyond P6 failed. One encouraging facet of these cell lines is that they can be frozen away, and then thawed and grown for

several weeks. Although the precise timing of quiescence after freeze/thawing was not examined, these frozen/thawed cells also ceased to grow well once they were cultured beyond 6 weeks. Nonetheless, the storage of early passage endoderm lines provides a way to utilize these cell lines for extended periods of time. A future interest is to determine if the lifetime of these cultured endoderm lines can be extended after immortalization. A specific interest is to introduce human telomerase reverse transcriptase (hTERT) into these cells. This enzyme maintains the lengths of telomeres, a cause for senescence in cultured cells, and thereby increases the longevity of cells in culture [139].

PCR results showed that cell lines maintained an endoderm-like behavior throughout culture as transcripts that were expressed in P0 samples were also expressed in the endoderm cell lines. Both cell lines expressed *GATA4* and *GATA6*, but also *HHEX* and *THBD*, PE-specific transcripts. An increased expression of the VE-marker, VEGFA, was observed as lines reached later passages, indicating that cells may be differentiating. An increase in the TE-specific transcripts, IFNT and CDX2, may indicate that TE cells were present in cultures, but were only able to establish themselves once PrE began to quiesce. This potential for multiple cell types in outgrowth cultures also provide a need to establishing immortalized PrE cells lines so that cells can be selected clonally, thereby eliminating the presence of non-PrE cell types.

To summarize, these observations established that bovine embryonic outgrowths can be used for the efficient creation of endoderm cell lines. These cells contain a

variety of endoderm transcripts, and expression profiles suggest that the cells contain PrE, VE, and PE potential. It remains unknown if specific differentiation of these presumptive PrE into VE and PE are possible. These cultures also contain markers of TE and EPI, suggesting a mixture of embryonic and extraembryonic cell types may exist in these outgrowths. If this is the case, then immortalization and clonal selection procedures may be needed to isolate PrE lines for future study. Although there is much work that needs to be completed on this topic, the work presented herein provided new insights that bring us much closer to providing new ways to examine endoderm development and differentiation in cattle and to better understand how endoderm functions to promote the continuation of embryonic and extraembryonic development during early pregnancy.

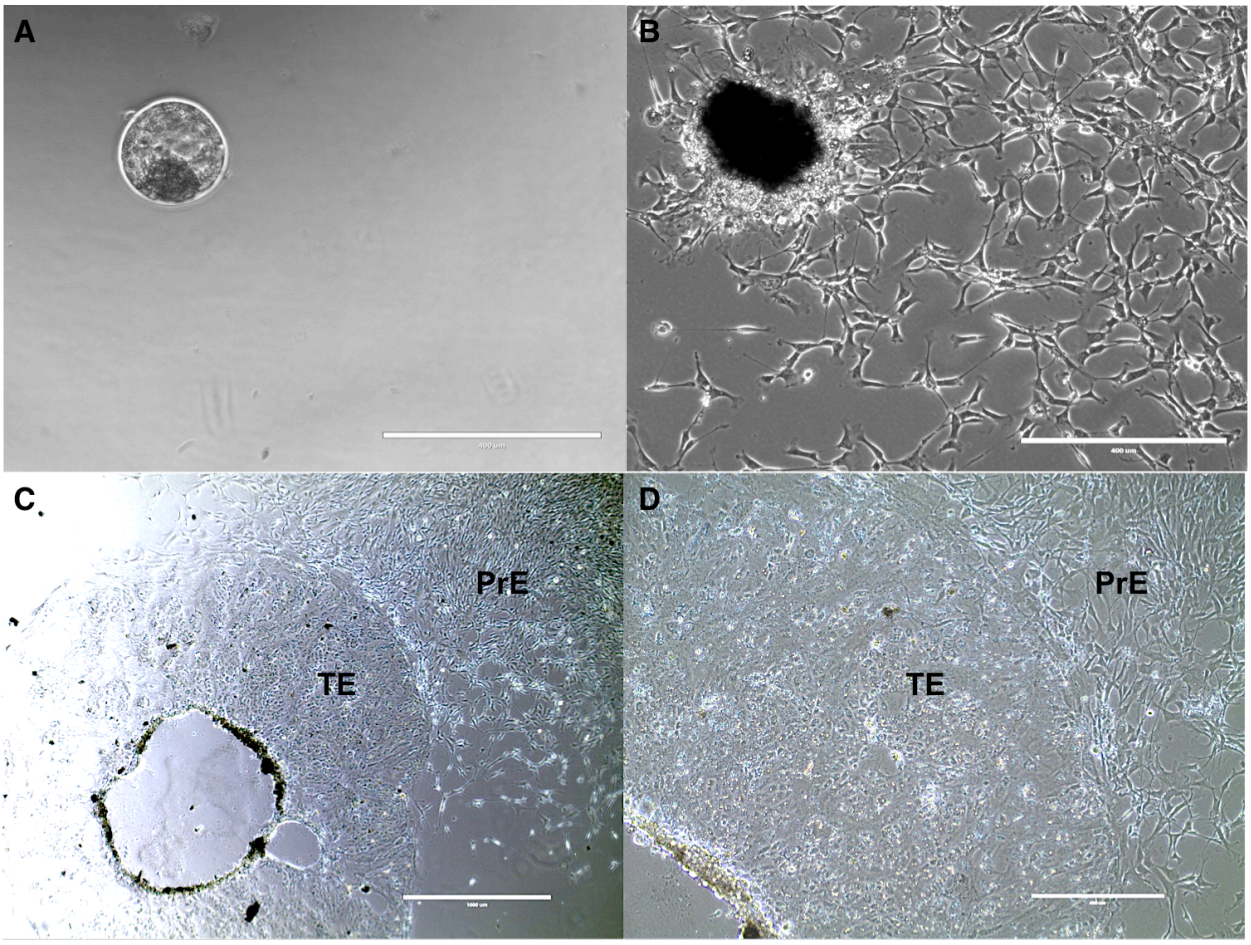

Figure 3-1 P0 outgrowths derived from *in vitro* produced bovine embryos. Day 8 blastocysts were transferred to 12-well plates (3.8 cm^2) coated with Matrigel™ Basement Membrane Matrix. Embryos were cultured in DMEM containing 5.5 mM glucose, 20% FBS, antibiotic/antimycotic mix, and 10 ng/ml recombinant bovine FGF2. Panel A: Day 8 blastocyst. Panel B: Day 15 embryo with endoderm outgrowth after FGF treatment. Panel C: Day 19 trophectoderm (TE) and primitive endoderm (PrE) outgrowths at 100x magnification. Panel D: Day 19 TE and PrE outgrowths at 200x.

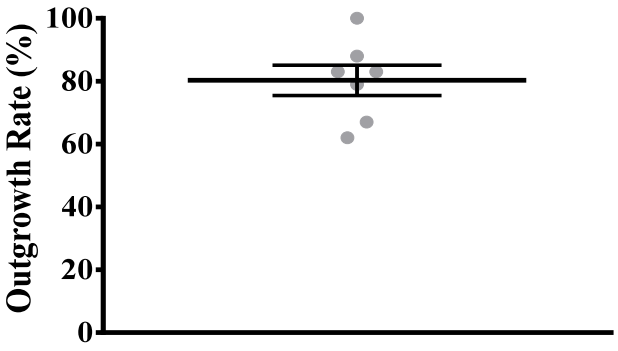

Figure 3-2 Incidence of outgrowth development from blastocysts treated with FGF2. Single blastocysts were transferred to 12-well plates (3.8 cm^2) coated with Matrigel™ Basement Membrane Matrix. Embryos were cultured in DMEM containing 5.5 mM glucose, 20% FBS, antibiotic/antimycotic mix, and 10 ng/ml recombinant bovine FGF2. On day 15 post-fertilization, outgrowths were examined (N=7 replicate studies).

Gene of interest	Lineage specification	Day 16/19	Day 21/23
GATA4	PrE	+	+
GATA6	PrE	+	+
CXCR4	PE	+	+
THBD	PE	+	+
HHEX	PE	+	+
BNIP1	VE	+	+
VEGFA	VE	+	+
CDX2	TE	+	+
IFNT	TE	+	+
NANOG	ICM	-	+
RPS9	Housekeeping	+	+

Figure 3-3 The presence (+) or absence (-) of lineage-specific transcripts in P0 bovine embryo outgrowths at two different collection points (day 16/19 and day 21/23). RNA was isolated from bovine P0 embryonic outgrowths at day 16, 19, 21, or 23 post-fertilization. Samples underwent qRT/PCR analysis to determine the presence or absence of lineage-specific markers (N= 6 day 16/19 samples, N=7 day 21/23 samples).

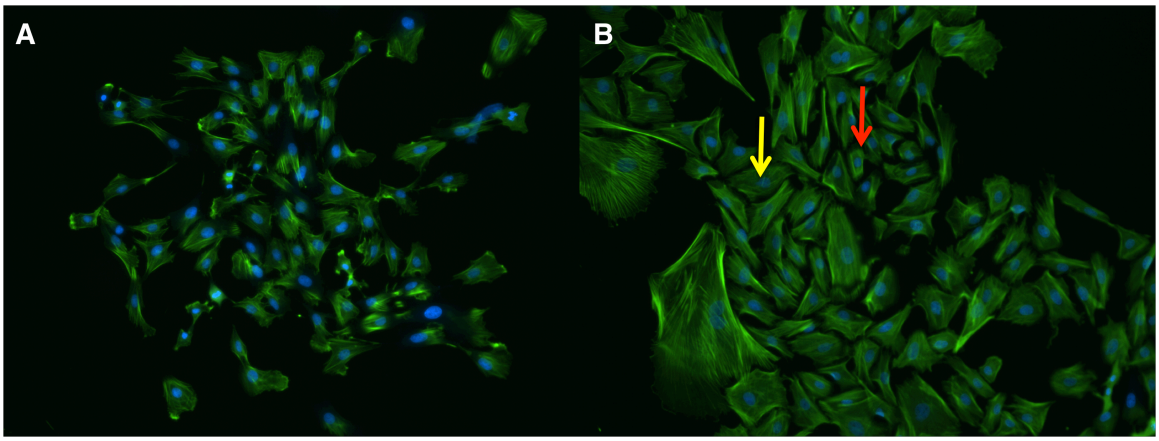

Figure 3-4 Fluorescent imaging of endoderm outgrowths stained with ActinGreen and DAPI. Endoderm cells were plated on 35mm culture dishes with 20mm glass bottoms, and given 3 days to attach to the plates. After 3 days, cells were fixed with 4% paraformaldehyde, and stained with ActinGreen and DAPI. Fluorescence was then observed at A) 100x and B) 200x magnification. The arrows indicate two potentially different cell types within the colony.

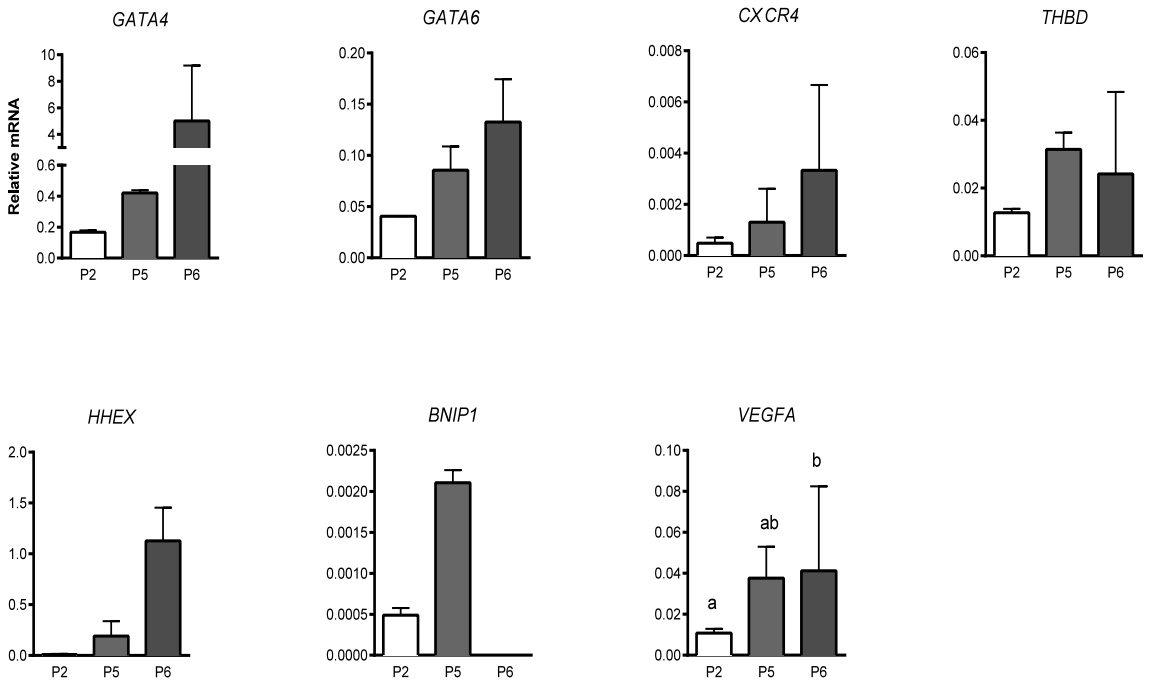

Figure 3-5 Relative mRNA expression of PrE-specific (*GATA4, GATA6*), PE-specific (*CXCR4, THBD, HHEX*), and VE-specific (*BNIP1, VEGFA*) transcripts. Endoderm outgrowths were continuously cultured in DMEM supplemented with 10% FBS, and antibiotic/antimycotic mix. RNA was collected at passages (P) 2, 5, and 6. P2 is representative of actively dividing cells, and P5 and P6 represent cells nearing quiescence. Differing superscripts denote significant differences in transcript expression between passages ($P<.05$).

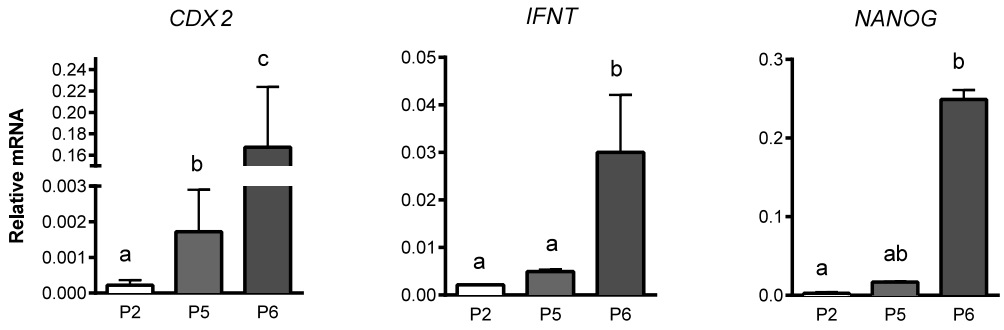

Figure 3-6 Relative mRNA expression of TE-specific (*CDX2, IFNT*) and ICM-specific (*NANOG*) transcripts. Endoderm outgrowths were continuously cultured in DMEM supplemented with 10% FBS, and antibiotic/antimycotic mix. RNA was collected at passages (P) 2, 5, and 6. P2 is representative of actively dividing cells, and P5 and P6 represent cells nearing quiescence. Differing superscripts denote significant differences in transcript expression between passages ($P<.05$).

Interpretive Summary

During early gestation, dairy cows experience a high rate of pregnancy failure. Significant losses can be observed within the first few weeks of pregnancy, and the timing of the losses coincides with TE and PrE lineage specification (days 6 to 8 of gestation), and IFNT production by the TE (day 6 to 21). It is probable that developmental errors during this time are to blame for pregnancy failures, however, knowledge of this period of embryonic growth is greatly lacking. Therefore, the goal of this work was to improve the understanding of early lineage specification events, and the actions of these cell types during bovine embryonic growth.

The first set of studies was performed to develop an ISRE-reporter system to be used as a substitute for conventional cytopathic assays when measuring IFNT in biological samples. Results showed the new assay had similar sensitivity to conventional assays, and made great improvements in the ease of performance, particularly because BSL2 conditions are nonessential. The ease of the new assay makes it ideal for use in several upcoming studies in this lab. One such study is examining the effects of various uterine factors on IFNT production, where the assay will be used in conjunction with q-RT/PCR analysis. The assay would benefit this work because a change in gene expression does not always equate to changes in protein production. Using both q-RT/PCR and the ISRE assay, we can be sure uterine factors are affecting IFNT protein production. Other opportunities to use the assay include examining IFNT in uterine flushes of pregnant cows under different nutritional conditions,

as maternal nutrition plays a direct role in pregnancy outcomes. The assay would allow researchers to make comparisons of IFNT production between different nutritional groups, and may reveal that altered IFNT resulting from increased/decreased maternal nutrition is a mechanism by which pregnancy outcomes are affected. Finally, the assay will be beneficial when assessing embryo quality *in vitro*. Currently our lab uses visual assessment to discern high quality embryos from lesser quality, though this method creates opportunity for human error and is highly objective. The assay will provide a method for ranking embryo viability based on IFNT production; an embryo producing higher amounts of IFNT is considered to be higher quality as it suggests the TE is more functional. This would ensure that only the highest quality embryos are transferred into recipient animals, or used for research purposes.

The next set of studies focused on establishing a bovine PrE cell line from embryonic outgrowths. Currently, no true bovine PrE cell line exists, so the development of such a line is anticipated to provide a great impact on future studies of embryogenesis in cattle. Results showed great improvements in outgrowth production, and also defined the behaviors of these outgrowths under continuous culture conditions. Future work with these lines includes immortalization to increase their longevity in culture, which will remove any time constraints on experiments caused by the current cells entering a quiescent state so quickly. Immortalization of the cell lines will also allow for cloned selection, which would eliminate the presence of multiple cell types within cultures. The cell lines can also be used to study interactions between TE and PrE during embryogenesis. The TE and PrE grow in direct contact with each other during

embryogenesis, and it is probable they communicate via chemical and physical interactions. These interactions have not been extensively studied in the bovine model, so these studies would improve the current understanding of the behaviors of these cells types. Both the new bioassay and PrE cell lines provide countless opportunities to improve pregnancy success in cattle. The information we gain from these tools may provide insight into specific factors that can be supplemented into embryo culture media to promote TE, IFNT, and PrE development, and thus improve embryo quality. It is also possible that the bioassay and PrE cell lines will lead to the creation of a new method for monitoring pregnancy, by tracking concentrations of factors that are found to promote IFNT and PrE production. As a whole, this work provides new methods for the study of early pregnancy, and has the potential to be used as a tool to decrease the incidence of pregnancy loss in cattle.

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Appendix A: Luciferase-Based ISRE Assay Protocol

ROUTINE HANDLING OF MDBK CELLS

MDBK Growth Medium:

- 450 ml high glucose DMEM (Life Technologies)
- 50 ml heat-treated FBS
- 5 ml ABAM or Pen/Strep (Life Technologies)

MDBK Cell Freezing Medium

- 95% DMEM + FBS
- 5% DMSO

Cell Thawing

1. Thaw MDBK cells in vial in water bath set to 37°C.
2. Sterilize vial by spraying with 70% ETOH, then wipe and let dry briefly in the cell culture hood.
3. Add entire contents of vial + 15 to 20 ml growth medium to a flask.
4. Place flask in 37°C incubator, and allow cells to incubate for 1 to 2 days before changing medium or passaging.

Changing Medium

1. Warm medium in water bath for 15 to 30 minutes.
2. Aspirate old medium.
3. Add 15 to 20 ml medium to the flask.

Cell Passaging

1. Warm cell growth medium and trypsin solution (0.05% or 0.25% in ETDA; Life Technologies) in 37°C water bath. Note: trypsin should be aliquoted and frozen in 2 to 10 ml aliquots. Once thawed, it is useful for 2 to 3 weeks.
2. Aspirate current medium from flask.
3. Rinse cells twice with 10 ml DPBS (Dulbecco's PBS; Life Technologies).
4. Add 2 ml trypsin to flask, and place in 37°C incubator for 2 to 5 minutes. *Be sure cells have become dislodged by gently tapping flask against hand*
5. Add 8 ml growth medium to flask.
6. Add cells and growth medium to new flasks in desired concentrations. Normally a 1:5 to 1:20 passage ratio is used for passage every 2 to 4 days, respectively.

MDBK Cell Freeze-Backs

1. Warm cell growth medium and trypsin in 37°C water bath.
2. Aspirate current medium from flask.
3. Rinse cells twice with 10 ml DPBS.
4. Add 2 ml Trypsin to flask, and place in 37°C incubator for 2 to 5 minutes.
5. Add 4 ml growth medium to flask.
6. Add contents of 2 flasks to a 15 ml conical tube.
7. Centrifuge tubes at 0.5 x 1,000 rcf (500 g) for 2 minutes.
8. Aspirate medium from tubes while avoiding cell pellet.
9. Add 5 ml of freezing medium to each tube, and break up cell pellet through gentle pipetting.
10. Add 1 ml of cells to each cryovial.
11. Place tubes in -80°C for 4 to 16 h.
12. Transfer tubes to liquid nitrogen tank.

COMPLETING THE ISRE-BASED IFN ASSAY

ISRE Assay

Day 1

1. Trypsinize 1 flask of MDBK cells that are >85% confluent.
2. Add growth medium for a final volume of 10 ml.
3. Add 100 μl of cell preparation to each well of one 96-well polystyrene plate with opaque walls and optically clear bottoms (Corning; 3610)
4. Incubate plate for 4 to 8 hours in 37°C incubator.
5. During the 4 h incubation, begin organizing and diluting standards and samples in a separate 96-well plate that lacks cells.
6. Add 100 μl growth medium to wells in first row of a separate 96-well plate.
7. In well A1, add 5 μl of the IFNA Standard and 45 μl of medium (i.e. 500 IU; aka 3,333 IU/ml). Repeat pipet to mix.
8. Transfer 50 μl from well A1 to well A2, and mix by repeat pipetting.
9. Continue with this 3:1 serial dilution of the standard across row A. Stop when you reach well A11. Well A12 will serve as the negative control.
10. The remaining wells will be used to prepare samples. Several things must be considered for these samples:
    a. Mix your samples with growth medium in at least an equal volume of growth medium (e.g. 25 μl of sample with 25 μl of growth medium).
    b. It is advised that the final volume is 60 μl or greater (preferably 100 μl).
    c. Several dilutions of each sample may be needed to ensure reading that fall within the dynamic part of the standard curve. Suggest at least completing 2 to 3 serial dilutions at 5:1 or 10:1 for all samples.
    d. Pilot studies are recommended to determine the sample dilution requirements.

11. After 4 hours, aspirate medium from plate containing cells.
12. Add 50 μl of sample from each well of the sample preparation plate to its corresponding well on the cell plate.
13. Incubate plate in 37°C incubator overnight.

Day 2

1. Thaw One-Glow Luciferase Assay Substrate (Promega Corp.; E6120) on bench-top. Repeated freeze-thawing events do not seem to interfere with outcomes if it is thawed slowly (i.e. not in the water bath)
2. Once thawed, remove cell plate from the incubator and add 50 μl Luciferase reagent to each well (leave the medium in the wells; final volume will be 100 μl).
3. Place plate on shaker platform and mix for 10 minutes.
4. Read plate. Steady levels of Luciferase can be measured for up to 6 h after adding the Luciferase reagent.

Concentration Adjustments

1. Enter data into an Excel spreadsheet.
2. Create a line graph of the IFN STD readings and determine max. and min. values
3. Create a line graph using the values falling between the max and min, and transform the x-axis to a logarithmic scale.
   *Note: x-values should be IFN-alpha IU/ml (3333, 1111,...)
   y-values should be the luciferase readings
4. Insert a logarithmic trend line with the equation and R^2 value.
5. Using the logarithmic equation, insert the luciferase reading as the y-value and solve for x.
   *Note: Values falling outside the 80-20% rule should be noted.
6. Adjust concentrations for dilution factors.
7. Calculate SEM of replications.

Preparation of the IFNA Standard

*** human recombinant interferon-alpha-A (EMD Millipore; #IF007)

1. Reconstitute IFN-alpha based on antiviral international units (IU).
2. For IU reconstitution:
   a. Add growth medium to a final concentration of 1 x 10^6 IU/ml.
   b. Separate into 100 ul aliquots and store at -80°C.
   c. When needed, thaw one vial of IFN-alpha stock and add 900 μl growth medium. This will produce a 100,000 IU/ml stock solution. Store this stock at 4°C for up to 1 month.