2019

Investigation of bovine conceptus galectins and the effect of bovine conceptus secretory proteins on the endometrial epithelial transcriptome

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INVESTIGATION OF BOVINE CONCEPTUS GALECTINS AND THE EFFECT OF
BOVINE CONCEPTUS SECRETORY PROTEINS ON THE ENDOMETRIAL EPITHELIAL
TRANSCRIPTOME

HEATHER L. BALDWIN

Thesis submitted
to the Davis College of Agriculture, Natural Resources and Design
at West Virginia University

in partial fulfillment of the requirements for the degree of

Master of Science in
Reproductive Physiology

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Division of Animal and Nutritional Sciences

Morgantown, WV

2019

KEYWORDS: Cattle, elongation, endometrium, conceptus, interferon tau, galectin

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ABSTRACT

INVESTIGATION OF BOVINE CONCEPTUS GALECTINS AND THE EFFECT OF BOVINE CONCEPTUS SECRETORY PROTEINS ON THE ENDOMETRIAL EPITHELIAL TRANSCRIPTOME

Heather L. Baldwin

Pregnancy loss in cattle is greatest in the first 30 days of gestation. Near d15 of gestation, bovine conceptuses elongate and express LGALS1, an immunosuppressive glycan-binding protein believed to be essential for implantation. However, other members of the bovine galectin family, including LGALS3, LGALS4, LGALS7, LGALS8, LGALS9 and a galectin-13-like gene (LGALS13L), also may be expressed by the elongating conceptus. Therefore, galectin expression in elongating, d16 bovine conceptuses and intercaruncular endometrium as well as elongating, d14 ovine conceptuses and intercaruncular endometrium was assessed by RT-qPCR. Angus heifer and Texel ewes underwent estrous cycle synchronization and either inseminated or not bred. On d16 (heifers) or d14 (ewes) after synchronized estrus (d0), reproductive tracts of pregnant (heifers, n=4; ewes, n=3) and cyclic (heifers, n=4; ewes, n=3) animals were removed. Conceptuses and intercaruncular endometrial samples were then collected. Conceptuses and intercaruncular endometrium samples were individually snap frozen and stored at -80°C until RT-qPCR. Bovine and ovine conceptus gene expression was normalized to control genes GAPDH and YWHAZ, and endometrial gene expression was normalized to control genes H3F3A and RNF11. A general linear model procedure (PROC GLM) in SAS was used to test for differences. For the conceptus data, alpha was set at 0.05, and a Tukey's test was included in the analysis. Conceptus data are presented as non-transformed means $\pm$ standard deviation (SD) of the mean. Endometrial data are presented as non-transformed least square means (LSM) $\pm$ standard error of the mean (SEM) and $P \leq 0.05$ was considered significant. In bovine conceptuses, expression of LGALS3 (24.47 ± 6.41) and LGALS1 (21.80 ± 2.07) was similar, yet, greater than all other galectins measured ($\alpha = 0.05$). Expression of LGALS8 (1.73 ± 0.27) was 12-fold less than LGALS1, however, LGALS8 expression was greater than LGALS9 (0.77 ± 0.77), LGALS13L (0.30 ± 0.10), LGALS4 (0.08 ± 0.01) and LGALS7 (0.02 ± 0.01) ($\alpha = 0.05$). In bovine intercaruncular endometrium, expression of LGALS1, LGALS8, and LGALS9 were greatest. Compared to cyclic heifers, endometrial expression of LGALS7 was less (5.80 ± 2.35 and 2.62 ± 2.35 ; cyclic and pregnant, respectively; $P \leq 0.05$), while expression of LGALS9 (9.26 ± 2.25 and 24.56 ± 2.25 ; cyclic and pregnant, respectively; $P \leq 0.001$) and LGALS3 were greater (2.62 ± 0.52 and 4.40 ± 0.52 ; cyclic and pregnant, respectively; $P \leq 0.05$) during pregnancy. In sheep conceptuses, expression of LGALS3 (4.30 ± 0.78), LGALS1 (1.90 ± 0.25) and LGALS8 (1.68 ± 0.25) were similar. Compared to all other galectins measured, LGALS4 (0.20 ± 0.10) had the lowest level of expression ($\alpha = 0.05$). In ovine intercaruncular endometrium, expression of LGALS3, LGALS8, and LGALS15 were greatest. Compared to cyclic ewes, expression of LGALS7 was greater in pregnant ewes (0.81 ± 0.24 and 2.25 ± 0.24 ; cyclic and pregnant, respectively; $P \leq 0.05$) while LGALS4 (3.10 ± 0.38 and 1.78 ± 0.38 ; cyclic and pregnant, respectively; $P = 0.06$) tended to be less during pregnancy and

LGALS15 tended to be greater during pregnancy (104.30 ± 234.20 and 591.35 ± 234.20 , $P = 0.07$; cyclic and pregnant, respectively). In conclusion, elongating bovine and ovine conceptuses express various members of the galectin gene family, and select galectin genes are differentially expressed in the endometrium during pregnancy. The galectins may have important functions during early pregnancy in ruminants.

Interferon-tau (IFNT), secreted by the elongating bovine conceptus, is the maternal recognition of pregnancy signal that suppresses the endometrial luteolytic mechanism by modifying expression of genes in specific cell types within the endometrium. The conceptus also secretes galectin proteins, specifically galectin-3. This study assessed the effect of IFNT (Experiment 1) and LGALS3 (Experiment 2) on bovine endometrial epithelial and fibroblast cell transcriptomes when the mid-luteal phase endometrial cells were cultured in a 3D environment. In Experiment one, epithelial cells, cultured in Transwell inserts above the fibroblast cells, were treated with RPMI media alone (control; $n=4$) or treatment media (RPMI with 5% FBS and 1% ABAM) containing 100 ng of recombinant ovine IFNT ($n=3$) for 6 h. In Experiment two, cells were treated with either RPMI media alone (Control; $n=5$), pregnant ovine uterine flush fluid, theoretically containing INFT, diluted with treatment media 1:1 ($n=4$), or treatment media (RPMI with 5% FBS and 1% ABAM) containing either 100 or 1000 ng of recombinant mouse LGALS3 ($n=5$) for 6 h. Total RNA was extracted from both cell types and the transcriptomes were assayed by RNA Sequencing and RT-qPCR. Interferon-tau regulated 673 and 83 genes (differentially expressed genes or DEGs) in epithelial and fibroblast cells, respectively ($P \leq 0.001$; FDR $P \leq 0.05$). Many of the DEGs identified in the epithelial (205) and fibroblast cells (64) were previously reported as IFNT and bovine conceptus-induced DEGs in intact bovine endometrium, including well-known ISGs such as ISG15, MX1, MX2, OAS1, and OAS2. Surprisingly, the ZC3HAV1 gene, which was recently found to be up-regulated in intact bovine endometrium in response to the conceptus, was only up-regulated in fibroblast cells despite applying IFNT to the apical surface of the epithelial cells in 3D culture. The RNA-Seq analysis of the bovine endometrial cells treated with mouse LGALS3 protein resulted in the identification of a single gene, RPL23A, that was upregulated in epithelial cells treated with 100 ng of LGALS3 ($P \leq 0.001$; FDR $P \leq 0.05$). This study supports the idea that the 3D culture of bovine endometrial cells may be utilized to study early conceptus-maternal crosstalk and identify endometrial cell type-specific responses to the conceptus.

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LIST OF ABBREVIATIONS

μg	microgram
μL	microliter
2D	two-dimensional
3D	three-dimensional
ABAM	antibiotic-antimycotic
AIJ	ampulla-isthmic junction
AKT	protein kinase B
APOL3	apolipoprotein L3
ATP8B4	ATPase phospholipid transporting 8B4
BATF2	basic leucine zipper ATF-like transcription factor 2
BCL2A1	Bcl-2-related protein A1
BNC	binucleate cells
bp	base pair
BP	biological processes
BSA	bovine serum albumin
BTM	beta-2 microglobulin
C	celcius
CH	corpus hemorrhagicum
CIDR	controlled intravaginal drug releasing device
CL	corpus luteum
cm	centimeter
CRD	carbohydrate recognition domain
CSF	colony-stimulating factors
CST3	cystatin-3
CTSL	cathepsin-L
CXCR4	C-X-C motif chemokine receptor 4
d	day
DEG	differentially expressed gene
dGE	deep glandular epithelium
DMRTA1	doublesex- and mab-3-related transcription factor A1

E	primer efficiency
E2	estradiol
ESR1	estrogen receptor alpha
EtOH	ethyl alcohol
FBS	fetal bovine serum
FF	flush fluid
FOXS1	forkhead box S1
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBP2	guanylate-binding protein 2
GHSR	growth hormone secretagogue receptor
GLM	generalized linear model
GnRH	gonadotropin releasing hormone
GO	gene ontology
GSK-3 β	glycogen synthase kinase-3 β
H	hour
H3F3A	H3 histone family member 3A
HBSS	hank's balanced salt solution
HSH2D	hematopoietic SH2 domain containing
IACUC	Institutional Animal Care and Use Committee
IDO1	indoleamine 2,3-dioxygenase
IFGBP1	insulin-like growth factor-binding protein 1
IFGR	interferon gamma receptor
IFI44	interferon induced protein 44
IFI44L	interferon induced protein 44 like
IFI6	interferon alpha-inducible protein 6
IFIT3	interferon induced protein with tetratricopeptide repeats 3
IFNAR	interferon alpha receptor
IFNG	interferon gamma
IFNT	interferon-tau
IGF-1	insulin-like growth factor 1

IHC	immunohistochemistry
IL-1	interleukin-1
IL-10	interleukin-10
IP10	interferon gamma-inducible protein 10
IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
IRF9	interferon regulatory factor 9
ISG	interferon stimulated gene
ISG15	ISG15 ubiquitin like modifier
ISGF3	interferon-stimulated gene factor-3
ITG	integrin
IVF	<i>in vitro</i> fertilization
Jak-STAT	janus-associated kinases and signal transducer and activator of transcription
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIF5C	kinesin family member 5C
LE	luminal epithelium
LGALS1	galectin-1
LGALS12	galectin-12
LGALS13L	galectin-13-like
LGALS14	galectin-14
LGALS15	galectin-15
LGALS2	galectin-2
LGALS3	galectin-3
LGALS3BP	galectin-3 binding protein
LGALS4	galectin-4
LGALS6	galectin-6
LGALS7	galectin-7
LGALS7B	galectin-7B
LGALS8	galectin-8
LGALS9	galectin-9
LH	luteinizing hormone

LIF	leukemia inhibitory factor
Log10	log base 10
logFC	log fold change
LPS	lipopolysaccharide
LSM	least square means
MAP6	microtubule associated protein 6
MAPK	mitogen-activated protein kinases
MCHR1	melanin concentrating hormone receptor 1
MHC	major histocompatibility complex
Min	minutes
mL	milliliter
mm	millimeter
MMPs	Matrix metalloproteinases
MMP-1	metalloproteinase-1
MX1	MX dynamin like GTPase 1
MX2	MX dynamin like GTPase 2
NFκB	nuclear factor kappa B
ng	nanogram
NRQ	normalized relative quantity
OAS	oligoadenylate synthetase
OAS1	2'-5'-oligoadenylate synthetase 1
OAS2	2'-5'-oligoadenylate synthetase 2
oIFNT	ovine interferon-tau
OTR	oxytocin receptor
P4	progesterone
PAG	pregnancy associated glycoproteins
PARM1	prostate androgen-regulated mucin-like protein 1
PBS	phosphate buffered saline
PF	persistent follicle
PG	prostaglandin
PGE2	prostaglandin E2

PGF	prostaglandin F2 alpha
PGI2	prostaglandin I2
PGR	progesterone receptor
PI3K	phosphatidylinositol 3-kinase
PIF	preimplantation factor
PL	placental lactogen
PLAC8	placenta-specific 8
PPIA	peptidylprolyl isomerase A
PROC GLM	general linear model procedure
PRRT3	proline-rich transmembrane protein 3
PTGS2	prostaglandin synthase 2
RIN	RNA integrity number
RNA-Seq	RNA Sequencing
RNF11	ring finger protein 11
RPL23A	60S ribosomal protein L23A
RPMI	Roswell Park Memorial Institute 1640
RQ	relative quantity
RSAD2	radical S-adenosyl methionine domain containing 2
RT-qPCR	Quantitative real-time PCR
s	seconds
SAMD9	sterile alpha motif domain containing 9
SD	standard deviation
SDHA	succinate dehydrogenase complex flavoprotein subunit A
SEM	standard error of the least squares means
sGE	surface glandular epithelium
siRNA	small interfering RNA
SLC15A3	solute carrier family 14 member 3
SLC25A19	solute carrier family 25 member 19
SLC25A28	solute carrier family 25 member 28
SLC25A30	solute carrier family 25 member 30
SOLD1	Ly-6 domain 1

Sqrt	square root
SSC4D	scavenger receptor cysteine-rich family member with 4 domains
STAT1	signal transducer and activator of transcription 1
STAT2	signal transducer and activator of transcription 2
TEER	transepithelial electrical resistance
TMC	trophoblast mononucleate cells
TNF $_{\alpha}$	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TNFSF10	TNF superfamily member 10
TNFSF13B	TNF superfamily member 13b
TYK	tyrosine kinsase-2
UGKO	uterine gland knockout
UV	ultra-violet
Wnt	Wingless-related integration site
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZAP	zinc-finger antiviral protein
ZBP1	Z-DNA binding protein 1
ZC3HAV1	zinc finger CCCH-type antiviral protein 1
$\Delta\Delta Cq$	delta-delta quantification cycle

CHAPTER ONE

LITERATURE REVIEW

Introduction

The beef cow/calf industry experiences an annual loss exceeding \$1.2 billion, with the largest contributing factor being embryonic loss [1]. The highest incidence of pregnancy loss in cattle occurs during the embryonic period of gestation, with 40% occurring between day (d) 8 and 17 [2-4]. The critical period for maternal recognition of pregnancy in cattle is d15-16 meaning that, in this time period, the conceptus begins to elongate in structure and secretes a substance to communicate with the dam the presence of a pregnancy and to convert the corpus luteum (CL) of the estrous cycle to a CL of pregnancy [5]. This secretory protein of the bovine trophectoderm is called interferon tau (IFNT) and its presence stimulates the expression of interferon-stimulated genes (ISGs) in the endometrium. The endometrial ISGs are considered classical or nonclassical and alter the endometrium in preparation for embryonic attachment and a successful gestation. Integrin proteins are imperative to attachment as they serve as a ligand for extracellular matrix compounds such as fibronectin. Galectins, specifically LGALS3, have been shown to interact with integrins to promote antiapoptotic effects during the period of implantation in humans and could promote adhesion between the conceptus and endometrium [6]. The purpose of this chapter is to review the literature that has contributed to the current understanding of the establishment of pregnancy in cattle, the origins of embryonic loss, and the possible involvement of LGALS3 during early pregnancy in cattle.

The Bovine Estrous Cycle

The female cow will reach puberty at approximately one year and will begin her reproductive cycle. The cycle is referred to as the estrous cycle with the time point of being sexually receptive termed “estrus”. Approximately every 21d, a cyclic cow or heifer has the chance to become pregnant as the cycle is continuous. The estrous cycle is defined as the period from one standing estrus to the next and consists of four stages; proestrus, estrus, metestrus, and diestrus [7]. Each estrous cycle can be divided into two phases, the follicular and luteal phases. The follicular phase consists of 20% of the estrous cycle and includes both proestrus and estrus. Proestrus continues from d17 to d20 of the bovine estrous cycle.

The beginning of the follicular phase, proestrus, is a result of luteolysis and is driven by alternations in the endocrine profile. Luteolysis marks the beginning of the follicular phase and is characterized by the regression of a functional CL due to uterine produced prostaglandin F₂ alpha (PGF). Progesterone (P₄) synthesized by the CL develops after the expulsion of an oocyte from a follicle and prevents further ovulation. Regression of the CL and subsequent decline of circulating P₄ allows for gonadotropin-releasing hormone (GnRH) to be released from the hypothalamus at a higher amplitude and frequency. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are released from the anterior pituitary. The regulation of the two differs as FSH is controlled by negative feedback from inhibin and estrogen, both originating from developing follicles. The pulse frequency of LH is controlled by the pulses of GnRH. The FSH aids in the development of follicles during proestrus by recruiting a cohort of follicles at the start of every follicular wave. Sirois and Fortune (1988) developed a model of bovine follicular development and regression by utilizing ultrasonography. Findings suggested that follicular development in heifers occurs in wave-like patterns while during each wave, a selected follicle

increases in size and asserts dominance on other developing follicles [8]. The selection process begins several days in advance to the dominant follicle reaching a preovulation diameter and causes regression of other developing follicles. The LH aids in selection which is the further development of the dominant follicle. Increased follicular development results in elevated inhibin production from granulosa cells which inhibits further FSH secretion. Following either atresia of the developing follicle or ovulation of the oocyte, FSH levels will once again rise to initiate another follicular wave [7].

The production of estradiol (E2), in large concentrations, will result in positive feedback on hypothalamic GnRH secretion leading to the preovulatory LH surge. This will trigger “standing estrus” meaning the female will become sexual receptive while exhibiting behavioral signs such as lordosis, increased movement, and the mounting of other animals. In cattle, estrus, which is a result of the LH surge, lasts approximately 15-18 h and ends at ovulation on d0 marking the end of the follicular phase. Ovulation typically takes place 30 h after the initiation of estrus [7].

The luteal phase, which begins after ovulation, consists of metestrus and diestrus and continues from d0 to d17 of the estrous cycle. Triggered by the preovulatory LH surge, the CL is formed from a newly ovulated follicle following luteinization in which the theca interna and granulosa cells of the follicle differentiate into small and large luteal cells, respectively. A corpus hemorrhagicum (CH) is the structure present immediately after ovulation as a result of the rupture of blood vessels as the oocyte complex is released from the follicle. The CH will then transition into a functional CL between d3 to d5 of the estrous cycle. Metestrus is characterized by the development of a functional CL and the transition to elevated P4 levels and declining E2. During this time, the first polar body appears as the oocyte undergoes meiosis. Corpus luteum

mass is directly correlated with CL P4 secretions levels as both plateau in the middle of the cycle [9].

Thecal and granulosa cells intermix post-ovulation and both contain the capability to produce P4. The P4 targets the hypothalamus to suppress GnRH and consequently, LH and FSH, by decreasing GnRH receptors located on gonadotrophs in the anterior pituitary. The uterine environment is influenced by P4 by aiding in endometrial secretions, termed histotroph, as well as restricting contractility of the myometrium. In addition, P4 has the ability to aid in mammary gland development. Granulosa cells form large luteal cells while thecal cells form small luteal cells. Large luteal cells, as a result of ovulation, possess secretory granules that contain oxytocin which will be critical for luteolysis [10].

The end of the luteal phase is marked by the occurrence of luteolysis which is induced by endometrial PGF. Luteolysis is characterized by a 1-3d regression of the CL resulting in reduced blood P4 levels. The PGF originates in the uterine epithelium and travels to the ovary to initiate luteolysis through a vascular countercurrent exchange between the uterine vein and ovarian artery [11]. This system enables PGF to be transported to the ovary avoiding dilution and metabolism that would occur in general circulation [7]. A large portion of PGF secreted by the uterine epithelium travels through the utero-ovarian vein and diffuses into the ovarian artery. The PGF can initiate apoptosis in luteal cells. As a result, blood P4 concentrations decrease and the cycle will transition into a new follicular phase characterized by elevated E2 levels.

Early Bovine Pregnancy

Once the physiological and hormonal profiles are precise, the oocyte will ovulate—allowing the oocyte to migrate to the oviduct. In cattle, past research has shown that the number

of ovulations occurring in the right compared to the left ovary is not proportionate with the right ovary having a greater ovulation rate [12, 13]. Following ovulation, the infundibulum captures the ovulated oocyte and permits the oocyte to travel to the ampulla-isthmic junction (AIJ) for possible fertilization. During natural mating, spermatozoa travel from the site of deposition, the cranial vagina, through the reproductive tract, to the AIJ [7].

If fertilization is successful, the presence of both the male and female pronuclei within the oocyte's cytoplasm confirms the existence of an ootid. The male pronucleus is formed once the sperm nucleus enters the oocyte's cytoplasm. The male pronucleus then undergoes decondensation allowing the female and male pronuclei fuse together to form a single nucleus. The resulting zygote is composed of a single cell [7]. Within 24 h, the single-celled embryo will undergo cytokinesis and will cleave into a two-celled embryo with two blastomeres. Blastomere cleavages will continue, where the two-cell embryo will cleave into a four-cell embryo followed sequential cleavages. It is also important to note that early embryonic development, until d5, occurs within the oviduct which has unique secretions in comparison to the uterine horns. The oviduct provides an optimal environment for the developing conceptus by providing a specific combination of amino acids, energy substrates, and ions [14-16]. Past research has also found that the presence of oviductal cells or oviduct conditioned media during culture improves embryo viability [17].

Near d4, the embryo becomes a morula. Visually, the morula is identified as a solid mass of cells which cannot be easily distinguished from one another and quantified. During this time, the developing conceptus, which refers to the embryo body and extra-embryonic tissues, is transferred from the oviduct into the uterus. Tight junctions between the outer cells of the morula aid in the accumulation of fluid within the intercellular spaces inside the morula. Sodium is

pumped into the intercellular space and, consequently, water accumulates. Blastocyst formation occurs around d7 of pregnancy in cattle. The blastocyst contains a fluid-filled cavity termed a blastocoele which consists of two distinct cell populations. One population forms the inner cell mass, which will give rise to the body of the developing embryo. The other cell population forms the outer layer and is referred to as trophoblast cells which will develop into the chorion of the placenta [7]. Lastly, between d7-8 of pregnancy, the blastocyst will hatch from the zona pellucida. At this point, the blastocyst is exposed to the uterine secretions. Expulsion of the blastocyst is a result of increasing internal pressure due to a combination of early embryonic growth, the enzymatic degradation of the zona pellucida, and contraction of the conceptus [7].

Once hatched from the zona pellucida, the embryo undergoes dramatic morphological change, increasing in size from around 0.15 mm in diameter to over 60 mm in total length due to the process of elongation [18]. The bovine embryo will double in length every day between d9-16 of gestation [19-21]. The sphere-shaped blastocyst will transform into an ovoid conceptus on approximately d12. Finally, the conceptus will transition into a tubular structure on approximately d13-14 of gestation [22]. The conceptus will span both uterine horns by d18 of gestation, later attaching to the uterine surface [7]. Alterations of the uterine environment, including histotroph gene expression, are mediated by an increased concentration of P4 which promotes rapid conceptus growth [23]. Uterine histotroph consists of enzymes, growth factors, cytokines, hormones, transport proteins, and other substances [20, 24-27]. During this time, the extraembryonic membranes of the pre-attachment embryo rapidly develop and are comprised of the yolk sac, chorion, amnion, and allantois. Bovine pre-attachment blastocysts are characterized by having a filamentous and thread-like structure near d16-17 [22].

As the development of the embryo progresses beyond the blastocyst stage, a dependency

on the maternal uterine environment develops. Bovine blastocysts can be produced by *in vitro* fertilization (IVF) technology; however, the uterine environment is essential for the elongation of the developing conceptus [28, 29]. *Ex vivo*, outside of the uterus, ruminant blastocysts are unable to elongate as histotroph from endometrial glands is essential for this process [30, 31]. Gray and coauthors (2002) utilized uterine gland knockout (UGKO) sheep to investigate the role of endometrial secretions on elongation. The UGKO ewes were generated by utilizing synthetic progestin which results in the lack of uterine glands after postnatal chronic exposure in ewe lambs [31]. In sheep, conceptuses normally elongate around d14 of gestation. Day 14 conceptuses from UGKO ewes were observed to be fragmented, growth-retarded, and tubular. In addition, it was concluded that development was interrupted in embryos from knock-out gland ewes due to lack of endometrial glands opposed to alterations in the expression of molecules that impact adhesiveness of the luminal epithelium [30].

Maternal Recognition of Pregnancy

Reciprocal communication takes place between the embryo and the uterine environment. Increased levels of steroid hormones, P4 and E2, affects uterine gland gene expression promoting elongation and growth of the conceptus. Progesterone also controls the expression of genes that aid in uterine receptivity to the conceptus. Uterine receptivity is essential for embryonic growth, development, and attachment [20, 32]. In addition, the conceptus will release proteins, steroids, and other factors that affect endometrial cell gene expression. Conceptus IFNT, a cytokine, causes modifications to endometrial PGF production that disrupts luteolysis allowing continuous P4 synthesis [33]. This process is termed maternal recognition of pregnancy and must occur between d15-16 of gestation for pregnancy to successfully continue. Trophoblast

cells are characterized into two groups: trophoblast mononucleate cells (TMC) and trophoblast binucleate cells (BNC) [34]. The TMCs are responsible for the secretion of the maternal recognition of pregnancy factor in ruminants. IFNT will then inhibit the transcription of the oxytocin receptor (OTR) in the LE/sGE of the uterus by first silencing estrogen receptor alpha (ESR1) expression [33, 35]. In the absence of oxytocin, luteolytic pulses of PGF cannot occur, thus prolonging the CL lifespan. Comparing pregnant to cyclic animals, basal levels of PGF and prostaglandin E2 (PGE2) in pregnant animals remain elevated due to continued uterine prostaglandin synthase 2 (PTGS2) expression [35]. The silencing of ESR1 by IFNT disables E2 from inducing endometrial epithelial progesterone receptor (PGR) transcription which allows stromal cells expressing PGR to regulate expression of ISGs [36].

Ulbrich et al. (2009) quantified prostaglandins (PGs) in the uteri of preimplantation pregnancy stage heifers in comparison to non-pregnant control heifers. It was found the presence of an embryo influenced the intrauterine environment of d15 pregnant heifers to consist of higher concentrations of prostaglandin I2 (PGI2) and secondly, PGF, both of which were greater than PGE2 [37]. Overall, clear evidence was found that the ratio of PGF/PGE2 was not altered between cyclic and pregnant heifers as the embryo contributes to the PG environment. Likely, PGI2 plays a role in the permeability of maternal vasculature. Both PGE2 and PGI2 have been found to be critical during the regulation of implantation and decidualization in other species including humans and mice [38, 39].

Interferon-tau is a type I interferon. Type I interferons are important in most viral defenses. Type I interferons bind the interferon alpha receptor (IFNAR) to signal a response biologically while the single Type II interferon family member, interferon gamma (IFNG) binds

interferon gamma receptor (IFNGR) [40]. The pregnancy recognition signal, IFNT, will differentially impact gene expression of cells in the luminal epithelium (LE), deep glandular epithelium (dGE), surface glandular epithelium (sGE), and stroma [36, 41]. The ISGs and are suggested to play various roles in the differentiation of the endometrium in preparation for pregnancy. There are two distinct classifications of ISGs, classical and non-classical, which are spatially stimulated by IFNT within the stroma/dGE and LE/sGE, respectively [42, 43]. Type I and II IFNs initiate the expression of classical ISGs through stimulation of janus-associated kinases and signal transducers and activators of transcription (Jak-STAT) pathways resulting in phosphorylation of their respective receptors activating STAT.

There is a greater understanding of IFNT mediated ISG effects in the ovine endometrium compared to cattle. The working hypothesis is as follows: conceptus-derived IFNT binds its receptor, IFNAR, in the LE/sGE. There, interferon regulatory factor 2 (IRF2), which is a transcriptional repressor, is up-regulated. The IRF2 inhibits ISGs which are considered classically activated such as STAT1, STAT2, interferon regulatory factor 9 (IRF9), beta-2 microglobulin (B2M), ISG15 ubiquitin-like modifier (ISG15), major histocompatibility complex (MHC), and oligoadenylate synthetase (OAS). However, IFNT is able to activate genes in the non-classical category of ISGs such as cystatin-3 (CST3), cathepsin-L (CTSL), and galectin-15 (LGALS15) in the LE/sGE of sheep through a currently unknown biological cell-signaling pathway [42]. Inversely, dGE and stromal cells lack expression of IRF2 resulting in activation of JAK1 and tyrosine kinase-2 (Tyk2), and consequently, STAT2. Once STAT2 is phosphorylated, STAT1 is able to become phosphorylated as well. With STAT1 and 2 activation, interferon-stimulated gene factor-3 (ISGF3) and interferon regulatory factor-1 (IRF1) transcription factors become activated allowing for the transactivation of classical ISGs [42, 44]. The LE and sGE

may lack classical ISG expression during early pregnancy as a method of defense against the immune rejection of the partially genetically dissimilar conceptus [45, 46].

The BNCs of the trophoblast appear between d18 and 20 of gestation in cattle and migrate from the chorionic tissue to the maternal endometrium where they secrete factors such as placental lactogen (PL) and pregnancy-associated glycoproteins (PAGs). The BNCs also secrete PG, along with estrogens, during preimplantation development. The BNC derived PAGs are believed to play a role in the regulation of placentation, mammatogenesis, steroidogenesis, and immune function [47-50]. Other vital trophoblastic secreted molecules include Bcl-2-related protein A1 (BCL2A1) which is BNC derived and is an imperative anti-apoptotic factor involved in cell maintenance [51]. Bovine TMCs and BNCs, along with maternal tissues, have been found to contain secreted protein of Ly-6 domain 1 (SOLD1) which plays various roles including reconstruction of the post-partum uterus, fetal and placental growth, and placental expulsion. Further, investigators found SOLD1 is involved in trophoblast cell migration and induced invasion [52, 53].

Bovine Embryonic Attachment

Implantation of the developing ruminant conceptus is rather non-invasive and consists of the process by which the fetal membranes attach to the maternal endometrial epithelium. Bovine embryonic attachment occurs near d21 of gestation, a time when the allantois formation becomes apparent macroscopically [54, 55]. Post-elongation, the conceptus will begin to attach to the LE while trophoblastic projections become incorporated into the openings of the maternal uterine glands [56]. The driving force of preparation of the endometrium for attachment of the developing conceptus is P4 [35, 57]. Implantation is accompanied by increased production of

secretions by the epithelial cells within the uterine lumen.

Past research in cattle and sheep has found certain histotroph components to be of significant importance during the attachment phase [30, 42]. Successful implantation requires a normally developed blastocyst, a receptive endometrium, and corresponding communication between the two—a process which is orchestrated by various cytokines and growth factors such as interleukin-10 (IL-10), leukemia inhibitory factor (LIF), colony-stimulating factors (CSF), interleukin-1 (IL-1), and insulin-like growth factor 1 (IGF-1) [58, 59]. Members of the family of proteins called integrins (ITGs) are imperative for successful implantation [60]. Integrins serve as a receptor for fibronectin, vitronectin, laminin, collagen type IV, and osteopontin—all of which compose the extracellular matrix [60-62]. Interferon gamma-inducible protein 10 (IP10), LGALS15, and insulin-like growth factor-binding protein 1 (IGFBP1), all of which are components of the uterine histotroph, have been described to induce ITGs during the attachment of the trophectoderm to uterine tissue [61, 63-65].

Growth factors that are endometrial and/or conceptus derived, such as tumor necrosis factor alpha (TNF α), initiate direct communication between the maternal and trophoblastic cells. The pro-inflammatory cytokine, TNF α , is involved in various cellular activities including cell differentiation, survival, and tissue homeostasis. The TNF α has the capability to bind two distinct receptors, TNF receptor 1 (TNFR1) or TNF receptor 2 (TNFR2), which can have dissimilar functions. Interestingly, TNFR1 activation is involved in cell apoptosis while TNFR2 can be involved in either cell proliferation or apoptosis. Along with TNFR2, TNF α protein and mRNA have been found to be expressed in pregnant and cyclic endometria of cattle and expressed in endometrial and placental tissues of other species including humans during early

and late gestation [66, 67]. It is speculated that nuclear factor kappa B (NFκB), a transcription factor that is activated by TNFα, is down-regulated in the blastocyst to suppress immune function during blastocyst formation. The endometrium is believed to regulate TNFα concentrations via controlling the secretion of TNFα into the histotroph as well as regulating the embryo's transcription of both TNFα and TNFR2 mRNA [67]. TNFα has been shown to stimulate PGF synthesis in endometrial stromal cells and IFNT has been shown to have an inhibitory effect on TNFα [68].

Preimplantation factor (PIF) is a peptide found to be secreted by murine, human, and bovine embryos, as well as placental tissue. Additionally, PIF has been found to be in human maternal general circulation [69, 70]. Trophoblastic PIF is expressed largely in the first and second trimesters and may be an indication of viable embryos that are *in vitro* produced by determining culture medium levels [71]. Human decidua cells cultured with PIF exhibited a role in immune regulation, adhesion, and regulation of apoptotic processes [72].

Early Pregnancy Loss in Cattle

Embryonic mortality is defined as the loss of pregnancy from the time of conception to d45 of gestation in cattle with early embryonic loss occurring from d0-27 and late embryonic loss occurring from d28-45 of gestation [73]. Most pregnancy losses occur in the first 50 days of gestation with losses before d20 attributing to 75% of all reproductive failures [74, 75]. Revenue from both dairy and beef farms depend on reproductive success. Therefore, it is important to address the causes of early embryonic loss to minimize financial loss. Reported fertilization rates in beef cattle are high, with values ranging from 93-96% when insemination occurs at the proper time in relation to ovulation [76-78]. Most reproductive failure following insemination occurs

post-fertilization. Methods known to minimize loss during the first eight days of pregnancy include alleviating heat stress, inflammatory diseases, body condition loss, and by increasing preovulatory follicular development progesterone levels as this time period comprises 20-50% of all embryonic loss [18]. Researchers addressed the role of a persistent follicle (PF) on early embryonic loss and found evidence that an oocyte from a PF is capable of becoming fertilized but exhibited a disability to grow into a 16-cell stage embryo [76]. The reduction in fertility is due to biochemical and morphological alterations that take place within a follicle that remains dominant for an extended period of time [75]. Along with an altered endocrine profile, studies have shown the occurrence of PF in cattle is accompanied by altered follicular steroid and gonadotropin receptor expression [79-81].

Galectin-3

Galectin-3 (LGALS3) is located on chromosome 10 in cattle and is a member of a large family of β -galactoside-binding animal lectins that have numerous physiological functions. Lectins bind carbohydrates and result in cell agglutination while playing vital roles in pathogen defense, cell trafficking, and immune regulation [82, 83]. Currently, 15 members have been identified in mammals [84]. Galectins are ancient members of a lectin family which are characterized by having conserved amino acid sequences with at least one carbohydrate recognition domain (CRD) and the ability to recognize β -galactoside structures located on cell surface glycoconjugates [85, 86]. Galectin family members have been categorized into three groups based on the number and organization of CRDs present: 1) the prototype, consisting of a single CRD, 2) the chimera type, whose only member is LGALS3 and consists of one CRD along with a non-lectin domain, and 3) the tandem repeat which consists of a short peptide linked

by two CRDs [82, 87, 88].

Galectin-3 is a 29 to 35 kDa protein and consists of a 110-130 amino acid N-terminal domain connected to glycine and proline-rich repeats, along with a single C-terminal CRD [87]. A conformational change occurs when LGALS3 or its CRD interact with carbohydrate ligands leading to rearrangement of the backbone loops located nearby its binding site. Multiple ligands of LGALS3 have been identified and include extracellular glycoproteins and glycolipids [89, 90]. N-terminal domain and CRD have been shown to interact in the formation of LGALS3 multimers as LGALS3 possess bivalent and multivalent binding properties [87, 91]. It is also unique in that it is the only chimera-type galectin found in vertebrates and has been discovered in various cell types including tumor cells, macrophages, activated T cells, epithelial cells, and fibroblasts. The localization of LGALS3 protein is dependent on the cell type as LGALS3 protein has been reported to be found in the cytoplasm or secreted through non-classical pathways [87, 92]. Galectin-3 does not contain an N-terminal signal sequence and becomes externalized through an endoplasmic reticulum-Golgi complex independent mechanism [93]. Also, LGALS3 may translocate to the nucleus or become secreted via a non-classical pathway of secretion following its cytoplasmic ribosomal synthesis. [94-96]. Intracellularly, LGALS3 has been shown to interact with nuclear and cytoplasmic proteins leading to the modulation of signaling pathways [86]. In addition, intracellular LGALS3 mediates anti-apoptotic effects by inhibiting apoptosis caused by nitrogen-free radicals [97]. Extracellular LGALS3 binds to oligosaccharides located on the surface of cells and promotes apoptosis by the release of cytochrome c and caspase-3 activation [98].

Galectin-3 has functions in the regulation of the immune response and may be involved

in the development of cancer and heart disease. Galectin-3 is also involved in various biological processes including cell adhesion, cell activation, chemoattraction, proliferation, cellular differentiation, and apoptosis [84, 87, 99]. Regarding immunology, LGALS3 is a regulator of inflammation and is secreted by macrophages, monocytes, dendritic cells, eosinophils, mast cells, natural killer cells, and both activated B and T cells [89, 100].

Cancer researchers have determined that LGALS3 promotes tumor cells growth in certain cancer types while promoting anti-apoptotic effects on tumor cells in various other cancers [101]. Contrarily, in cancers such as breast, non-melanoma skin, prostate, endometrial, and leukemia cancers, LGALS3 exerts tumor suppression effects on cancerous cells [102]. In the case of endometrial and breast cancers, a down-regulation of LGALS3 is associated with amplified aggressiveness while in pancreatic cancer, LGALS3 up-regulation is linked to cancer cell proliferation and invasion [102-104]. A study utilizing LGALS3 small interfering RNA (siRNA) found the knockdown resulted in a reduction in migration of cancer cells, invasion, cell proliferation, anchorage-independent colony formation, and tumor growth in mice with prostate cancer [105]. Signaling pathways of LGALS3 vary between cancers as well. Research has shown in cases of melanoma and gastric cancer, LGALS3 promotes tumor growth through the up-regulation of metalloproteinase-1 (MMP-1) and by the regulation of mitogen-activated protein kinases (MAPK) pathway in lung cancer [41, 106, 107]. In human breast cancer, LGALS3 has been shown to activate wingless-related integration site (Wnt) signaling, which is characterized by nuclear accumulation of β -catenin—a major component of the Wnt pathway [108-110]. In human colon cancer cells, via the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) pathway, LGALS3 regulates glycogen synthase kinase-3 β (GSK-3 β) phosphorylation leading to the degradation of β -catenin [108].

Expression of LGALS3 in myocardial cells of healthy humans and rodents is low, however, LGALS3 protein up-regulation has been observed during cardiac stressors leading to cardiac fibrosis and ultimately, heart failure [111]. Expression levels are found to correlate with progression and severity of cardiac damage, therefore, levels are utilized as a biomarker for heart failure [112]. It is hypothesized that *LGALS3* expression in heart failure is triggered hormonally by aldosterone or angiotensin II. Also, the combination of LGALS3 and aldosterone or angiotensin II instigates sclerosis, fibrosis, and hypertrophy of the renal system, cardiac tissue, as well as vascular tissue [113, 114]. Cardiac fibroblast proliferation is triggered by LGALS3 which induces detrimental collagen I deposition that will impact the diastolic and systolic functions of the heart [99].

Galectin-3 in Reproduction

Recently, LGALS3 was identified to play an important part in the preparation of the endometrium for implantation with a presence in the endometrium and trophoblast cells. Given the resemblances of tumor invasion and the migration of trophoblast cells, investigators have addressed the possible roles of LGALS3 at the maternal-fetal interface regarding implantation [115, 116]. Previous work suggests *LGALS3* expression is regulated by different mechanisms in placental tissues compared to uterine expression. After conducting immunohistochemistry (IHC) studies on pregnant murine uteroplacental tissue samples, it was concluded that LGALS3 is synthesized by all cell types within the murine uteroplacental samples. Levels of LGALS3 mRNA remained constant in murine uterine tissues until d12 of gestation and declined thereafter until parturition. Interestingly, in placental tissues, LGALS3 mRNA levels remained constant after d12 of gestation until term [115]. In bovine tissues, LGALS3 protein has been identified in

the cervix, oviduct, uterus, atretic follicles, and CL [117]. Specifically, in the nonpregnant bovine uterus, LGALS3 has been localized via IHC in some epithelial cells as well as macrophages located in the submucosa but were absent in endometrial glands [117]. In the bovine CL, LGALS3 expression was elevated at the regressed state in comparison to all other luteal stages and was localized on steroidogenic cells in the CL [118].

Successful implantation or attachment requires tight regulation of endometrial receptivity preparations including cellular proliferation proceeded by differentiation [119]. The expression of cell surface molecules alters as uterine receptivity develops though not a lot is known about molecular interactions occurring at the maternal-fetal interface. Galectins have been found to increase cell-adhesion by associating surface glycoproteins on opposing cells in addition to strengthening other intercellular adhesion molecules [120].

The Role of Galectin-3 in Apoptosis

A study utilizing the human epithelial endometrial derived cell line, RL95-2, addressed the effects of intracellular LGALS3 on apoptosis. Apoptosis inducers were utilized to determine if expression levels of LGALS3 were altered and it was found *LGALS3* expression was increased in apoptotic conditions [6]. To address whether the upregulation of *LGALS3* was pro-apoptotic, a siRNA technique was used to silence *LGALS3* in RL95-2 epithelial cells. It was observed that in the presence of apoptosis inducers, RL95-2 cells with *LGALS3* siRNA were more sensitive to the induced apoptosis. It was then suggested that the upregulation of *LGALS3* during the time of implantation in the human menstrual cycle may result in endometrial antiapoptotic effects [6]. In addition, *LGALS3* was postulated to be an antiapoptotic factor under P4 and E2 regulation. When RL95-2 cells were treated with *LGALS3* siRNA, the previously exhibited antiapoptotic roles of

P4 and E2 were eliminated. Through an underdetermined relationship, P4 and E2 are proposed to indirectly or directly promote *LGALS3* expression and, consequently, antiapoptotic effects [6]. Further, when studying women with endometriosis, decreased endometrial *LGALS3* expression was observed while P4 and E2 regulated *LGALS3* expression in endometrial epithelial cells. Ultimately, the lack of *LGALS3* expression was thought to have caused resistance to a P4 uterine environment leading to endometriosis-related infertility [121].

Integrins are significant during the preimplantation period as they play a major role in cell adhesion. Via surface interactions with extracellular matrix ligands, integrins have been found to play a role in the embryo-endometrium cross talk in humans [122, 123]. Extracellular *LGALS3* binds to β -1 integrin and induces apoptosis. Bovine luteal cells express β -1 integrin and contain glycans to which *LGALS3* will bind. Hashiba et al. (2014) found that PGF stimulated secreted *LGALS3* from cultured luteal steroidogenic cells and *LGALS3* plays a structural luteolytic role through binding to β -1 integrin [118]. Induced by *LGALS3*, a form of programmed cell death, termed anoikis, occurs in the bovine luteal steroidogenic cells due to inhibition of β -1 integrin-extracellular matrix binding [118, 124, 125].

3D Cell Culture

In the past, two-dimensional (2D) monoculture techniques have been utilized to investigate cellular processes of bovine epithelial and fibroblast cells [126]. To better simulate the columnar arrangement of epithelial cells *in vivo*, a three-dimensional (3D) culture allows epithelial cells to maintain a physiological polarity *in vitro* due to tight junctions unlike the traditional 2D cell culture techniques [127, 128]. Tight junctions are imperative to epithelial cellular functions because the passage of ions and molecules paracellularly is regulated by tight

junctions [129, 130]. The 3D transwell culture system enables the coculture of two cells types which physiologically interact *in vivo*. Transepithelial electrical resistance (TEER), a measurement which reflects epithelial integrity, can be measured via an epithelial volt-ohm meter [127]. The following studies investigated the expression of galectins in bovine conceptuses and in the endometrium. Further, the effect of IFNT and *LGALS3* on the endometrial epithelium and fibroblast transcriptomes in a 3D cell culture system was investigated.

CHAPTER TWO

GALECTIN GENE EXPRESSION IN BOVINE AND OVINE ELONGATING CONCEPTUSES AND PREGNANT AND CYCLIC ENDOMETRIUM

ABSTRACT

Pregnancy loss in cattle is greatest in the first 30 days of gestation. Near d15 of gestation, bovine conceptuses elongate and express *LGALS1*, an immunosuppressive glycan-binding protein believed to be essential for implantation. However, other members of the bovine galectin family, including *LGALS3*, *LGALS4*, *LGALS7*, *LGALS8*, *LGALS9* and a galectin-13-like gene (*LGALS13L*), also may be expressed by the elongating conceptus. Therefore, galectin expression in elongating, d16 bovine conceptuses and intercaruncular endometrium as well as elongating, d14 ovine conceptuses and intercaruncular endometrium was assessed by RT-qPCR. Angus heifer and Texel ewes underwent estrous cycle synchronization and either inseminated or not bred. On d16 (heifers) or d14 (ewes) after synchronized estrus (d0), reproductive tracts of pregnant (heifers, n=4; ewes, n=3) and cyclic (heifers, n=4; ewes, n=3) animals were removed. Conceptuses and intercaruncular endometrial samples were then collected. Conceptuses and intercaruncular endometrium samples were individually snap frozen and stored at -80°C until RT-qPCR. Bovine and ovine conceptus gene expression was normalized to control genes *GAPDH* and *YWHAZ*, and endometrial gene expression was normalized to control genes *H3F3A* and *RNF11*. A general linear model procedure (PROC GLM) in SAS was used to test for differences. For the conceptus data, alpha was set at 0.05, and a Tukey's test was included in the analysis. Conceptus data are presented as non-transformed means $\pm$ standard deviation (SD) of

the mean. Endometrial data are presented as non-transformed least square means (LSM) $\pm$ standard error of the mean (SEM) and $P \leq 0.05$ was considered significant. In bovine conceptuses, expression of *LGALS3* (24.47 ± 6.41) and *LGALS1* (21.80 ± 2.07) was similar, yet, greater than all other galectins measured ($\alpha = 0.05$). Expression of *LGALS8* (1.73 ± 0.27) was 12-fold less than *LGALS1*, however, *LGALS8* expression was greater than *LGALS9* (0.77 ± 0.77), *LGALS13L* (0.30 ± 0.10), *LGALS4* (0.08 ± 0.01) and *LGALS7* (0.02 ± 0.01) ($\alpha = 0.05$). In bovine intercaruncular endometrium, expression of *LGALS1*, *LGALS8*, and *LGALS9* were greatest. Compared to cyclic heifers, endometrial expression of *LGALS7* was less (5.80 ± 2.35 and 2.62 ± 2.35 ; cyclic and pregnant, respectively; $P \leq 0.05$), while expression of *LGALS9* (9.26 ± 2.25 and 24.56 ± 2.25 ; cyclic and pregnant, respectively; $P \leq 0.001$) and *LGALS3* were greater (2.62 ± 0.52 and 4.40 ± 0.52 ; cyclic and pregnant, respectively; $P \leq 0.05$) during pregnancy. In sheep conceptuses, expression of *LGALS3* (4.30 ± 0.78), *LGALS1* (1.90 ± 0.25) and *LGALS8* (1.68 ± 0.25) were similar. Compared to all other galectins measured, *LGALS4* (0.20 ± 0.10) had the lowest level of expression ($\alpha = 0.05$). In ovine intercaruncular endometrium, expression of *LGALS3*, *LGALS8*, and *LGALS15* were greatest. Compared to cyclic ewes, expression of *LGALS7* was greater in pregnant ewes (0.81 ± 0.24 and 2.25 ± 0.24 ; cyclic and pregnant, respectively; $P \leq 0.05$) while *LGALS4* (3.10 ± 0.38 and 1.78 ± 0.38 ; cyclic and pregnant, respectively; $P = 0.06$) tended to be less during pregnancy and *LGALS15* tended to be greater during pregnancy (104.30 ± 234.20 and 591.35 ± 234.20 , $P = 0.07$; cyclic and pregnant, respectively). In conclusion, elongating bovine and ovine conceptuses express various members of the galectin gene family, and select galectin genes are differentially expressed in the endometrium during pregnancy. The galectins may have important functions during early pregnancy in ruminants.

INTRODUCTION

Galectins are a large family of β -galactoside-binding animal lectins that have numerous physiological functions. Lectins bind carbohydrates and result in cell agglutination while playing vital roles in pathogen defense, cell trafficking, and immune regulation [82, 83]. Currently, 15 members of the galectin family have been identified in mammals [84]. Galectins are ancient members of a lectin family, which are characterized by having conserved amino acid sequences with at least one CRD and the ability to recognize β -galactoside structures on cell surface glycoconjugates [85, 86]. Galectins also interact with proteins independently of carbohydrates—acting in an autocrine manner to inhibit cell growth and division [133]. The galectin family of proteins has been shown to play a crucial role in regulating tumor and placental angiogenesis [134, 135]. In addition, galectins play a large role immunologically in inflammation, adaptive and innate immune responses, and immune tolerance [136, 137]. Elongating bovine conceptuses express galectin-1 (*LGALS1*) which has also been associated with the viability of d7 bovine blastocysts. Expression of *LGALS1* was found to be elevated in *in-vivo* produced blastocysts compared to less viable *in-vitro* produced d7 bovine blastocysts [138]. Further, the endometrium of sheep produces *LGALS15* after d10 of gestation to aid in trophectoderm migration and attachment. However, *LGALS15* is not found in cattle [64].

Galectin family members have been categorized into three groups based on the number and organization of CRDs present. The prototype group, consisting of a single CRD, includes *LGALS1*, 2, 5, 7, 10, 11, 13, 14, and 15. The chimera type group, whose only member is *LGALS3*, consists of one CRD along with a non-lectin domain. The tandem repeat group, which include a short peptide linked by two CRDs, members include *LGALS4*, 6, 8, 9, and 12 [82, 87,

88]. According to published transcriptomic and proteomic data and published bovine genome sequences in NCBI, cattle have genes encoding *LGALS1*, galectin-2 (*LGALS2*), *LGALS3*, galectin-4 (*LGALS4*), galectin-7 (*LGALS7*), galectin 7B (*LGALS7B*), galectin-8 (*LGALS8*), *LGALS9*, galectin-12 (*LGALS12*), and galectin-13Like (*LGALS13L*) while the ovine genome contains galectin genes for *LGALS1*, *LGALS2*, *LGALS3*, *LGALS4*, *LGALS7*, *LGALS8*, *LGALS9*, *LGALS12*, galectin-14 (*LGALS14*), and *LGALS15* (<https://www.ncbi.nlm.nih.gov/genbank/> version 19.12.2017, 23.30.00).

Alterations of the uterine environment, including histotroph gene expression, are mediated by an increased concentration of P4 which promotes rapid conceptus growth [23]. Uterine histotroph consists of enzymes, growth factors, cytokines, hormones, transport proteins, and other substances [20, 24-27]. The bovine conceptus will double in length every day between d9-16 of gestation [19-21]. Bovine pre-attachment blastocysts are characterized by having a filamentous and thread-like structure, which arises around d16-17 [22]. The conceptus will span both uterine horns by d18 of gestation, later attaching to the uterine surface [7]. Early conceptuses secrete factors, including proteins and steroids, that aid in alteration of the maternal endometrium [131, 132].

The aim of this study was to characterize galectin gene expression in the early bovine and ovine conceptus during the elongation phase of development as well as to characterize their expression in the endometrium in response to the conceptus.

MATERIALS AND METHODS

Animals and Tissue Collection

All procedures were in compliance with the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* and approved by the West Virginia University Institutional Animal Care and Use Committee (WVU IACUC). All animals were housed at the West Virginia University Animal Science Farm (Morgantown, WV). Angus and Hereford heifers were subjected to a Co-Sync + 7d controlled intravaginal drug releasing device (CIDR; Zoetis, Parsippany-Troy Hills, NJ) protocol to synchronize estrus [139]. Approximately 52-56 h post CIDR removal, heifers received GnRH and were either inseminated (n=4) with semen from an Angus bull or not bred (n=4). Estrus was synchronized in Texel ewes using a 5d CIDR program [140]. Ewes were then either placed with a Texel ram to be naturally bred (n=3) or not placed with the ram (n=3) 24 hours after CIDR removal. To obtain pregnant and cyclic reproductive tissues, heifers and ewes were slaughtered at a local abattoir 16 and 14 days later, respectively. Within 45 min, all reproductive tracts were flushed using 20 mL of 1X PBS (Thermo Fisher, Waltham, MA). Conceptuses were then collected and intercaruncular endometrial samples were collected from the uterine horn ipsilateral to the CL. All tissue samples were immediately snap-frozen and stored at -80°C until RNA extraction.

RNA Extraction

RNA was extracted from bovine (n=4) and ovine (n=3) conceptuses and endometrium. A 5-mm steel bead (Qiagen, Hilden, Germany), washed in RNaseZap (Thermo Fisher), was placed in each screw-cap tube containing the conceptus along with 350 µL of lysis buffer. Screw-cap tubes were placed in a Mini-Beadbeater-24 (Biospec Products, Bartlesville, Oklahoma) and

homogenized for 30 s at maximum speed. The lysate was transferred to a homogenizer spin column (Omega Bio-Tek, Inc., Norcross, GA) along with an additional 350 μ L of lysis buffer. Columns were centrifuged for 2 min at maximum speed. The RNA was extracted from the lysate according to recommendations from the manufacturer of the E.Z.N.A. DNA/RNA kit (Omega Bio-Tek, Inc.).

Intercaruncular endometrium samples (<50 mg) were placed in a screw-cap tube containing a single steel bead (Qiagen) and 1.0 mL of Trizol (Thermo Fisher). Screw-cap tubes were then placed in a Mini-Beadbeater-24 (Biospec Products) and homogenized for 2 min at maximum speed. After centrifuging, 0.1 mL of chloroform was added to the lysate, vigorously shaken, and allowed to rest for 3 min. Samples were then transferred to a precooled centrifuge (4°C) and spun at 12,000 x g for 15 min. The aqueous supernatant from the tubes was removed, combined with 1.5 x volumes of 100% ethanol, and gently mixed. The E.Z.N.A Total RNA Kit 1 (Omega Bio-Tek, Inc.) was then used to extract RNA following the manufacturer's recommendations. The concentration of RNA and integrity was determined using NanoDrop (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) and Bioanalyzer (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA). The RNA was stored at -80°C until cDNA was synthesized.

Reverse Transcription and RT-qPCR

Following the manufacturer's recommendations, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 500 ng of RNA and nuclease-free water was added to 10 μ L of master mix to reach a total volume of 20 μ L. No-reverse transcriptase control samples were prepared. Following a 2 h cDNA synthesis

at 37°C, 5 µL of each sample was pooled and the remaining cDNA was diluted 1:20 using nuclease-free water. Pooled cDNA was used to generate 7 standards (1:4 serial dilutions). Standards were used to calculate primer efficiencies ($E = [10^{(-1/\text{slope})} - 1]$). The slope is a plot of observed Ct values versus the 7 standard serial dilutions. Percent efficiency was then obtained by dividing E by 2 and multiplying by 100.

Quantitative real-time PCR was utilized to measure the expression of galectin genes. The CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA) was used to perform RT-qPCR reactions. All primers were designed using Primer3Plus (Version 2.4.2) unless otherwise noted. Primer sets for bovine and ovine cDNA were identical unless otherwise noted. Sample RT-qPCR reactions were carried out in 20 µL reactions in duplicate [10 µL of SYBR Green Master Mix (Catalog Number: A25743; Applied Biosystems), 1.2 µL of forward and reverse primer mix (1 µM final concentration), 2.6 µL nuclease-free water, and 5µL of cDNA template (6.6 ng RNA equivalent)]. RT-qPCR reaction of no-reverse transcriptase controls and no-template controls were included. Thermo-cycling conditions were as follows: 50°C for 2 min, 95°C for 2 min, which was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A disassociation analysis was included for each primer set and the presence of a single, sharp peak was confirmed.

The qbase+ package (Biogazelle; Zwijnaarde, Belgium) was used to select reference genes. Average RT-qPCR Ct values were imported into qbase+ software, which was used to analyze real-time data and generate the normalized relative quantity (NRQ) of genes of interest using a generalized delta-delta quantification cycle method ($\Delta\Delta Cq$; also known as $\Delta\Delta$ cycle threshold (Ct))[141]. A panel of eight prospective reference genes was analyzed (*GAPDH*, *ACTB*, *RPL18*, *PPIA*, *YWHAZ*, *RNF11*, *H3F3A*, *SDHA*) across all conceptus cDNA samples and

a subset of 12 endometrium cDNA samples. Appropriate reference genes were selected using the qbase+ geNorm function [141]. Bovine and ovine conceptus target gene expression was normalized to two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) (geNorm $M \leq 0.5$). The endometrial expression for both heifers and ewes was normalized to ring finger protein 11 (*RNF11*) and H3 histone family member 3A (*H3F3A*) (geNorm $M \leq 0.5$) [142]. Conceptus and endometrium gene expression was calculated using the following equation: Relative quantity (RQ) = $2^{-(35-Ct)}$. The RQ values were divided by the normalization factor (geometric mean of control gene RQ) to obtain the normalized relative quantity (NRQ). The NRQ values were then multiplied by a factor of 100. RT-qPCR amplicon size was validated using gel electrophoresis (0.8% agarose gel, 1X Tris/Borate/EDTA Buffer, and 0.5 µg/mL ethidium bromide) and a 50 bp DNA ladder (New England BioLabs, Ipswich, MA). Gels were visualized under ultra-violet (UV) light using the DigiDoc-It Imaging System (Analytik Jena, Jena, Germany). Amplicons from RT-qPCR on cDNA from conceptuses were visualized using gel electrophoresis to ensure primer specificity (Figure 2.3).

Immunohistochemistry

An elongating d15 bovine conceptus was obtained during a previous study [143]. After flushing the conceptus from the reproductive tract, the conceptus was placed in 4% buffered formalin phosphate (Thermo-Fisher) overnight and embedded in 2% agarose before processing. Conceptuses were then embedded in paraffin and sections (4 µm) were mounted on microscope slides which were deparaffinized using xylene, and were rehydrated through a series of EtOH washes, and boiled for 6 min in 0.01M sodium citrate buffer as an epitope retrieval technique.

Slides were allowed to cool for 45 min on ice and then were washed twice for 5 min in 1 X PBS. The tissue on slides was then enclosed using a wax pen and sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Slides were washed in 1 X PBS for 5 min and then blocking serum provided with the Rabbit IgG Vectastain Elite ABC-Peroxidase Kit (Cat. Number: PK-6101, Vector Laboratories, Inc., Burlington, CA) was added to each slide for 20 min to block non-specific binding. Slides were rinsed with 1 X PBS and then the Streptavidin/Biotin Blocking Kit (Vector Laboratories, Inc.) was used to block endogenous streptavidin and biotin. Following a rinse with 1 X PBS, slides were incubated with StartingBlock (PBS) Blocking buffer (Thermo Fisher) for 30 min. A primary rabbit anti-bovine polyclonal antibody (SAB2101338, Sigma Aldrich, St. Louis, MO) directed towards the N terminal region of *LGALS3*, was diluted in 1 X PBS (1:500) and 500 μ L of the solution was added to a serial section of the conceptus. Rabbit IgG, at the same concentration as the primary antibody, was used as an antibody control and 500 μ L added to IgG control slides at the same concentration as the primary antibody. The primary antibody and antibody control were left on slides overnight at 4°C. The following morning, a biotinylated secondary antibody, included in the Vectastain Elite ABC-Peroxidase Kit, was added to slides according to the manufacturer's recommendations. Following a 30 min incubation with the secondary antibody solution, slides were rinsed with 1 X PBS for 15 min. Slides were then covered with Vectastain Elite ABC reagent for 30 min and once again, rinsed with 1 X PBS for 15 min. ImmPACT DAB peroxidase substrate (SK-4105, Vector Laboratories) was added to tissue sections and allowed to incubate for 2-5 min. Sections were rinsed in running tap water for 5 min followed by 9 seconds in hematoxylin (GH5216, Sigma Aldrich). Sections were immediately rinsed with double distilled water for 2 min. Slides were then dipped in Scott's tap water and placed in double distilled

water. Slides were dehydrated in graded alcohol followed by xylene. Coverslips were mounted over tissues using Permount (Thermo Fisher) and images were taken using a Zeiss Axio Lab.A1 microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

Relative expression data were analyzed using the general linear model procedure (PROC GLM) in the statistical analysis software (SAS Institute Inc.; Cary, NC). For the conceptuses, the analyses tested for differences in relative galectin family members. For the endometrium, the analysis tested for an effect of pregnancy status on gene expression of select galectin family members. All data were log base 10 transformed. Conceptus data are presented as the non-transformed means $\pm$ standard deviation of the mean (SD). Alpha was set at 0.05 and a Tukey's test was included in the analysis of conceptus gene expression. Endometrial data are presented as non-transformed least squares means (LSM) $\pm$ standard error of the mean (SEM). *P*-values represent the transformed data and $P \leq 0.05$ was considered significant.

RESULTS

According to GenBank, *LGALS1*, 2, 3, 4, 7, 8, 9, 12, and 13like are present in the bovine genome while genes for *LGALS1*, 2, 3, 4, 7, 8, 9, 12, 14, and 15 are listed as being present in the ovine genome in GenBank. Therefore, we investigated the expression of galectins in elongating bovine and ovine conceptuses and pregnant and cyclic intercaruncular endometrium. Representative images of elongating bovine and ovine conceptuses obtained are shown in Figure 2.2.

Bovine Day 16 Conceptus and Endometrium Galectin Gene Expression

The expression of IFNT was investigated via RT-qPCR in d16 elongating bovine conceptuses (1293.24 ± 420.87 ; mean $\pm$ SD) as well as the third-most stable reference gene, *H3F3A* (78.91 ± 17.19 ; mean $\pm$ SD). Expression of galectins in d16 bovine conceptuses, depicted in Figure 2.3, were found to include *LGALS1* (21.80 ± 2.07 ; mean $\pm$ SD), *LGALS3* (24.47 ± 6.41 ; mean $\pm$ SD), *LGALS4* (0.08 ± 0.01 ; mean $\pm$ SD), *LGALS7* (0.02 ± 0.01 ; mean $\pm$ SD), *LGALS7B* (0.01 ± 0.005 ; mean $\pm$ SD), *LGALS8* (1.73 ± 0.27 ; mean $\pm$ SD), *LGALS9* (0.77 ± 0.77 ; mean $\pm$ SD), and *LGALS13L* (0.29 ± 0.10 ; mean $\pm$ SD).

Intercaruncular endometrium of pregnant and cyclic heifers expressed *LGALS1* (25.66 ± 2.15 and 30.10 ± 2.15 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS3* (2.62 ± 0.52 and 4.40 ± 0.52 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$), *LGALS4* (5.47 ± 0.66 and 4.64 ± 0.66 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS7* (5.80 ± 2.35 and 2.62 ± 2.35 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$), *LGALS7B* (5.04 ± 2.59 and 10.04 ± 2.59 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS8* (21.01 ± 3.95 and 25.24 ± 3.95 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS9* (9.26 ± 2.25 and 24.56 ± 2.25 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.001$), and *LGALS13L* (0.24 ± 0.07 and 0.14 ± 0.07 ; cyclic and pregnant, respectively; LSM $\pm$ SEM) (Figure 2.4). Expression of *LGALS1*, *LGALS8*, and *LGALS9* were greatest. Compared to cyclic heifers, endometrial expression of *LGALS7* was less (5.80 ± 2.35 and 2.62 ± 2.35 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$), while expression of *LGALS9* (9.26 ± 2.25 and 24.56 ± 2.25 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.001$) and *LGALS3* were greater (2.62 ± 0.52 and 4.40 ± 0.52 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$) during pregnancy.

Ovine Day 14 Conceptus and Endometrium Galectin Gene Expression

We were unable to detect gene expression of *LGALS2* and *LGALS12* in the ovine elongating conceptuses nor the endometrium of pregnant and cyclic ewes which could be due to primer sets that were not efficient or the tissue did not express these genes. The expression of IFNT was investigated via RT-qPCR in d14 elongating ovine conceptuses (Figure 2.5; 3296.74 ± 1090.66 ; mean $\pm$ SD) as well as the third-most stable reference gene, *H3F3A* (35.71 ± 1.43 ; mean $\pm$ SD). Expression of galectins in d14 ovine conceptuses, depicted in Figure 2.5, was found to include *LGALS1* (1.90 ± 0.25 ; mean $\pm$ SD), *LGALS3* (4.30 ± 0.78 ; mean $\pm$ SD), *LGALS4* (0.19 ± 0.10 ; mean $\pm$ SD), *LGALS7* (0.04 ± 0.02 ; mean $\pm$ SD), *LGALS8* (1.68 ± 0.25 ; mean $\pm$ SD), and *LGALS13L* (1.33 ± 1.09 ; mean $\pm$ SD).

Intercaruncular endometrium of pregnant and cyclic ewes expressed *LGALS1* (0.83 ± 0.21 and 0.50 ± 0.21 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS3* (7.60 ± 1.94 and 12.36 ± 1.94 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), *LGALS4* (3.10 ± 0.38 and 1.78 ± 0.38 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P = 0.06$), *LGALS7* (0.81 ± 0.24 and 2.25 ± 0.24 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$), *LGALS8* (72.84 ± 17.45 and 96.60 ± 17.45 ; cyclic and pregnant, respectively; LSM $\pm$ SEM), and *LGALS15* (104.30 ± 234.20 and 591.35 ± 234.20 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P = 0.07$) (Figure 2.6). Expression of *LGALS3*, *LGALS8*, and *LGALS15* were greatest. Compared to cyclic ewes, expression of *LGALS7* was greater in pregnant ewes (0.81 ± 0.24 and 2.25 ± 0.24 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P \leq 0.05$) while *LGALS4* (3.10 ± 0.38 and 1.78 ± 0.38 ; cyclic and pregnant, respectively; LSM $\pm$ SEM; $P = 0.06$) tended to be less during pregnancy and *LGALS15* tended to be greater during pregnancy (104.30 ± 234.20 and 591.35 ± 234.20 ; LSM $\pm$ SEM; $P = 0.07$; cyclic and pregnant, respectively).

Localization of *LGALS3* Protein in an Elongating Bovine Conceptus

The presence and localization of *LGALS3* protein was investigated in a d15 elongating bovine conceptus. Figure 2.8 exhibits staining of *LGALS3* protein in the bovine conceptus but not in the isotype control slide, rabbit IgG. Staining appeared to be within both the trophoctoderm and endoderm cytoplasm.

DISCUSSION

Galectins have been suggested to play roles in the regulation of tumors and placental angiogenesis, and an immunological role through the regulation of inflammation, adaptive and innate immune responses, and immune tolerance [134-137]. Successful implantation or attachment requires tight regulation of endometrial receptivity preparations including cellular proliferation preceded by differentiation [119]. The expression profile of cell surface molecules alters as uterine receptivity develops. Galectins have been found to increase cell-adhesion by associating surface glycoproteins on opposing cells in addition to strengthening other intercellular adhesion molecules [120]. Galectins have been highly researched in human and mice reproductive biology. The role of many galectins during bovine and ovine pregnancy is currently undetermined. In this study, we aimed to better characterize galectin genes expressed by the early bovine and ovine conceptus during the elongation phase of development as well as to characterize their expression in the endometrium during pregnancy.

We were unable to detect gene expression of *LGALS2* and *LGALS12* in the bovine elongating conceptuses nor the endometrium of pregnant and cyclic animals which could be due to primer sets that were not efficient or the tissue did not express these genes. Early bovine

conceptuses have been previously reported to express *LGALS1* [144]. Data from the current study suggest that both the bovine and ovine elongating conceptuses express *LGALS1*. Bovine endometrial expression of *LGALS1* was greater than any other galectin investigated; however, expression was not affected by pregnancy status which supports previously published data by Okumu et al. (2013) [138]. Tirado-González et al. (2013) reported systemic levels of *LGALS1* were elevated during the first trimester of normal human pregnancy compared to women who subsequently suffered a miscarriage, and they suggested that circulating *LGALS1* could serve as an indicator of early successful pregnancy in humans. Their results supported that *LGALS1* is playing an immunoregulatory role during pregnancy through the modulation of human leukocyte antigen G expressed on trophoblast cells [145]. Expression of *LGALS1* also has been associated with migration of trophoblast stem cells as the knockout of *LGALS1* in trophoblast stem cells resulted in the down-regulation of matrix metalloproteinases (MMPs), which are associated with successful cytotrophoblast invasion. Overexpression of *LGALS1* resulted in the up-regulation of MMPs. Pre-eclampsia patients have a higher placental expression of *LGALS1* than normal patients suggesting that expression of *LGALS1* is a fetal response to elevated maternal systemic inflammation. Therefore, *LGALS1* was suggested to play a role in human maternal-fetal immune tolerance [147].

Investigators have addressed the possible roles of *LGALS3* at the maternal-fetal interface [115, 116]. Bovine and ovine embryos both expressed *LGALS3* at d16 and d14, respectively. Okumu et al. (2011) published that endometrial *LGALS3* expression was not different in pregnant and cyclic heifers [138]. Data from the current study suggest expression of *LGALS3* is higher in the endometrium of pregnant heifers. Previous work suggests *LGALS3* expression is regulated by different mechanisms in placental tissues compared to in the uterus. After

conducting immunohistochemistry (IHC) studies on pregnant murine uteroplacental tissue samples, it was concluded that *LGALS3* is synthesized by all cell types within the murine uteroplacental samples. Levels of *LGALS3* mRNA remained constant in murine uterine tissues until d12 of gestation and declined thereafter until parturition. Interestingly, in placental tissues, *LGALS3* mRNA levels remained constant after d12 of gestation until term [115]. In bovine tissues, LGALS3 protein has been identified in the cervix, oviduct, uterus, atretic follicles, and CL [117]. Specifically, in the nonpregnant bovine uterus, LGALS3 has been localized via IHC in epithelial cells as well as macrophages located in the submucosa but absent in endometrial glands [117]. In the bovine CL, *LGALS3* expression was elevated at the regressed state in comparison to all other luteal stages and was localized on steroidogenic cells in the CL [118].

In addition to *LGALS1* and *LGALS3*, d16 bovine conceptuses expressed *LGALS8*, *LGALS9*, and *LGALS13* while expression of *LGALS4*, *LGALS7*, and *LGALS7B* was minimal. Expression of *LGALS4* and *LGALS7B* was detected in the bovine endometrium; however, neither was affected by pregnancy status. Pregnancy increased endometrial expression of *LGALS9*. The endometrium of cyclic heifers expressed greater *LGALS7* in comparison to pregnant animals. First-trimester human placentas express *LGALS7* and *LGALS7* might play a role in adhesion during early pregnancy [148]. In addition, *LGALS7* was elevated from weeks 10-12 and 17-20 in the serum of women who developed pre-eclampsia later on in pregnancy [149].

Interestingly, *LGALS8* expression in the bovine endometrium was similar to that of *LGALS1* and *LGALS9*. In addition, expression of *LGALS8* was detected in ovine conceptuses and endometrial samples. Though the functions of *LGALS8* during early pregnancy have not been fully elucidated, *LGALS8* was expressed in similar patterns to *LGALS1* and *LGALS3* in the

human trophoblast during the first trimester [150]. In humans, uterodomes form on the plasma membrane during the window of implantation (mid-secretory phase) to allow for the blastocyst to implant, and *LGALS9* was highly expressed by uterodomes during this timeframe, suggesting *LGALS9* could serve as an endometrial marker of receptivity [151].

Specifically expressed in sheep, endometrial *LGALS15* has been hypothesized to be a regulator of blastocyst and trophoblast development. In addition to endometrial *LGALS15* in sheep, the d14 ovine conceptus expressed *LGALS15*. Past research reports that the treatment of ovine trophectoderm cells with *LGALS15* resulted in increased proliferation and migration. Through integrin binding, *LGALS15* aids in the attachment and migration of the trophectoderm [64].

CONCLUSIONS

Collectively, findings from this study confirm that various galectins are present in the elongating ruminant embryo and are differentially expressed by the endometrium during early pregnancy. Previous research on human and mice tissue has noted that the dysregulation of galectins leads to abnormal pregnancies and infertility [152]. As little research has focused on galectins in ruminant reproduction, future research should focus on characterizing the functions of specific galectins during early pregnancy in ruminants.

Table 2.1. GenBank accession number, gene name, primer direction, primer sequence, product size and percent amplification efficiency of cDNAs amplified during real time quantitative-polymerase chain reaction (RT-qPCR).

Bovine GenBank Acc. Number	Ovine GenBank Acc. Number	Gene	Primer	Primer Sequence (5'-3')	Product Size (bp)	Bovine Amp. Efficiency	Ovine Amp. Efficiency	Source
NM_175782.1	NM_001009287.1	<i>LGALS1</i>	Forward	GTGGCCGCAGACGCCAAGAG	99	90%	90%	[143]
			Reverse	ACGTCCCCATGCGCGTTGAAA				
NM_001102341.2	XM_004010664.4	<i>LGALS3</i>	Forward	CAGTGCAGAGGCGTCGGGAAA	121	97%	99%	This Study
			Reverse	CTGCCCCAGCAGGCTGGTTT				
NM_001034768.2	XM_027978255.1	<i>LGALS4</i>	Forward	TCATGGTCATGACGGAGCACTTCA	127	106%	107%	This Study
			Reverse	ATTGAAGCATCAGGTCGCCATCCA				
XM_002694975.5	XM_004015233.4	<i>LGALS7</i>	Forward	CTTAACGTCCCCACAAGA	136	102%	104%	This Study
			Reverse	ATCACTGCCTGGTTCCTCAC				
XM_002694977.4	---	<i>LGALS7B</i>	Forward	GAGCTTTAATGTCCCCACA	139	101%	---	This Study
			Reverse	ATCACTGCCTGGTTCCTCAC				
NM_001045954.2	XM_027962309.1	<i>LGALS8*</i>	Forward	TCGTGATCATGGTCCTGAAA	141	96%	98%	This Study
			Reverse	TGAAGCCACAGAGTGAATG				
NM_001015570.3	XM_004012486.3	<i>LGALS9</i>	Forward	TCAGCTTCCAGCCTCCAGGG	86	96%	93%	[153]
			Reverse	TCCAGGGGCGCTGTGTATGGT				
---	NM_001009238.1	<i>LGALS15</i>	Forward	GGTGGATTTCGGCACTGGTA	98	---	93%	This Study
			Reverse	TCCCGTCCGTGAAAGTGTTT				
XM_024979086.1	---	<i>LGALS13L</i>	Forward	GGTGGATTCTGCACGGGTA	81	97%	---	This Study
			Reverse	ATCACCACCATGCTGTTCGT				
NM_001168279.1	NM_001123400.1	<i>IFNT</i>	Forward	TGCAGGACAGAAAAGACTTTGGT	69	91%	91%	[154]
			Reverse	CCTGATCCTTCTGGAGCTGG				

*To amplify ovine *LGALS8*, a separate forward primer was used with the following sequence (3'-5'): TCGTGATCATGGTGCTG

Figure 2.1. Images of a d16 bovine (A) and d14 ovine (B) conceptus.

Figure 2.2. Galectin RT-qPCR products visualized on a 0.8% agarose gel and a 50 bp DNA ladder. Amplicons for cattle (C) and sheep (S) galectins side by side.

Figure 2.3. Quantitative real-time PCR analysis of galectins in d16 bovine conceptuses. Data presented are the means $\pm$ SD. Expression of the genes was normalized to the two control genes, *GAPDH* and *YWHAZ*. Relative expression of IFNT and the control gene, *H3F3A*, are also presented. (a-h) Means with the same letter are not statistically different ($\alpha = 0.05$).

Figure 2.4. Quantitative real-time PCR analysis of galectins in pregnant (n=4) and cyclic (n=4) d16 bovine intercaruncular endometrium. Galectin expression was normalized to two control genes, *H3F3A* and *RNF11*. Data are presented as LSM $\pm$ SEM.

Figure 2.5. Quantitative real-time PCR analysis of galectins in d14 ovine conceptuses. Data presented are the means $\pm$ standard deviation. Expression of the genes was normalized to the two control genes, *GAPDH* and *YWHAZ*. Relative expression of IFNT and the control gene, *H3F3A*, are also presented. (a-g) Means with the same letter are not significantly different ($\alpha = 0.05$).

Figure 2.6. Quantitative real-time PCR analysis of galectins in pregnant (n=3) and cyclic (n=3) d14 ovine intercaruncular endometrium. Galectin expression is relative to two control genes, *H3F3A* and *RNF11*. Data are presented as LSM $\pm$ SEM.

Figure 2.7. (B and D) Galectin-3 (LGALS3) protein in an elongating d15 bovine conceptus. (A and C) Absence of DAB staining in IgG isotype control serial section of the conceptus. Images were taken on a Zeiss Axio Lab.A1 microscope at 10 X (A-B) and 20 X (C-D) magnification.

CHAPTER THREE

EFFECT OF INTERFERON TAU AND GALECTIN-3 ON BOVINE ENDOMETRIAL EPITHELIAL AND FIBROBLAST CELL TRANSCRIPTOMES IN 3D CELL CULTURE

ABSTRACT

Interferon-tau (IFNT), secreted by the elongating bovine conceptus, is the maternal recognition of pregnancy signal that suppresses the endometrial luteolytic mechanism by modifying expression of genes in specific cell types within the endometrium. The conceptus also secretes galectin proteins, specifically galectin-3. This study assessed the effect of IFNT (Experiment 1) and LGALS3 (Experiment 2) on bovine endometrial epithelial and fibroblast cell transcriptomes when the mid-luteal phase endometrial cells were cultured in a 3D environment. In Experiment one, epithelial cells, cultured in Transwell inserts above the fibroblast cells, were treated with RPMI media alone (control; n=4) or treatment media (RPMI with 5% FBS and 1% ABAM) containing 100 ng of recombinant ovine IFNT (n=3) for 6 h. In Experiment two, cells were treated with either RPMI media alone (Control; n=5), pregnant ovine uterine flush fluid, theoretically containing INFT, diluted with treatment media 1:1 (n=4), or treatment media (RPMI with 5% FBS and 1% ABAM) containing either 100 or 1000 ng of recombinant mouse LGALS3 (n=5) for 6 h. Total RNA was extracted from both cell types and the transcriptomes were assayed by RNA Sequencing and RT-qPCR. Interferon-tau regulated 673 and 83 genes

(differentially expressed genes or DEGs) in epithelial and fibroblast cells, respectively ($P \leq 0.001$; FDR $P \leq 0.05$). Many of the DEGs identified in the epithelial (205) and fibroblast cells (64) were previously reported as IFNT and bovine conceptus-induced DEGs in intact bovine endometrium, including well-known ISGs such as *ISG15*, *MX1*, *MX2*, *OAS1*, and *OAS2*. Surprisingly, the *ZC3HAV1* gene, which was recently found to be up-regulated in intact bovine endometrium in response to the conceptus, was only up-regulated in fibroblast cells despite applying IFNT to the apical surface of the epithelial cells in 3D culture. The RNA-Seq analysis of the bovine endometrial cells treated with mouse LGALS3 protein resulted in the identification of a single gene, *RPL23A*, that was upregulated in epithelial cells treated with 100 ng of LGALS3 ($P \leq 0.001$; FDR $P \leq 0.05$). This study supports the idea that the 3D culture of bovine endometrial cells may be utilized to study early conceptus-maternal crosstalk and identify endometrial cell type-specific responses to the conceptus.

INTRODUCTION

The beef cow/calf industry experiences an annual loss exceeding \$1.2 billion, with the largest contributing factor being embryonic loss [1]. The highest incidence of pregnancy loss in cattle occurs during the embryonic period of gestation, with 40% occurring between d8 and d17 [2-4]. Bovine embryos will elongate from approximately 2 mm on d13 into an approximately 6 cm filamentous structure, spanning the length of the uterine horn by d19 [19, 20, 30]. During this time, the conceptus secretes a substance to communicate to the maternal endometrium its presence and prolong progesterone (P4) production by the CL [5]. This secretory protein, IFNT, is derived from the bovine mononuclear trophoderm. The presence of IFNT stimulates the

expression of ISGs in addition to blocking the pulsatile secretion of endometrial PGF from the uterine horn and ultimately, luteolysis [42]. Luteolysis, the functional and structural regression of the CL, is prevented due to the paracrine release of IFNT from the conceptus which acts to modify endometrial PGF through the silencing of uterine epithelial cell estrogen receptor alpha (*ESR1*) and thus, epithelia oxytocin receptor (*OTR*) [155]. Overall, luteal P4 is maintained and supports gestation until the establishment of placental P4.

Interferon-tau is a type I interferon. The type I interferons bind IFNAR to signal a biological response. There is a single Type II interferon family member, IFNG, which binds the IFNGR [40]. The IFNT acts on the endometrium and modifies endometrial cell gene expression. The pregnancy recognition signal, IFNT, affects the differential expression of genes in the LE, dGE, sGE, and stroma [36, 41]. The genes affected, ISGs, are categorized in two distinct classifications, classical and non-classical ISGs, which are spatially stimulated by IFNT within the stroma/dGE and LE/sGE, respectively [42, 43]. Type I and II IFNs initiate the expression of classical ISGs through stimulation Jak-STAT pathways resulting in phosphorylation of their respective receptors activating STAT. Classical ISGs expressed in the stroma/dGE in response to IFNT include *STAT1* and *STAT2*, *IRF1*, *ISG15*, and 2'-5'-oligoadenylate synthetase 1 (*OAS1*) [45, 156, 157]. Transcriptomic analysis of ruminant endometrium has led to the identification of non-classical ISGs which are stimulated by IFNT and are primarily induced by P4 spatially in the LE/sGE [20, 41]. Genes include *CST3*, *CTSL*, and *LGALS15* in sheep [42]. In the LE/sGE, interferon regulatory factor-2 (*IRF2*) prevents the IFNT from regulating gene expression of classical ISGs.

Mathew et al. (2019) published data identifying conceptus and IFNT induced DEGs

[143]. Endometrial explants were cocultured with d15 conceptuses from artificial insemination or IVF origin, or 100 ng of IFNT, or media alone. Transcriptome differences were analyzed via RNA-Seq and RT-qPCR. Conceptus induced, IFNT-dependant DEGs were identified through the grouping of explant DEGs that were differentially expressed in response to all three treatment groups as well as DEGs similar between the IFNT and AI conceptus treated explants [143].

Galectins are a large family of β -galactoside-binding animal lectins that have numerous physiological functions. Lectins bind carbohydrates and result in cell agglutination while playing vital roles in pathogen defense, cell trafficking, and immune regulation [82, 83]. Galectins are ancient members of a lectin family which are characterized by having conserved amino acid sequences with at least one CRD and the ability to recognize β -galactoside structures located on cell surface glycoconjugates [85, 86]. Elongating bovine embryos have been found to express galectin-1 (*LGALS1*) and *LGALS3*. Recently, *LGALS3* was identified to play an important part in the preparation of the endometrium for implantation with a presence in the endometrium and trophoblast cells. Given the resemblances of tumor invasion and the migration of trophoblast cells, investigators have addressed the possible roles of *LGALS3* at the maternal-fetal interface regarding implantation [115, 116]. After conducting IHC studies on pregnant murine uteroplacental tissue samples, it was concluded that *LGALS3* is synthesized by all cell types within the murine uteroplacental samples. Levels of *LGALS3* mRNA remained constant in murine uterine tissues until d12 of gestation and declined thereafter until parturition. Interestingly, in placental tissues, *LGALS3* mRNA levels remained constant after d12 of gestation until term [115]. In bovine tissues, *LGALS3* protein has been identified in the cervix, oviduct, uterus, atretic follicles, and CL [117]. Specifically, in the nonpregnant bovine uterus, *LGALS3* has been localized via IHC in epithelial cells as well as macrophages located in the

submucosa but were absent in endometrial glands [117].

In the past, 2D monoculture techniques have been utilized to investigate cellular processes of bovine epithelial and fibroblast cells [129, 130]. The 3D transwell culture system published by MacKintosh et al. (2013) enables the coculture of two cells types, which would normally interact *in vivo* [127]. MacKintosh et al. utilized bovine endometrial epithelial and fibroblast cells in this system to investigate the effects of lipopolysaccharide (LPS) in a post-partum uterine disease [127]. To better simulate the columnar arrangement of epithelial cells *in vivo*, a 3D culture allows epithelial cells to maintain a physiological polarity *in vitro* due to tight junctions unlike traditional 2D cell culture techniques [127, 128]. Tight junctions are imperative to epithelial cellular functions because the passage of ions and molecules paracellularly is regulated by tight junctions. A measurement that reflects epithelial integrity, TEER, can be measured via an epithelial volt-ohm meter [127].

This aim of this study was to examine the effect of IFNT and LGALS3 on bovine endometrial epithelial and fibroblast cell transcriptomes when cells are cultured in a 3D environment.

MATERIALS AND METHODS

Primary Cell Isolation, Culture, and Treatment

Bovine reproductive tracts utilized to isolate endometrial cells were collected from a local abattoir. The tracts were chosen based on the appearance of a mid-luteal phase CL and the absence of either pregnancy and/or infection [158]. Cells were isolated from different animals for the IFNT Experiment (Experiment 1; n=3-4) and LGALS3 Experiment (Experiment 2; n=4-5). The reproductive tracts were immediately placed on ice and transported to the laboratory. Upon

arrival at the laboratory, reproductive tracts were rinsed with water, and the broad ligament and the cervix were removed. The remaining reproductive tract was sprayed with 70% ethyl alcohol (EtOH), and the uterine horn ipsilateral to the CL was removed with a scalpel. The tract was then placed in a cold dissecting tray lined with 70% EtOH soaked paper towels and sprayed once again with 70% EtOH. A cut was made along the anti-mesometrial side of the uterine horn ipsilateral to the CL.

Bovine endometrial epithelial and fibroblast cells were isolated from uterine horn ipsilateral to the CL [127]. Briefly, the exposed endometrium was rinsed with 1X phosphate buffered saline (PBS) (Gibco, Gaithersburg, MD) containing 1% antibiotic-antimycotic (ABAM) (Gibco). Thin intercaruncular endometrial strips were dissected and placed in 25 mL of hank's balanced salt solution (HBSS) (Gibco). Endometrial strips were washed twice in 25 mL of HBSS and trimmed into 3-5 mm pieces and washed in a final HBSS wash (25 mL) containing 1% ABAM. Endometrial strips were then incubated for 10 min at 38.8°C in atmospheric oxygen and 5% CO₂. The wash was then drained from the conical tube and 40 mL of filter-sterilized enzyme solution [49.5 mL HBSS, 25 mg Collagenase II (Sigma-Aldrich), 50 mg bovine serum albumin (BSA) (Sima-Aldrich), 700 µL Trypsin-EDTA-HBSS solution (Sigma-Aldrich; Gibco), and 125 µL DNase I (Sigma-Aldrich)] was added. The endometrium in enzyme solution was placed in a water bath at 38.8°C for 1 h with manual agitation every 10 min.

Following incubation, the endometrium in enzyme solution was filtered through a 100 µM filter placed over a 40 µM filter (Corning Inc.; Tewksbury, MA). The filtered solution was placed with 5 mL of prewarmed (38.8°C) HBSS with 10% fetal bovine serum (FBS) (VWR International, Radnor, PA). The 40 µM filter, containing sheets of epithelial cells, was then overturned and washed with 30 mL of pre-warmed (38.8°C) Roswell Park Memorial Institute

1640 (RMPI) (Gibco), 5% FBS (VWR International), and 1% ABAM (Gibco) (complete media). Fifteen mL aliquots of the cell suspension were transferred into T75 flasks (Greiner Bio-One, Monroe, NC) and cells were allowed to attach for 48 h at 38.8°C in atmospheric oxygen and 5% CO₂. Fibroblasts, in filtered solution, were centrifuged at 700 x g for 7 min at 25°C. The supernatant was aspirated and 5 mL of prewarmed (38.8°C) sterile water was added to the pellet and lightly vortexed for 30 s. After, 45 mL of HBSS (Gibco) containing 10% FBS (VWR International) was added to the cells which were then filtered once again through a 40 µM filter. Flow through was centrifuged at 700 x g for 7 min at 25°C. The supernatant was aspirated and 45 mL of complete media was added to re-suspend pellet. Fifteen mL aliquots were added to T75 flasks which were placed in the incubator at 38.8°C in atmospheric oxygen and 5% CO₂. After 18 h, fibroblasts in flasks were washed with 10 mL of prewarmed 1X PBS (Gibco) and 15 mL of complete media was added. Every 2d, both fibroblast, and epithelial flasks were rinsed with 1X PBS (Gibco) and 15 mL of fresh complete media was added until the cells reached confluency. Once approximately 80% confluent, cells were dislodged from the flask with 1.5-2 mL of Accutase (Sigma-Aldrich) and passed into new T75 flasks in complete media.

Cells were counted using a hemocytometer and once the epithelial cell concentration of 3×10^5 cells/mL was reached, cells were removed using 2-3 mL of Accutase (Sigma-Aldrich) and placed in polyethylene terephthalate transwell cell culture inserts with a pore size of 0.4 µm (Millipore Sigma, Burlington, MA). Before the addition of epithelial cells, transwell inserts were coated with 25-30 µL of Matrigel (Corning Inc.) with ice-cold RPMI (Gibco) at a 1:8 dilution. Transwell inserts were placed in a 24-well plate containing 800 µL of complete media. Cells were cultured in transwell inserts for 9d. On d9 (day of treatment), TEER was calculated using

the following formula: $TEER (\Omega cm^2) = (sample\ resistance - blank\ resistance) \times membrane\ area$. Resistance values were obtained by using a Millicell ERS-Voltohmmeter (Millipore Sigma). The 3D cultures were conducted as previously described [127]. The day prior to treatment, 1 mL of fibroblast cells at the concentration of 1×10^5 cells/mL were plated per well in new 24 plates. Fibroblasts were allowed to adhere and 18 h later, the cells were washed with 1 X PBS (Gibco) and new media was added [treatment media; RPMI (Gibco), 5% FBS (VWR International), and 1% ABAM (Gibco)]. The transwell inserts containing epithelial cells were then added to the fibroblast cells and new treatment media was added to the transwell inserts.

Experiment One

After 2 h, new treatment media alone (Control) or treatment media containing 100 ng of recombinant ovine IFNT (oIFNT), kindly donated by Dr. Gilles Charpigny of INRA, Biologie du Développement et Reproduction (Jouy en Josas, France), was added to well inserts containing epithelial cells for 6 h [143]. Fibroblast cell media was not changed. The experiment was repeated at least 4 times (cells from 4 different uteri). However, the oIFNT treatment for one uterus was lost, therefore, n=3-4. Both treatments were applied to three 3D cell cultures from each uterus.

Experiment Two

After 2 h, 300 μ L of new treatment media alone (Control), d14 pregnant ovine flush fluid (FF) (diluted 1:1 with treatment media), or treatment media containing either 100 or 1000 ng of recombinant mouse LGALS3 protein (Catalog Number: 9039-GA; R&D Systems, Minneapolis, MN) was added to well inserts containing epithelial cells for 6 h. The ovine pregnant uterine

flush fluid, theoretically containing IFNT, was obtained during a previous study by flushing the uterine horn ipsilateral to the CL of a d14 pregnant ewe with 20 mL of 1 X PBS (Thermo Fisher). The presence of a conceptus confirmed pregnancy. Fibroblast media was not changed. The experiment was replicated with cells from five different uteri. The FF treatment from one replication was lost, therefore, n=4-5 uteri. All treatments were applied to three 3D cell cultures from each uterus.

RNA Isolation and RNA Sequencing (RNA-Seq)

Total RNA was extracted from both cell types, separately, using the Qiagen RNeasy Kit (Qiagen) for Experiment one while the E.Z.N.A. Total RNA Kit I (Omega Bio Tek, Inc.) was used to collect RNA for Experiment two. Both kits were used according to the manufacturer's recommendations. Briefly, the media was collected and the membrane from transwell inserts was removed using a scalpel, placed in lysis buffer, and agitated using a pipette tip. Lysis buffer was added to fibroblast cells in wells and gently scraped using a pipette tip to obtain fibroblast RNA. The three RNA samples per treatment for both cells types were pooled. The concentration of RNA and integrity was determined using NanoDrop (NanoDrop ND-1000, NanoDrop Technologies) and Bioanalyzer (2100 Bioanalyzer, Agilent Technologies), respectively. The RNA integrity number (RIN) values obtained from the Bioanalyzer ranged from 8.6-10.0. The RNA was stored at -80°C until cDNA was synthesized.

RNA library preparation and sequencing was performed by the University of Missouri DNA Core facility as previously described by Moraes et al. [159]. The raw sequences (fastq) underwent adapter removal and quality trimming utilizing Trimmomatic [160]. The quality reads were then mapped to the bovine reference genome ARS-UCD1.2 using Hisat2 mapper, a

sensitive and fast alignment program of next-generation sequencing data [161]. The sorted binary alignment maps and the NCBI gene annotation of the ARS-UCD1.2 assembly were subjected to FeatureCounts [162] to quantify read counts of genes for each sample. Differential gene expression analysis between sample groups was performed by robustly fitting the expression data to a weighted generalized linear model (GLM) using edgeR robust [163].

To perform a Gene Ontology (GO) analysis of statistically significant transcripts generated by RNA-Seq data, DAVID Bioinformatics Resource 6.8 was utilized [164]. Pathway analyses included were an enriched biological process (BP) in addition to the Kyoto Encyclopedia of Genes and Genomes (KEGG). During the annotation, the GO Direct category was used which provides mappings directly annotated by the source database and the *Homo sapien* background was used during the analysis. The GO Direct and KEGG categories were considered enriched when $P \leq 0.05$.

Reverse Transcription and Quantitative real-time PCR (RT-qPCR)

Following the manufactures recommendations, cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, 500 ng of RNA and nuclease-free water was added to 10 μ L of master mix to reach a total volume of 20 μ L. No-reverse transcriptase control samples were also prepared. Following a 2 h cDNA synthesis at 37°C, 5 μ L of each sample was pooled and the remaining cDNA was diluted 1:20 using nuclease-free water. Pooled cDNA was used to generate 7 standards (1:4 serial dilutions). Standards were used to calculate primer efficiencies (E) using the following equation: $E = [10^{(-1/\text{slope})} - 1]$. The slope is a plot of observed Ct values versus the 7 standard serial dilutions. Percent efficiency was then obtained by dividing E by 2 and multiplying by 100.

Quantitative real-time PCR was utilized to examine gene expression differences in response to treatment and to verify the data obtained from RNA-Seq. The CFX96 Real-Time PCR machine (Bio-Rad Laboratories) was used to perform RT-qPCR reactions. All primers were designed using Primer3Plus (Version 2.4.2) unless otherwise noted. Sample RT-qPCR reactions were carried out in 20 μ L reactions in duplicate [10 μ L of SYBR Green Master Mix (Catalog Number: A25743; Applied Biosystems), 1.2 μ L of forward and reverse primer mix (1 μ M final concentration), 2.6 μ L nuclease-free water, and 5 μ L of cDNA template (6.6 ng RNA equivalent)]. The RT-qPCR reaction of no-reverse transcriptase controls and no-template controls were included. All samples from an experiment were assayed in a single 96-well plate. Thermo-cycling conditions were as follows: 50°C for 2 min, 95°C for 2 min, which was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation analysis was included for each primer set and the presence of a single, sharp peak was confirmed.

The qbase+ package (Biogazelle) was used to analyze and calculate relative gene expression values. Average RT-qPCR Ct values were imported into qbase+ software, which was used to analyze real-time data and generate the normalized relative quantity (NRQ) of genes of interest using a generalized delta-delta quantification cycle method ($\Delta\Delta C_q$; also known as $\Delta\Delta CT$)[141]. A panel of eight prospective reference genes was analyzed (*GAPDH*, *ACTB*, *RPL18*, *PPIA*, *YWHAZ*, *RNF11*, *H3F3A*, *SDHA*) across a subset of 12 random cDNA samples and two reference genes were selected using the qbase+ geNorm function [141]. Expression was normalized to two housekeeping genes, succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and ring finger protein 11 (*RNF11*) in the oIFNT Experiment and the two genes, peptidylprolyl isomerase A (*PPIA*) and *SDHA*, in the LGALS3 Experiment. These reference genes were found to be the most stably expressed across the subset of samples for each

experiment (geNorm $M \leq 0.5$) [142]. Sizes of RT-qPCR amplicons were validated using gel electrophoresis (0.8% agarose gel, 1X Tris/Borate/EDTA Buffer, and 0.5 $\mu\text{g/mL}$ ethidium bromide) and a 50 bp DNA ladder (New England BioLabs). Gels were visualized under ultra-violet (UV) light using the DigiDoc-It Imaging System (Analytik Jena).

Statistical Analysis

Log-transformed sample NRQ data were analyzed using the proc GLM in the statistical analysis software (SAS Institute Inc.). The analysis tested the effect of recombinant oIFNT (Experiment one) or recombinant mouse LGALS3 (Experiment two) on endometrial cell gene expression. The epithelial and fibroblast NRQ data were analyzed separately. Residual data were scrutinized for normality using the PLOTS= (diagnostics residuals) statement. If residuals were not normally distributed, they were square root (sqrt) transformed. Data are presented as non-transformed least square means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Statistical significance was declared at $P \leq 0.05$.

RESULTS

Experiment One

Differentially Expressed Genes in Response to oIFNT

The RNA-Seq analysis identified 673 and 83 DEGs (up- or down-regulated) in the epithelial and fibroblast cells, respectively, in response to treatment with 100 ng of oIFNT in the 3D culture system ($P \leq 0.001$; FDR $P \leq 0.05$; Figure 3.2). Epithelial cell data consisted of 632 and 41 DEGs that were up- and down-regulated, respectively. Fibroblast cell data identified 83 DEGs that were up-regulated and 0 DEGs that were down-regulated. To potentially identify

conceptus IFNT responsive genes specifically in epithelial and fibroblast cells, data were compared to recently published RNA-Seq data of bovine endometrial explants incubated with an elongating, d15 bovine conceptus and explants treated with 100 ng/mL of recombinant oIFNT. The DEGs, published by Mathew et al. (2019) were considered to be conceptus induced, IFNT dependent DEGs (Figure 3.2) [143]. Comparison of DEGs between epithelial cells and fibroblast cells treated with 100 ng/mL of oIFNT in a 3D environment with the conceptus induced, IFNT dependent DEGs resulted in the identification of 224 DEGs in common with explants and epithelial cells or fibroblast cells in 3D culture (Figure 3.2). Sixty-nine DEGs were in common between all three groups (Figure 3.2). Explants shared 154 DEGs with epithelial cells only and a single gene was differentially expressed in explants and fibroblast cells only (Figure 3.2). All DEGs in common between explants (Mathew et al. 2019) and endometrial cells in 3D culture from the current study were up-regulated [143].

Epithelial-Derived DEGs in Response to oIFNT

Analysis of RNA-Seq data resulted in the identification of 632 genes that were up-regulated and 41 genes that were down-regulated in bovine endometrial epithelial cells in response to treatment with 100 ng/mL of oIFNT ($P \leq 0.001$; FDR $P \leq 0.05$; Figure 3.2). Epithelial cells shared 223 up-regulated genes with explant data and 154 of these DEGs were up-regulated only in the epithelial cells during 3D culture. The top 10 genes with the greatest log fold change (logFC) of the 223 DEGs shared between explants and epithelial cells were kinesin family member 5C (*KIF5C*; logFC 8.66), hematopoietic SH2 domain containing (*HSH2D*; logFC 7.86), ATPase phospholipid transporting 8B4 (*ATP8B4*; logFC 7.52), indoleamine 2,3-dioxygenase (*IDO1*; logFC 7.18), forkhead box S1 (*FOXS1*; logFC 7.17), growth hormone

secretagogue receptor (*GHSR*; logFC 5.67), prostate androgen-regulated mucin-like protein 1 (*PARM1*; logFC), apolipoprotein L3 (*APOL3*; logFC 5.34), melanin concentrating hormone receptor 1 (*MCHRI*; logFC 4.88), and guanylate-binding protein 2 (*GBP2*; logFC 4.52) (Figure 3.2; Table 3.2). The top 20 DEGs in common out of the 223 DEGs are listed in Table 3.2 with their respective epithelial log fold change differences and fibroblast log fold change differences, if applicable. Of the 223 DEGs, 69 were also up-regulated in both epithelial and fibroblast cells in response to oIFNT (Figure 3.2).

Further analysis of DEGs shared between explants and epithelial cells through RT-qPCR validated that genes including MX dynamin like GTPase 1 (*MX1*) (Figure 3.3; 1.92 ± 15.45 and 59.62 ± 17.83 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.05$), *MX2* (Figure 3.4; 0.59 ± 37.32 and 680.18 ± 43.1 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), *OAS1* (Figure 3.5; 0.80 ± 10.34 and 39.03 ± 11.94 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.05$), galectin-3 binding protein (*LGALS3BP*; Figure 3.6; 0.72 ± 0.40 and 2.79 ± 0.50 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), galectin-9 (*LGALS9*; Figure 3.7; 3.07 ± 2.99 and 36.78 ± 3.45 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), *OAS2* (Figure 3.8; 0.59 ± 1.06 and 9.97 ± 1.22 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.01$), *BATF2* (Figure 3.9; 0.59 ± 1.03 and 8.51 ± 1.19 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), and TNF superfamily member 13b (*TNFSF13B*; Figure 3.12; 0.48 ± 0.80 and 12.7 ± 0.93 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$) were significantly upregulated in the oIFNT treated epithelial cells. Microtubule associated protein 6 (*MAP6*) was found to be significantly down-regulated after oIFNT treatment by RNA-Seq (logFC -6.50; $P \leq 0.001$; FDR $P \leq 0.05$) and RT-qPCR validation of this gene exhibited *MAP6* expression in

response to IFNT was less than in control cells, although not significantly (Figure 3.11; 1.86 ± 0.30 and 0.35 ± 0.35 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P = 0.17$).

Fibroblast-Derived DEGs in Response to oIFNT

The RNA-Seq data identified 83 up-regulated and 0 down-regulated transcripts in fibroblast cells treated with oIFNT in the 3D cell culture ($P \leq 0.001$; FDR $P \leq 0.05$). Fibroblast cells shared 69 DEGs with both explants and epithelial cells, all of which were up-regulated. Of the 69 genes, the following were the top 10 greatest up-regulated in fibroblast cells: *OAS2* (logFC 9.63), interferon induced protein 44 like (*IFI44L*; logFC 8.66), *RSAD2* (logFC 8.35), *ZBP1* (logFC 8.06), *MX2* (logFC 7.83), *MX1* (logFC 7.77), *PLAC8* (logFC 7.75), *IFI44* (logFC 7.63), TNF superfamily member 10 (*TNFSF10*; logFC 7.50), and sterile alpha motif domain containing 9 (*SAMD9*; logFC 7.46). A single transcript (also recently reported as a conceptus and oIFNT induced DEG in endometrial explants), zinc finger CCCH-type antiviral protein 1 (*ZC3HAV1*; logFC 0.87) was differentially expressed in fibroblast cells (up-regulated) but not epithelial cells ($P \leq 0.05$) [143].

Validation of RNA-Seq data through RT-qPCR confirmed genes including *MX1* (Figure 3.3; 0.01 ± 0.14 and 5.28 ± 0.17 Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), *MX2* (Figure 3.4; 0.01 ± 1.61 and 4.37 ± 1.85 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.01$), *OAS1* (Figure 3.5; 0.04 ± 0.95 and 10.86 ± 1.09 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), *LGALS3BP* (Figure 3.6; 0.58 ± 0.07 and 1.18 ± 0.08 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.01$), *LGALS9* (Figure 3.7; 0.04 ± 0.15 and 0.79 ± 0.18 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.05$), *OAS2* (Figure 3.8; 0.25 ± 0.13 and 1.62 ± 0.15 ; Control and oIFNT, respectively; LSM $\pm$ SEM; $P < 0.001$), *BATF2* (Figure 3.9;

0.30 ± 0.25 and 1.55 ± 0.29; Control and oIFNT, respectively; LSM ± SEM; $P < 0.01$), and *TNFSF13B* (Figure 3.12; 0.33 ± 0.35 and 1.48 ± 0.41; Control and oIFNT, respectively; LSM ± SEM; $P < 0.05$) were increased in response to oIFNT treatment. Expression of *ZC3HAV1* in epithelial cells was unaltered by the presence of oIFNT protein in culture, while in fibroblast cells, expression of this gene increased due to oIFNT (Figure 3.13; Control and oIFNT, respectively; LSM ± SEM; $P < 0.05$).

Gene Ontology Analysis

The gene ontology analysis of the 223 up-regulated epithelial DEGs in response to oIFNT identified 97 enriched biological processes ($P \leq 0.001$; Table 3.4). The top 5 enriched pathways include the following: 1) type I interferon signaling pathway, 2) defense response to virus, 3) interferon-gamma mediated signaling pathway, 4) response to virus, and 5) negative regulation of viral genome replication ($P \leq 0.001$; Table 3.4). The KEGG pathway analysis found 12 enriched pathways ($P \leq 0.05$; Table 3.4). The top 5 pathways include 1) influenza A, 2) herpes simplex infection, 3) hepatitis C, 4) measles, 5) RIG-1-like receptor signaling pathway ($P \leq 0.001$; Table 3.4).

The 70 oIFNT-stimulated DEGs in fibroblast cells, which includes the 69 shared with the epithelial cells and *ZC3HAV1*, were identified to be associated with 43 biological processes. The top 5 enriched pathways include 1) defense response to virus, 2) type I interferon signaling, 3) response to virus, 4) negative regulation of viral genome replication, and 5) interferon-gamma-mediated signaling pathway ($P < 0.001$; Table 3.5). The KEGG pathway analysis found 7 enriched pathways ($P \leq 0.05$; Table 3.5). The top 5 pathways include 1) influenza A, 2) herpes

simplex infection, 3) measles, 4) RIG-I-like receptor signaling, and 5) hepatitis C ($P \leq 0.001$; Table 3.5).

Experiment Two

The Effect of Mouse-LGALS3 on Bovine Endometrial Epithelial and Fibroblast Cells in 3D Culture

The effect of a recombinant mouse LGALS3 protein on bovine endometrial epithelial and fibroblast cells in 3D culture was analyzed by RNA-Seq. Results indicated a single gene, 60S ribosomal protein L23A (*RPL23A*), was up-regulated in epithelial cells (log FC 4.05). The transcriptome of fibroblast cells was not affected by LGALS3 treatment. The gene, *RPL23A*, was analyzed through RT-qPCR, however, an increase in expression of this gene was unable to be validated using this method (Table 3.6). The cells that received the lower concentration of LGALS3 protein (100 ng) and the control-treated cells were analyzed through RNA-Seq. The relative expression of other genes including C-X-C motif chemokine receptor 4 (*CXCR4*), doublesex- and mab-3-related transcription factor A1 (*DMRTA1*), *ISG15*, *LGALS3BP*, leukemia inhibitory factor (*LIF*), proline-rich transmembrane protein 3 (*PRRT3*), and scavenger receptor cysteine-rich family member with 4 domains (*SSC4D*) was assessed through RT-qPCR to see whether a higher concentration of LGALS3 (1000 ng) affected their expression. Of the genes analyzed, neither concentration of LGALS3 affected their relative expression (Table 3.6).

The Effect of Day 14 Pregnant Ovine Flush Fluid on Endometrial Epithelial and Fibroblast Cell Transcriptomes Cultured in 3D

The ovine flush fluid treatment up-regulated expression of *CXCR4* (5.12 ± 0.98 and 9.50 ± 1.10 ; Control and FF, respectively; LSM $\pm$ SEM; $P < 0.01$), *ISG15* (0.50 ± 5.31 and 130.27 ± 5.94 ; Control and FF, respectively; LSM $\pm$ SEM; $P < 0.001$), *LGALS3BP* (0.98 ± 0.16 and 4.12 ± 0.18 ; Control and FF, respectively; LSM $\pm$ SEM; $P < 0.001$), and *LIF* (4.27 ± 0.77 and 7.11 ± 0.86 ; Control and FF, respectively; LSM $\pm$ SEM; $P < 0.05$) as indicated by RT-qPCR (Table 3.6).

DISCUSSION

The aim of this study was to identify IFNT and LGALS3 induced DEGs in the bovine endometrial epithelium and fibroblast cells in a 3D cell culture system. To date, several studies have addressed endometrial genes stimulated by the elongating ruminant conceptus particularly in sheep, however, literature is lacking information examining differential expression within endometrial epithelial and/or stromal cells, specifically, during early bovine pregnancy [143, 165, 166].

Endometrial cells of the uterus regulate maternal-conceptus communication through the differential expression of genes. The endometrium also contains other cells including glands, endothelial cells, and immune cells. Past research has noted that endometrial epithelial and fibroblast cells, which largely comprise the endometrium, have different expression profiles [38, 167-169]. During this preimplantation and conceptus elongation in ruminants, genes differentially expressed in fibroblasts have been documented to be associated with maternal

immunosuppression to protect the semi-allogenic conceptus [42, 165]. The JAK-STAT signaling pathway is activated via IFNT to stimulate classical ISGs during the elongation phase in the ruminant endometrium [170]. Epithelial and fibroblast cells treated with IFNT in the 3D cell culture had 69 differentially expressed transcripts in common with bovine endometrial explants treated with IFNT or an elongating Day 15 conceptus. There were 154 DEGs shared specifically between the epithelial cells and explants that were not shared with the fibroblasts. On the other hand, there was one DEG shared between the fibroblast cells and explants but not with the epithelial cells [143].

3D Cell Culture

Epithelial and fibroblast cell-specific responses to IFNT have not been elucidated, particularly in a 3D cell culture system. Recently, 3D cell culture models have been utilized to investigate cellular communication mechanisms and responses. Mackintosh et al. (2013) found the coculture of bovine endometrial epithelial and stromal cells enabled the polarization of epithelial cells. The 3D cultures were treated with oxytocin and arachidonic acid to assay the physiological function of the model. The cells were also treated with lipopolysaccharide (LPS) to mimic a bacterial infection. In the present study, we assessed the use of a 3D culture system to study maternal-conceptus crosstalk.

The IFNT was administered apically to the 3D culture. The RNA-Seq data identified 673 DEGs in epithelial cells and 83 DEGs in fibroblast cells ($P \leq 0.001$; FDR $P \leq 0.05$), therefore, apical treatment to the 3D system elicited a response in both cell types. We do not know if the IFNT traveled through the epithelium layer to modify the fibroblast cell transcriptome or if the epithelial cells secreted factors basolaterally in response to IFNT to modify fibroblast cell gene

expression. Considering conceptus IFNT can leave the endometrium and travel systemically, it is possible that IFNT traveled either paracellularly or transcellular to reach the fibroblast cells [171, 172].

Classical and Non-Classical ISGs stimulated by IFNT

Endometrial transcriptomic reported by Forde et al. (2011) of d16 cyclic and pregnant heifers consisted largely of classical ISGs including *B2M*, interferon alpha-inducible protein 6 (*IFI6*), *ISG15*, *MX2*, *MX1*, *OAS1*, and *STAT1* [168]. The bovine endometrium has also been found to respond earlier than d16 as d8 blastocysts and d15 conceptuses have been documented to alter endometrial gene expression in bovine endometrial explants cultured with explants [143, 153]. The 3D cultured bovine endometrial epithelial and fibroblast cells exhibited a significant upregulation of classical ISGs including *IFIT1*, *ISG15*, *MX2*, *OAS2*, and *RSAD2* in response to IFNT treatment (Table 3.3). These genes were also recently reported to be up-regulated genes in explants treated with IFNT or a conceptus [143]. The gene, *RSAD2*, is believed to be involved in the development of an antiviral endometrial environment via the inhibition of an innate immune response [173, 174].

The role of various classical ISGs is still undetermined. The endometrial up-regulation of the classical ISG, *ISG15*, is associated with infection prevention and is hypothesized to protect the developing conceptus from a negative inflammatory response [175]. In sheep, *ISG15*, *IFIT1*, and *RSAD2* have been identified to be up-regulated by IFNT in the endometrial glandular epithelium and fibroblast cells after d14 of gestation [41, 42]. This agrees with data from the current study as these genes were found to be up-regulated by IFNT in both epithelial and

fibroblast cells as well as by the coculture of endometrial explants with an elongating conceptus and IFNT.

Factors, including IFNT and P4, stimulate ovine endometrial genes in a cell-specific manner. Expression of IFNT and P4 responsive genes, including the non-classical ISGs, *LGALS15*, and *CTSL*, were shown to be upregulated in the epithelium by d14/15 of both pregnant and cyclic sheep, however, expression then resided by d16 in cyclic sheep. This led to the conclusion that *LGALS15* and *CTSL* are stimulated by P4, IFNT extends their expression [167, 176]. Bovine and ovine IFNT responsive endometrial genes vary as *LGALS15*, a gene highly upregulated during early pregnancy in sheep is not present in cattle [177]. Non-classical ISGs are hypothesized to influence proliferation, migration, attachment, and adhesion of the trophectoderm to support elongation of the conceptus [169]. The gene, *LGALS3BP*, has been previously reported to be abundant in both pregnant and cyclic d16 uterine luminal fluid and endometrium of heifers in comparison to d13 [138, 168, 178]. Galectin-9 was upregulated in epithelial and fibroblast cells in response to IFNT treatment, while *LGALS3BP* was found to only be up-regulated in epithelial cells (Table 3.2). Both *LGALS3BP* and *LGALS9* are also reported as being stimulated by a conceptus and IFNT in bovine endometrial explants [143]. During implantation, *LGALS9* has been suggested to play an immunomodulatory role through the regulation of endometrial leukocytes in humans [151]. The recently classified non-classical ISG, *LGALS9* has been suggested to play an immunomodulatory role through the regulation of endometrial leukocytes during human implantation [151]. Through interaction with *LGALS3*, *LGALS3BP* forms cell aggregates and is involved in cell adhesion [179].

In the ovine model, *IRF2* acts to suppress the transcriptional effects of IFNT on luminal epithelial cells, thereby inhibiting the up-regulation of many endometrial classical ISGs [45].

Classical ISGs contain at least a single IFN-stimulated response element (ISRE) in their promoter of which *IRF2* acts to inhibit transcriptional activity [169]. Induced by IFNT, IRF1 is hypothesized to increased classical ISG expression in the stroma. Both *IRF1* and *IRF2* were up-regulated in the epithelial cells in response to IFNT (logFC 0.76 and 0.66, respectively; Table 3.2).

Members of the family of proteins known as SLC transporters are important during conceptus elongation in ruminants as they transport endometrial amino acids into the uterine lumen for nourishment [25, 26]. It is believed that the effects of IFNT and P4 trigger an increase in amino acids to be transported to the uterine lumen [180]. Solute carrier family 14 member 3 (*SLC15A3*) is an amino acid transporter that transports lysosome histidine and peptides and was found to be elevated in bovine gestational d18 endometrial tissue [165]. Groebner et al. (2011) found mRNA expression of *SLC15A3* increased in response to recombinant bovine IFNT treatment to cocultured bovine glandular epithelial and stromal cell. The stromal cells exhibited a significant 177-fold increase of *SLC15A3* while glandular epithelial cells had a 36-fold increase [181]. Data from the current study show the up-regulation of *SLC15A3* in the epithelial and fibroblast cells due to IFNT (logFC 6.29 and 7.06, respectively; Table 3.2). Previously, *SLC15A3* was identified to be IFNT-dependent along with other SLC transporters including the mitochondrial transporter of an unknown solute, solute carrier family 25 member 30 (*SLC25A30*), and the mitochondrial thiamine pyrophosphate transporters, solute carrier family 25 member 28 (*SLC25A28*) and solute carrier family 25 member 19 (*SLC25A19*) [143]. The transporters *SLC25A30*, *SLC25A28*, and *SLC25A19* were all up-regulated by IFNT in 3D cultured epithelial cells in the current study (logFC 1.34, 2.54, and 1.27, respectively; Table 3.2).

Despite the apical IFNT treatment to the 3D culture of bovine endometrial epithelial and fibroblast cells, IFNT affected fibroblast gene expression as exhibited by the up-regulation of the gene *ZC3HAV1* in the fibroblast cells alone. The gene, *ZC3HAV1*, is an ortholog of zinc-finger antiviral protein (*ZAP*) which degrades viral RNA through direct binding [182]. Zinc finger CCCH-type antiviral protein 1 has antiviral properties as the CCCH zinc-finger motif binds viral RNA directly [183, 184]. Ozawa et al. (2012) identified *ZC3HAV1* as a gene that is up-regulated in the inner cell mass of d8 *in vitro* produced blastocysts in comparison to the trophectoderm [185]. The role of IFNT induced *ZC3HAV1* within bovine endometrial stromal cell is unknown.

Forde et al. (2015) found *LGALS3* mRNA was expressed by both the d16 endometrium and conceptus but was more abundant in the d16 conceptus [178]. Therefore, we attempted to test the function of *LGALS3* in the bovine endometrium using a 3D cell culture system. Bovine endometrial epithelial and fibroblast cells in the 3D culture were treated with 100 and 1000 ng of recombinant mouse *LGALS3* protein. Compared to Control epithelial cells, epithelial cells treated with *LGALS3* had greater expression of a single gene, *RPL23A* (logFC 4.05). However, *RPL23A* gene expression differences were unable to be detected when assayed by using RT-qPCR (Table 3.6). Six pseudogenes for *RPL23A* are present in the bovine genome, therefore, it is possible we did not amplify the correct transcript by RT-qPCR. The cells that received the lower concentration of *LGALS3* (100 ng) were analyzed by RNA Seq, we investigated the gene expression all samples to examine if the higher dose of *LGALS3* (1000 ng) resulted in significant gene expression differences. Of the genes examined, we were unable to detect differences between either *LGALS3* treatment (100 and 1000 ng) and the Control-treated epithelial and fibroblast cells. A previous study successfully utilized recombinant *LGALS3* protein from humans and an unidentified mammal *in vitro* to elicit a response on cultured bovine

luteal cells [118]. However, we conclude that recombinant mouse LGALS3 protein did not elicit a cellular response in the bovine cells. Further studies utilizing recombinant bovine LGALS3 may conclude a role for LGALS3 during early pregnancy during cattle. Recombinant bovine LGALS3 was not available to test the responsiveness of the endometrium cells in experiment two, therefore, we treated the cells with uterine FF collected from d14 pregnant sheep. Theoretically, the ovine FF contained IFNT produced by the conceptus. The endometrial cells treated with the ovine FF did increase the expression of IFNT stimulated genes (*ISG15* and *LGALS3BP*; Table 3.6) indicating the cells were responsive. Interestingly, *CXCR4* and *LIF*, genes are known to be important for implantation in mammals were also increased in the endometrial cells in response to pregnant ovine uterine FF (Table 3.6). The role *LGALS3* plays in the endometrium and early ruminant embryo requires further investigation.

CONCLUSIONS

Findings from this study support the utilization of 3D culture of bovine endometrial cells to study early conceptus-maternal crosstalk and identify endometrial cell type-specific responses to the conceptus. We identified 154 IFNT-responsive genes specifically in the endometrial epithelial cells that were previously found to be upregulated in total endometrium treated with IFNT and d15 elongating bovine conceptuses [143]. A single gene, *ZC3HAV1*, also found to be upregulated in total bovine endometrium treated with IFNT and d15 bovine conceptuses, was upregulated in the fibroblast but not the epithelial cells. Sixty-nine genes were differentially expressed in both epithelial and fibroblast cells. Expression of specific IFNT responsive genes within specific tissue compartments of the endometrium may have important functions during

maternal recognition of pregnancy and placentation in ruminants. The role of IFNT responsive genes and uncharacterized conceptus secretory proteins, such as galectins, needs further investigation.

Table 3.1. GenBank accession number, gene name, primer direction, primer sequence, product size, percent amplification efficiency, and source of primer of cDNAs amplified during real-time quantitative polymerase chain reaction (RT-qPCR).

GenBank Acc. Number	Gene	Primer	Primer Sequence (5'-3')	Product Size (bp)	Amp. E. (%)	Source
NM_178320	<i>PPIA</i>	Forward	CATACAGGTCCTGGCATCTTGTC	108	--	[153]
		Reverse	CACGTGCTTGCCATCCAACC			
NM_174178	<i>SDHA</i>	Forward	ACTTCACCGTTGATGGCAA	59	--	[153]
		Reverse	GCAGAAATCGCATCTGAAA			
NM_001040516.1	<i>RPL19</i>	Forward	GAAAGGCAGGCATATGGGTA	86	--	[153]
		Reverse	TCATCCTCCTCATCCAGGTT			
NM_174814.2	<i>YWHAZ</i>	Forward	ACCTACTCCGGACACAGAACATC	65	--	[143]
		Reverse	CCAGTTTGGCCTTCTGTACCA			
NM_001077953.1	<i>RNF11</i>	Forward	TCCGGGAGTGTGTGATCTGTATGAT	131	--	[143]
		Reverse	GCAGGAGGGGCACGTGAAGG			
NM_001034034.2	<i>GAPDH</i>	Forward	TTCTACTGGCGCTGCCAAGG	107	--	[143]
		Reverse	GATCCACAACAGACACGTTGGG			
NM_173979.3	<i>ACTB</i>	Forward	CAGCAGATGTGGATCAGCAAGC	91	--	[143]
		Reverse	AACGCAGCTAACAGTCCGCC			
NM_001014389.2	<i>H3F3A</i>	Forward	CATGGCTCGTACAAAGCAGA	136	--	[143]
		Reverse	ACCAGGCCTGTAAACGATGAG			
NM_001029846.1	<i>OAS1</i>	Forward	CCCGGCGGACCTACAGGAA	84	95	[168]
		Reverse	TCCAGCCAGACCAAAGCCGC			
XM_010801645.3	<i>MX1</i>	Forward	CGAGCCGAGTCTCCAAATG	114	96	[153]
		Reverse	CAACTCTTGCCACGATACC			
NM_001077900.1	<i>STAT1</i>	Forward	GCATTAGTCAGGGCCAAATTGTTACAG	139	96	[153]
		Reverse	GCCAGATACAGGAAGCTTGCAC			
NM_173941.2	<i>MX2</i>	Forward	GGGCAGCGGAATCATCACCCG	102	93	[143]
		Reverse	AGCTGCTGCGTAATGTTGCGGTA			
NM_001015570.3	<i>LGALS9</i>	Forward	TCAGCTTCCAGCCTCCAGGG	86	97	[153]
		Reverse	TCCAGGGGCGCTGTGTATGGT			
NM_001046316.2	<i>LGALS3BP</i>	Forward	CAACTGCAGACACGACAAGG	88	95	[153]
		Reverse	AGGGATTTCCGCCAGATAGGT			
NM_174301.3	<i>CXCR4</i>	Forward	AAGGCTCAGAAGCGCAAG	102	102	[186]
		Reverse	GAGTCGATGCTGATCCCAAT			
NM_174366.1	<i>ISG15</i>	Forward	CCAACCAGTGCTGCAGAGA	76	97	[143]
		Reverse	CCCTAGCATCTTCACCGTCA			
NM_001024557.1	<i>OAS2</i>	Forward	TGGATAACACCTTGCTGGCTG	82	93	This Study
		Reverse	GGTCCAGGTGACTCGTTCTG			
NM_001101866.2	<i>IDO1</i>	Forward	CACCCCAAAGAAGTTTGCCG	80	99	This Study
		Reverse	GCTGAATGCCAGGAGAACA			
NM_001114506.1	<i>TNFSF13B</i>	Forward	GGGACGAAGTGAAGTCTGGTG	147	96	This Study
		Reverse	TCTTAGCATCTTCCCGGGGT			
XM_005216258.4	<i>MAP6</i>	Forward	ATAAGCCAACCCAGCTGAC	82	110	This Study
		Reverse	GGAGGTTCCCTTGAAGGGCTC			
NM_001192561.2	<i>BATF2</i>	Forward	ATCTCCTGCACAGCTGTCAC	132	106	This Study
		Reverse	AGGAACTCTAGAGGGGCAGG			
XM_003586006.5	<i>ZC3HAV1</i>	Forward	AGGCCTTTTGTGACCCCAAA	119	97	This Study
		Reverse	TGTGACGGATGAAGGTGTGG			
XM_003587616.5	<i>PRRT3</i>	Forward	GAGGCCTGGGAAGTCAGTTC	98	97	This Study
		Reverse	GATGGGAGATCTGACGGCTG			
NM_001192640.1	<i>DMRTA1</i>	Forward	CACGGCATTAACTTAGCGCC	115	92	This Study
		Reverse	TGACACAAATTCGCGCATGA			
NM_001045958.2	<i>RPL23A</i>	Forward	ATGAAGATGGCGCCGAAG	110	88	This Study
		Reverse	TGGCTGTGGACACCTTTCAA			
XM_015475541.2	<i>LIF</i>	Forward	GGGACAACCTAACAGCAGTG	91	96	This Study
		Reverse	GCACAGCTTGTCAGGTTG			

Figure 3.1. The 3D cell culture system used to coculture bovine endometrial epithelial and fibroblast cells. Epithelial cells were seeded on a transwell insert coated with Matrigel (Corning Inc.) and fibroblast cells were located below inserts. The oIFNT or LGALS3 was administered apically.

Figure 3.2. A Venn diagram (Venny 2.1 BioinfoGP) categorizing differentially expressed genes (DEGs) in response to oIFNT among bovine endometrial intercaruncular explants and bovine endometrial epithelial fibroblast cells cultured in a 3D system. Explants, treated with oIFNT and d15 bovine conceptus [143], shared 224 transcripts with endometrial epithelial and fibroblast cells. Of those DEGs, 154 were specific to the epithelium while a single transcript, *ZC3HAV1*, was exclusive to the fibroblast cells cultured in the 3D system. Explants treated with a conceptus or oIFNT shared 69 transcripts that were differentially expressed with both epithelial and fibroblast cells treated with oIFNT.

Table 3.2. Genes up- or down-regulated in the Venn diagram (Figure 2.1).

I					II			III	IV
Up	GTF2B	BCL2L14	RNF31	MITD1	Up	EIF2AK2	DHX58	Up	Up
GBP2	STAT2	PSMB8	PAPD7	TLR3	MX2	SP100	CASP7	ZC3HAV1	OAS1Z
GBP6	IRF8	PSMF1	SLC25A19	CASP8	IFIT3	IRF4	IFI27		LOC112441507
SLFN11	HSH2D	LEKR1	CCDC82	FBXO33	RSAD2	DTX3L			OAS1Y
IDO1	OPTN	NAMPT	IL15RA	RICTOR	IFIT1	PML			CCDC194
IFITM3	DAXX	IRF3	TRIM26	ABHD1	IFI44	TRIM5			SP140L
ACKR4	ATXN3	TAP2	CCDC6	RNF114	IFIT2	IRF9			LOC107132617
TNFSF13B	GNB4	CMTR1	TRIM16	SHISA5	ISG15	IRF7			CGAS
MLKL	RBCK1	PAPD4	PPP2R3C	PLEKHA7	GBP4	TIFA			C7H19orf66
MASTL	NMI	SCLY	HEATR5B	CDK18	IFI44L	XAF1			LOC112446427
SP110	PLSCR2	NT5C3A	IRF2	PDCD4	SAMD9	PARP12			TMPRSS2
KIF5C	GDAP2	CDKN2AIP	FAM111B	POLH	OAS2	IFIT5			
IFITM1	MOV10	RAB8B	JADE2	TRIM38	BST2	PARP10			
WARS	STAT1	ZNF607	DUSP11	C2	UBA7	PNPT1			
FOXS1	ATAD1	OGFR	YTHDC2	ARSH	USP18	SLC15A3			
ISG20	LGALS3BP	PSME2	TMEM268	XRN2	ZBP1	FAM3B			
GNGT2	AKAP7	ANKFY1	CHMP5	ELMO2	OAS1X	IFI16			
SLFN12	USP25	PXK	PLSCR4	IRF1	MX1	SP140			
APOL3	ANXA1	PSMA2	CTC1	RAB37	GBP1	TRANK1			
TMEM140	SLC25A28	LAP3	XPO1	TMEM106A	CMPK2	ADAR			
CDADC1	PARM1	DNAJC1	RBMS2	TREX1	RTP4	PLEKHA4			
CD274	NCOA7	SLC25A30	NFE2L3	DDX19B	RNF213	TRIM34			
GHSR	VCPIP1	SERTAD1	MAT2B		BATF2	CMTR2			
HES4	TRIM56	ERAP1	TAPBP		EPSTI1	TDRD7			
CASP4	GRINA	RIPK3	PRICKLE3		DDX58	LGALS9			
BCL2L15	SPATS2L	STK38L	NUB1		PARP14	PARP9			
DRAM1	ATP10A	TAPBP	DNPEP		TNFSF10	IFI6			
IFI35	NLRC5	CUL4B	KIFC3		HERC6	RBM43			
FAM212A	CNP	NR3C1	CD47		IFIH1	UBE2L6			
BOLA-DMB	CBLN3	RNF19B	RNF139		GBP5	RNASEL			
SASS6	ATP8B4	AIDA	B2M		HERC5	TRIM21			
BPNT1	MAD2L2	RBBP6	KAT2A		PLAC8	N4BP1			
MORC3	PSMB9		AZI2		ZNFX1	TAP1			
	BCL2L12		MCHR1			TRIM25			

V					VI			
Up	REGAKINE-1	FAM172B	TOR1AIP2	Down	Up	PCK1	LOC512672	SLC13A5
CLEC4F	TMEM74B	C4A	FYTTD1	KIT	CNGB1	SYT16	LOC101903126	ANKRD53
CCL8	SLC2A6	IP6K1	IFRG15	CHST15	SAA4	LOC107132327	LOC101905257	CCDC190
CXCL11	BL37L	STOML1	CFLAR	FAM101B	LYZ3	KIF26A	STAT4	LOC530929
CXCL10	C3AR1	FAS	PAPD5	PECAM1	PRM1	ABCC11	B3GNT3	LOC101902154
TIMD4	PTPRO	GSDMD	VPS13C	CD93	SOX10	ZC3H12D	LY6L	CPLX1
APOBEC3Z1	C21ORF91	TLE4	JAK2	AFAP1L1	LOC781494	MYAML2	TSPAN32	LOC112444847
OAS1	PDCD1	DYSF	TCEAL1	FAM124B	JAKMIP3	LOC100141258	ATP8A2	ISG12(B)
GBP7	CD53	DYNC1H1	USF1	APLNR	NUGGC	IL3RA	CLEC2D11	BST1
GVINP1	CLEC2D	RAPGEF3	MIA3	SOX18	MUC12	ANKRD35	GOLCA6L22	TACR1
CXCL9	POLD3	PARP8	USP34	PALD1	LOC511617	LUZP2	ERBB4	PLA2R1
IFI47	TFEC	SYNE2	SOCS2	VASH1	OASL	UPB1	PSCA	OVCH1
MYADML	C19ORF66	PLPP3	ABCB7	JAG1	BIRC7	HS3ST1	C5H22orf23	LOC512323
MB21D1	ABCG2	LIFR	DNAJC13		LOC787234	SLC9C1	THEM6	CSPG5
SECTM1	STARD8	RIPK2	GAB1		ADGRG7	C1R	CHST4	TAF4B
TNFSF13BL	MICB	KYAT3	BID		IFNL3	LRIT3	TRIM14	IL34
ISG12B	C3ORF38	ARHGEF3	MXD1		LOC512869	PLA2G7	LOC101905194	LOC782527
LOR	HEG1	SCARF1	TTC9C		BVES	CAPN8	LOC104970178	WDR17
CYP2J2	IL23A	ZC3H12C	PHF11		TMPRSS5	LOC112445995	TNFRSF9	SOCS1
TCF15	HORMAD1	ZCCHC6	UNC93B1		TNFSF4	BREH1	LOC112448863	SCN9A
KYNU	IL15	AIFM2	LDB2		SBK3	SLC23A2	DSC1	ADAM19
BL37	NEURL3	PSME1	PSMA8		ASB5	TAGAP	LOC515697	CYP2S1
P2RY6	PTPN11	ZNF628	PLA2G16		CNDP1	ARHGAP9	LPL	JPH2
CLEC10A	SIGLEC1	TGM2	CXORF21		FRAS1	CDHR4	LOC509972	RAB19
CDHR5	CD40	PDE5A	UNC45B		ACVR1C	ABCB5	LOC112446668	KRT42
RUBCN	PHACTR1	MTO1	ART3		SLC02B1	IFITM2	SCNN1B	MYO1A
REC8	ACOT9	MRPL32	C1ORF115		SLC22A10	CLDN16	GMNC	VWA5A
C1ORF109	TAL1	ECM1	NCF1		OXT	SULT6B1	LOC616948	FAM71A
ZNF366	FRMD4B	KIAA1033	IFI27L		ODF3L1	TGM7		ARID3C
BL36	ACSL5	PPFIBP1	ALS2		DPY19L2	C5		IQCD
TLR4	TSKU	SSAT-1	MAN1C1		LOC512440	MESP2		GANC
EHD3	ADGRF5	FUNDC1	C2ORF68		ELMOD1	CD34		COX7A1
LPCAT2	DECR2	PPA1			TRIM15	PDCD1LG2		RIPOR3
AGRN	CTSS	SLK			TRPC3	RPE65		
					GPR63	LOC521656		

VI Cont.

Up	DRAM2	DENND2D	LOC518495	ACOX3	HFE	FPGT	SPPL2A	NBEAL2
MASP1	FLT3LG	GRAMD1C	A2ML1	FBXO7	RNF39	WDR18	PDK3	SRD5A1
SIX1	IL18	ALDH9A1	TRAPPC2	ARL6IP5	DBNL	F2R	ZCCHC8	VPS33A
CCDC39	FAM172BP	CROT	LYPD6B	SARS	MTPAP	TMED8	SLC35C1	MLEC
SCNN1G	NKX3-1	CSRNP1	PRKAR1B	RFX5	DNMT1	SCP2	NARS	MEGF9
ZCCHC2	LY6E	TACSTD2	ZDHHC14	TAF5	TPRKB	TCOF1	KAT2B	NAV2
PMAIP1	BOLA-NC1	CLDN1	TICRR	APRT	EIF2AK1	MIER3	DESI1	UNC5B
TM6SF2	SFN	CLSTN2	TRMT13	STXBP3	SPTY2D1	MRPL44	HAUS3	TUBB3
EPHX2	TMEM171	FAM192A	CPOX	ZNF197	GALM	RNF135	PRKAB1	SAMD1
FBXO16	FUT10	YARS	ITPR2	SERINC2	SLC1A5	DENND4C	FGD4	BCAT1
PGR	PLA2G3	FAM129A	WIPF3	PSMB10	COA7	OSBPL8	ARHGEF16	ARVCF
INPP5F	MISP	AKAP6	GIPC2	PRKD2	TMEM255B	SNX6	ZMYM1	PPARA
IQUB	CALCOCO2	ZNF852	DCK	S1PR2	MTFP1	CENPO	CNOT9	PIAS2
NFIX	ERAP2	LIPA	SLC6A12	DDX24	PRMT9	SEC24B	UHRF2	MTFR1
ORC5	CCDC102A	MTR	ATP7A	GCHFR	TTC4	PRPSAP1	DNAJC8	TUBA1A
TCTN3	CSDC2	SAT1	TMEM41A	C16H1orf112	FYB2	NISCH	KCTD9	PTCH1
EHD4	ZUP1	TATDN3	GABARAPL1	ELF1	TSPYL1	SSBP3	PELI1	EML1
OVOL1	ARL6IP1	BSPRY	SLC25A12	RRM2B	PALM	ANAPC11	MCL1	AKAP12
CATSPERE	NEB	ALPK1	CAMK2D	HNF4G	SARNP	STRIP1	FAM168B	LMCD1
CIITA	LOC508153	NOSTRIN	APOBEC3H	TWISTNB	STARD3	FAM110C	HNRNP1L	ETV4
RAD21L1	CTSL	XRCC5	OSMR	ID4	DNAJC7	JDP2	KRT7	ALDH1A3
BTC	WDR86	C1H3orf38	LNPEP	LOC101905041	TXNRD1	PJA2	TRIM4	FITM2
LOC515736	VAMP5	SIDT2	TMEM144	B4GALT1	RCBTB1	MARCKSL1		SLC25A15
GDA	SAMHD1	CSRNP2	FAM76A	EMP1	SEPT7	CBLL1	Down	KLF13
DUSP15	C16H1orf115	LENG1	MOCS1	ACSS2	GYG1	STAMPB	DESI2	SCRN1
PLIN2	AP5B1	TCAF2	UBIAD1	PCGF5	TMEM230	KAT5	MBTPS1	FAM92A
ZFP36	ICAM1	RBMX2	ACP2	ADM2	NECTIN2	ZNF710	GNPAT	GVQW3
IL22RA1	ICAM2	MST1R	AP1AR	SNX4	AVEN	HGH1	GTF2A1	PCDHA5
FBXO45	INA	MUC1	EVA1A	VPS33B	BIRC3	CEPT	ZDHHC23	ZMYND10
LOC618733	CHST1	SLC27A1	C11H2orf68	POLR2K	MOCOS	GTPBP2	PSD4	HIST1H3A
ICA1L	TACR3	UTP23	PPAT	CGN	GPHN	TIPARP	PKD1	MAP6
HSPB11	FOXQ1	HK2	BTBD11	HKDC1	PLEKHO1	CSTF1	SESN3	
APOBEC1	GABRP	SEMA4A	BOLA	ADPGK	SCO1	ACADM	ENTR1	
DDIT3	THEM4	FCHSD2	HACD2	DNAJA1	APOPT1	MTMR4	NETO2	

Table 3.3. The logFC of the top 20 genes up-regulated in bovine endometrial epithelial cells and shared with explants treated with oIFNT and a d15 conceptus [143] (I and II of Figure 2.2). The fibroblast cell logFC is listed if the gene was also up-regulated in fibroblast cells.

GenBank Acc. Number	Gene	Epithelial Cell LogFC	Fibroblast Cell LogFC
XM_005227105.4	<i>BATF2</i>	11.88	2.80
XM_015473641.2	<i>MX2</i>	10.26	7.83
XM_002686295.6	<i>IFI44</i>	9.95	7.63
XM_015460598.2	<i>IFIT1</i>	9.65	3.56
NM_001024557.1	<i>OAS2</i>	9.51	9.63
XM_024993137.1	<i>PLAC8</i>	8.96	7.75
NM_001076127.2	<i>KIF5C</i>	8.66	--
XM_015474249.2	<i>ZBP1</i>	8.47	8.06
NM_001045941.1	<i>RSAD2</i>	8.46	8.35
NM_001075414.1	<i>IFIT3</i>	8.15	3.36
NM_174366.1	<i>ISG15</i>	7.94	6.90
XM_003586247.5	<i>HSH2D</i>	7.86	--
XM_024981673.1	<i>IFI27</i>	7.85	5.03
XM_024998051.1	<i>ATP8B4</i>	7.52	--
XM_015471926.2	<i>BST2</i>	7.25	6.47
NM_001101866.2	<i>IDO1</i>	7.18	--
NM_001099716.1	<i>FOXS1</i>	7.17	--
XM_003585891.5	<i>IFI44L</i>	7.09	8.66
NM_001206162.1	<i>IRF4</i>	6.97	5.73
NM_178108.2	<i>OAS1X</i>	6.78	7.35

Table 3.4. Gene ontology (GO) Biological Process Direct for 223 genes up-regulated in bovine endometrial epithelial cells treated with 100 ng of oIFNT in 3D culture (I and II of Figure 2.2).

Term	P-Value	Genes
GO Biological Process Direct		
Type I interferon signaling pathway	< 0.001	RNASEL, IFITM1, IFITM3, RSAD2, OAS2, IFI35, ISG20, ISG15, XAF1, MX1, MX2, SP100, BST2, STAT1, PSMB8, STAT2, IFIT3, IRF9, IFIT2, IFIT1, IFI27, IRF7, IRF8, IRF1, IRF2, IRF3, IRF4, GBP2, IFI6, ADAR
Defense response to virus	< 0.001	RNASEL, IFITM1, IFITM3, PML, TLR3, RSAD2, IFI44L, OAS2, ISG20, TRIM5, NLRC5, ISG15, MX1, MX2, DHX58, BST2, HERC5, TRIM25, IFI16, STAT1, STAT2, IRF9, IFIT3, TRIM56, IFIT2, IFIT1, TRIM34, IFIT5, IRF1, IRF3, SLFN11, EIF2AK2, GBP1, ADAR
Interferon-gamma-mediated signaling pathway	< 0.001	SP100, NMI, PML, TRIM26, TRIM25, OAS2, STAT1, TRIM21, B2M, IRF9, TRIM38, TRIM5, TRIM34, IRF7, IRF8, IRF1, IRF2, IRF3, IRF4, GBP2, GBP1
Response to virus	< 0.001	IFIH1, BST2, IFITM1, IFITM3, RSAD2, IFI44, OAS2, ISG20, DDX58, PSMA2, IFIT3, IFIT2, IFIT1, IRF7, EIF2AK2, MX1, MX2, DHX58, ADAR
Negative regulation of viral genome replication	< 0.001	RNASEL, IFITM1, BST2, IFITM3, RSAD2, PARP10, IFI16, ISG20, IFIT1, ISG15, EIF2AK2, MX1, ADAR
Negative regulation of type I interferon production	< 0.001	DDX58, NLRC5, IFIH1, ISG15, HERC5, UBA7, UBE2L6, TRIM25, IRF3, DHX58
Innate immune response	< 0.001	IFIH1, BST2, ANXA1, PML, HERC5, TLR3, TRIM26, TRIM25, IFI16, TRIM21, B2M, DDX58, NLRC5, TRIM5, CASP4, IFIT5, IRF7, C2, EIF2AK2, MX1, MX2, DHX58, ADAR, ZBP1
Protein polyubiquitination	< 0.001	DTX3L, HERC6, HERC5, RBBP6, RNF213, TRIM21, PSMB8, PSMB9, PSMA2, PSMF1, RNF114, PSME2, RNF139, RBCK1, RNF19B, RNF31
Response to interferon-alpha	< 0.001	BST2, IFITM1, IFITM3, EIF2AK2, MX2, ADAR
Antigen processing and presentation of endogenous peptide antigen via MHC class I	< 0.001	TAP2, TAP1, ERAP1, TAPBP, B2M
Response to interferon-beta	< 0.001	BST2, IFITM1, IFITM3, XAF1, STAT1
Positive regulation of I-kappa b kinase/NF-kappa b signaling	< 0.001	TRIM5, APOL3, TRIM38, TNFSF10, BST2, CASP8, SHISA5, RBCK1, TRIM25, IRF3, LGALS9, RNF31
Response to interferon-gamma	< 0.001	SP100, BST2, IFITM1, NUB1, IFITM3, TRIM21
Positive regulation of interferon-beta production	< 0.001	DDX58, IFIH1, IRF7, IRF1, TLR3, IRF3
Negative regulation of viral release from host cell	< 0.001	TRIM5, PML, TRIM26, TRIM25, TRIM21
ISG15-protein conjugation	< 0.001 < 0.001	ISG15, HERC5, UBA7, UBE2L6 TRIM5, IFITM1, IFITM3, TRIM26, TRIM25

Negative regulation of viral entry into host cell		
Response to type I interferon	< 0.001	TRIM56, SP100, ISG15, MX1
Apoptotic process	< 0.001	BCL2L15, BCL2L14, FAM3B, PML, NR3C1, STAT1, BCL2L12, DAXX, PDCD4, ELMO2, TNFSF10, CASP4, CASP7, CASP8, IRF1, IRF3, XAF1, MX1, DRAM1
Positive regulation of NF-kappa b transcription factor activity	< 0.001	TRIM5, TRIM38, RIPK3, RBCK1, TLR3, TRIM25, EIF2AK2, LGALS9, RNF31
Positive regulation of sequence-specific DNA binding transcription factor activity	< 0.001	DDX58, TRIM5, TRIM38, SP100, TRIM34, TRIM26, TRIM25, TRIM21
Positive regulation of interferon-alpha production	< 0.001	DDX58, IFIH1, IRF7, IRF3
Positive regulation of type I interferon production	< 0.001	IRF7, IRF1, IRF3, IFI16, DHX58, ZBP1
MDA-5 signaling pathway	< 0.001	IFIH1, IRF7, IRF3
Antigen processing and presentation of peptide antigen via MHC class I	< 0.001	TAP2, TAP1, ERAP1, TAPBP, B2M
Regulation of type I interferon production	< 0.001	TRIM56, IRF7, TREX1, TRIM21
I-kappa b kinase/NF-kappa b signaling	0.001	RIPK3, RBCK1, TIFA, TLR3, AZI2, RNF31
Proteasome-mediated ubiquitin-dependent protein catabolic process	0.001	PSMA2, PSMF1, TRIM38, ATXN3, PSME2, RNF139, PML, RBCK1, PSMB8, PSMB9
Regulation of activated T cell proliferation	0.001	CD274, RIPK3, IDO1
Cellular response to interferon-beta	0.001	PNPT1, IRF1, TLR3, STAT1
Translesion synthesis	0.001	POLH, ISG15, UBA7, UBE2L6, TRIM25
Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	0.001	PSMA2, PSMF1, PSME2, PSMB8, PSMB9, B2M
NIK/NF-kappab signaling	0.001 0.001	PSMA2, PSMF1, PSME2, RIPK3, PSMB8, PSMB9 ISG15, UBA7, UBE2L6

Modification-dependent protein catabolic process		
Detection of virus	0.001	DDX58, IFIH1, TLR3
Intracellular transport of viral protein in host cell	0.001	IFIT1, TAP2, TAP1
Regulation of mRNA stability	0.001	PSMA2, PSMF1, XPO1, RNASEL, PSME2, PSMB8, PSMB9
RNA catabolic process	0.002	PNPT1, OAS2, XRN2, ISG20
Regulation of adaptive immune response	0.002	IRF7, RIPK3, IRF1
Positive regulation of viral entry into host cell	0.003	TRIM38, LGALS9, TRIM21
Antigen processing and presentation of exogenous protein antigen via MHC class Ib, TAP-dependent	0.003	TAP2, TAP1, B2M
Tumor necrosis factor-mediated signaling pathway	0.003	PSMA2, PSMF1, TNFSF13B, PSME2, STAT1, PSMB8, PSMB9
Regulation of cellular amino acid metabolic process	0.003	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Response to cytokine	0.003	IFI27, SP100, CD274, PML, STAT1
Positive regulation of extrinsic apoptotic signaling pathway	0.004	TNFSF10, BCL2L14, PML, RBCK1
TRIF-dependent toll-like receptor signaling pathway	0.004	IRF7, CASP8, TLR3, IRF3
Protein trimerization	0.005	TRIM5, TRIM34, TRIM21
Positive regulation of type I interferon-mediated signaling pathway	0.005	NLRC5, IRF7, IRF3
RNA processing	0.006	PNPT1, RBMS2, YTHDC2, DUSP11, XRN2, ADAR
Ubiquitin-dependent protein catabolic process	0.006	PSMA2, PSMF1, ATXN3, USP18, NUB1, CUL4B, USP25, RNF213
JAK-STAT cascade	0.006	NMI, IL15RA, STAT1, STAT2
Negative regulation of innate immune response	0.007	NMI, IFI16, DHX58
T cell receptor signaling pathway	0.009	PSMA2, PSMF1, PSME2, RBCK1, PSMB8, PSMB9, RNF31

Viral process	0.010	KAT2A, XPO1, SP100, MORC3, RBCK1, SP110, DAXX, GTF2B, PSMB8, PSMB9
Regulation of innate immune response	0.010	IRF1, ERAP1, DHX58
Negative regulation of viral transcription	0.010	SP100, IFITM3, TRIM21
Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	0.010	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Immune response	0.013	GBP6, TNFSF10, TNFSF13B, IFITM3, CD274, IRF8, ACKR4, OAS2, GBP2, IFI6, TAPBP, B2M
Positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	0.013	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Histone H3 acetylation	0.015	KAT2A, JADE2, TRIM16, IRF4
Anaphase-promoting complex-dependent catabolic process	0.015	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Regulation of extrinsic apoptotic signaling pathway via death domain receptors	0.017	TNFSF10, SP100, CASP8
T cell activation	0.019	CASP8, IRF4, AZI2, HSH2D
Necroptotic process	0.019	RIPK3, TLR3, MLKL
Proteolysis involved in cellular protein catabolic process	0.020	PSMA2, CASP8, PSMB8, PSMB9
Defense response to protozoan	0.021	BATF2, IRF8, IRF4
Regulation of myd88-dependent toll-like receptor signaling pathway	0.024	IRF7, IRF1
Cytosol to ER transport	0.024	TAP2, TAP1
Organelle fission	0.024	MX1, MX2
Interferon-alpha production	0.024	IRF7, AZI2
Regulation of type III interferon production	0.024 0.024	DDX58, IFIH1 XRN2, ISG20

DNA catabolic process, exonucleolytic		
Wnt signaling pathway, planar cell polarity pathway	0.025	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Positive regulation of NF- kappa b import into nucleus	0.026	RBCK1, TLR3, LGALS9
Cellular response to interferon-gamma	0.031	GBP6, GBP5, TLR3, LGALS9
Myeloid cell differentiation	0.033	IRF8, PML, IFI16
Protein K48-linked deubiquitination	0.033	ATXN3, USP25, VCPIP1
Regulation of interferon- gamma production	0.035	ISG15, RIPK3
Cytoplasmic pattern recognition receptor signaling pathway in response to virus	0.035	DDX58, IFIH1
Zonula adherens maintenance	0.035	PLEKHA7, KIFC3
Interferon-beta production	0.035	TRIM56, IRF7
Response to tumor necrosis factor	0.036	NUB1, CASP8, YTHDC2
Positive regulation of interleukin-12 production	0.036	IRF8, TLR3, IDO1
Protein ubiquitination involved in ubiquitin- dependent protein catabolic process	0.037	HERC6, HERC5, TRIM25, CUL4B, RNF19B, RBBP6
Stimulatory C-type lectin receptor signaling pathway	0.037	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Positive regulation of viral genome replication	0.038	TRIM38, IFIT1, ADAR
Regulation of type I interferon-mediated signaling pathway	0.038	USP18, STAT1, STAT2
Inflammatory response	0.039	APOL3, NMI, CASP4, SP100, GBP5, ANXA1, TLR3, IFI16, PXX, LGALS9
Cellular response to DNA damage stimulus	0.039 0.044	CDKN2AIP, IRF7, DTX3L, IRF3, MASTL, RBBP6, CTC1 PNPT1, XRN2, ISG20

RNA phosphodiester bond hydrolysis, exonucleolytic		
Double-strand break repair	0.045	PARP9, DTX3L, PAPD7, MAD2L2
Defense response	0.046	TAP1, MX1, MX2, SP140
Negative regulation of canonical Wnt signaling pathway	0.047	PSMA2, PSMF1, PSME2, MAD2L2, PSMB8, PSMB9
Protein linear polyubiquitination	0.047	RBCK1, RNF31
Positive regulation of interleukin-10 secretion	0.047	CD274, LGALS9
Type I interferon biosynthetic process	0.047	IRF7, IRF3
Regulation of tumor necrosis factor-mediated signaling pathway	0.050	CASP8, RBCK1, RNF3
KEGG Pathways		
Influenza A	< 0.001	XPO1, IFIH1, RNASEL, PML, TLR3, RSAD2, TRIM25, OAS2, STAT1, STAT2, DDX58, IRF9, TNFSF10, IRF7, IRF3, EIF2AK2, MX1, ADAR
Herpes simplex infection	< 0.001	RNASEL, IFIH1, SP100, PML, TLR3, OAS2, STAT1, DAXX, STAT2, DDX58, IRF9, IFIT1, IRF7, TAP2, CASP8, TAP1, IRF3, EIF2AK2
Hepatitis C	< 0.001	DDX58, IRF9, RNASEL, IFIT1, IRF7, IRF1, TLR3, IRF3, OAS2, EIF2AK2, STAT1, STAT2
Measles	< 0.001	DDX58, IRF9, IFIH1, TNFSF10, IRF7, IRF3, OAS2, EIF2AK2, MX1, STAT1, STAT2, ADAR
RIG-I-like receptor signaling pathway	< 0.001	DDX58, IFIH1, ISG15, IRF7, CASP8, TRIM25, IRF3, AZI2, DHX58
Cytosolic DNA-sensing pathway	< 0.001	DDX58, IRF7, RIPK3, TREX1, IRF3, ZBP1, ADAR
Hepatitis B	0.002	DDX58, IFIH1, IRF7, CASP8, TLR3, IRF3, STAT1, STAT2
Proteasome	0.002	PSMA2, PSMF1, PSME2, PSMB8, PSMB9
Viral carcinogenesis	0.010	KAT2A, IRF9, SP100, IRF7, CASP8, IRF3, EIF2AK2, GTF2B
Pertussis	0.012	CASP7, IRF8, IRF1, IRF3, C2
Antigen processing and presentation	0.012	PSME2, TAP2, TAP1, TAPBP, B2M
Toll-like receptor signaling pathway	0.036	IRF7, CASP8, TLR3, IRF3, STAT1

Table 3.5. Gene ontology (GO) Biological Process Direct for (70) genes up-regulated in bovine endometrial fibroblast cells treated with 100 ng of oIFNT in 3D culture (II and III of Figure 2.2.).

Term	P-Value	Genes
GO Biological Process Direct		
Defense response to virus	< 0.001	RNASEL, ZC3HAV1, BST2, PML, HERC5, RSAD2, TRIM25, IFI44L, OAS2, IFI16, IRF9, IFIT3, IFIT2, TRIM5, IFIT1, ISG15, TRIM34, IFIT5, EIF2AK2, MX1, MX2, DHX58, GBP1, ADAR
Type I interferon signaling pathway	< 0.001	RNASEL, SP100, BST2, RSAD2, OAS2, IRF9, IFIT3, IFIT2, IFIT1, IFI27, ISG15, IRF7, IRF4, XAF1, MX1, MX2, IFI6, ADAR
Response to virus	< 0.001	IFIH1, BST2, ZC3HAV1, RSAD2, IFI44, OAS2, DDX58, IFIT3, IFIT2, IFIT1, IRF7, EIF2AK2, MX1, MX2, DHX58, ADAR
Negative regulation of viral genome replication	< 0.001	RNASEL, IFIT1, ISG15, ZC3HAV1, BST2, RSAD2, IFI16, PARP10, EIF2AK2, MX1, ADAR
Interferon-gamma-mediated signaling pathway	< 0.001	IRF9, TRIM5, SP100, TRIM34, IRF7, PML, TRIM25, OAS2, IRF4, TRIM21, GBP1
Innate immune response	< 0.001	IFIH1, BST2, ZC3HAV1, PML, HERC5, TRIM25, IFI16, TRIM21, DDX58, TRIM5, IFIT5, IRF7, EIF2AK2, MX1, MX2, DHX58, ADAR, ZBP1
Negative regulation of type I interferon production	< 0.001	DDX58, IFIH1, ISG15, HERC5, UBA7, UBE2L6, TRIM25, DHX58
ISG15-protein conjugation	< 0.001	ISG15, HERC5, UBA7, UBE2L6
Response to interferon-alpha	< 0.001	BST2, EIF2AK2, MX2, ADAR
Positive regulation of interferon-alpha production	< 0.001	DDX58, IFIH1, ZC3HAV1, IRF7
Negative regulation of viral release from host cell	< 0.001	TRIM5, PML, TRIM25, TRIM21
Positive regulation of type I interferon production	< 0.001	ZC3HAV1, IRF7, IFI16, DHX58, ZBP1
Positive regulation of sequence-specific DNA binding transcription factor activity	< 0.001	DDX58, TRIM5, SP100, TRIM34, TRIM25, TRIM21
Positive regulation of interferon-beta production	< 0.001	DDX58, IFIH1, ZC3HAV1, IRF7
Modification-dependent protein catabolic process	< 0.001	ISG15, UBA7, UBE2L6

Response to type I interferon	< 0.001	SP100, ISG15, MX1
Positive regulation of I-kappa b kinase/NF-kappa b signaling	< 0.001	TRIM5, TNFSF10, ZC3HAV1, BST2, TRIM25, LGALS9
Translesion synthesis	< 0.001	ISG15, UBA7, UBE2L6, TRIM25
Protein trimerization	< 0.001	TRIM5, TRIM34, TRIM21
Defense response	0.002	TAP1, MX1, MX2, SP140
Response to interferon-gamma	0.003	SP100, BST2, TRIM21
Protein polyubiquitination	0.004	DTX3L, HERC6, HERC5, RNF213, TRIM21
Regulation of type III interferon production	0.007	DDX58, IFIH1
Organelle fission	0.007	MX1, MX2
Cytoplasmic pattern recognition receptor signaling pathway in response to virus	0.011	DDX58, IFIH1
MDA-5 signaling pathway	0.011	IFIH1, IRF7
Positive regulation of NF-kappa b transcription factor activity	0.013	TRIM5, TRIM25, EIF2AK2, LGALS9
Response to cytokine	0.015	IFI27, SP100, PML
Peptide transport	0.018	TAP1, SLC15A3
Detection of virus	0.018	DDX58, IFIH1
Intracellular transport of viral protein in host cell	0.018	IFIT1, TAP1
Positive regulation of viral entry into host cell	0.025	LGALS9, TRIM21
Positive regulation of RIG-I signaling pathway	0.029	ZC3HAV1, DHX58
Cellular response to interferon-alpha	0.032	IFIT3, IFIT2
Response to interferon-beta	0.032	BST2, XAF1
Activation of innate immune response	0.036	TRIM5, IFI16
Activation of cysteine-type endopeptidase activity involved in apoptotic process	0.037	IFI27, TNFSF10, PML
Negative regulation of innate immune response	0.039	IFI16, DHX58
Positive regulation of mRNA catabolic process	0.043	ZC3HAV1, PNPT1

Regulation of type I interferon production	0.046	IRF7, TRIM21
Cellular response to virus	0.046	LGALS9, ADAR
Cellular response to exogenous dsRNA	0.046	IFIT1, ZC3HAV1
Negative regulation of viral transcription	0.046	SP100, TRIM21
KEGG Pathways		
Influenza A	< 0.001	IFIH1, RNASEL, PML, RSAD2, TRIM25, OAS2, IRF9, DDX58, TNFSF10, IRF7, EIF2AK2, MX1, ADAR
Herpes simplex infection	< 0.001	DDX58, IRF9, RNASEL, IFIT1, IFIH1, SP100, IRF7, TAP1, PML, OAS2, EIF2AK2
Measles	< 0.001	DDX58, IRF9, IFIH1, TNFSF10, IRF7, OAS2, EIF2AK2, MX1, ADAR
RIG-I-like receptor signaling pathway	< 0.001	DDX58, IFIH1, ISG15, IRF7, TRIM25, DHX58
Hepatitis C	< 0.001	DDX58, IRF9, RNASEL, IFIT1, IRF7, OAS2, EIF2AK2
Cytosolic DNA-sensing pathway	0.002	DDX58, IRF7, ZBP1, ADAR
Viral carcinogenesis	0.037	IRF9, SP100, IRF7, EIF2AK2

Figure 3.3. Quantitative real-time PCR analysis of *MXI* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *MXI* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.4. Quantitative real-time PCR analysis of *MX2* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *MX2* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.5. Quantitative real-time PCR analysis of *OAS1* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 n ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *OAS1* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.6. Quantitative real-time PCR analysis of *LGALS3BP* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *LGALS3BP* was greater in epithelial and fibroblast cells treated with oIFNT (*** P < 0.001; ** P < 0.01; * P < 0.05).

Figure 3.7. Quantitative real-time PCR analysis of *LGALS9* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *LGALS9* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.8. Quantitative real-time PCR analysis of *OAS2* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *OAS2* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.9. Quantitative real-time PCR analysis of *BATF2* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *BATF2* was greater in epithelial and fibroblast cells treated with oIFNT (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Figure 3.10. Quantitative real-time PCR analysis of *IDO1* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Expression of *IDO1* was not significantly elevated in oIFNT treated epithelial and fibroblast cells ($P > 0.05$).

Figure 3.11. Quantitative real-time PCR analysis of *MAP6* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Expression of *MAP6* decreased in oIFNT treated epithelial cells, however, not statistically ($P > 0.05$). In the fibroblast cells, there was not a trend ($P > 0.05$).

Figure 3.12. Quantitative real-time PCR analysis of *TNFSF13B* gene expression in bovine (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. Relative expression of *TNFSF13B* was greater in epithelial and fibroblast cells treated with oIFNT ($***P < 0.001$; $**P < 0.01$; $*P < 0.05$).

Figure 3.13. Quantitative real-time PCR analysis of *ZC3HAV1* gene expression in bovine endometrial (A) epithelial and (B) fibroblast cells cultured in 3D treated with either treatment media only (Control; n=4) or 100 ng/mL of oIFNT (n=3). Relative expression data are normalized over the geometric mean of reference genes, *RNF11* and *SDHA*. Data are presented as LSM $\pm$ SEM. The treatment of endometrial cells with oIFNT significantly increased *ZC3HAV1* expression exclusively in the fibroblast cells in comparison to the control-treated cells. Expression of *ZC3HAV1* was not different in epithelial cells ($P > 0.05$) ($***P < 0.001$; $**P < 0.01$; $*P < 0.05$).

Table 3.6. Quantitative real-time PCR analysis of the effect of 100 or 1000 ng of recombinant mouse *LGALS3* protein on 3D cultured epithelial and fibroblast cells. The effect of flush fluid of a d14 pregnant ewe (FF) on epithelial and fibroblast cells gene expression was also examined. Control cells received treatment media only. Relative expression data are normalized over the geometric mean of reference genes, *PPIA* and *SDHA*. Data are presented as LSM $\pm$ SEM.

Gene	Cell Type	Control	FF	LGALS3 (100 ng)	LGALS3 (1000 ng)	Trt.	Contrast
<i>RPL23A</i>	Epi.	1.44 $\pm$ 0.11	0.93 $\pm$ 0.12	1.04 $\pm$ 0.11	0.99 $\pm$ 0.11	NS	NS
	Fib.	1.07 $\pm$ 0.10	1.20 $\pm$ 0.11	1.02 $\pm$ 0.10	0.83 $\pm$ 0.10	NS	NS
<i>CXCR4</i>	Epi.	5.12 $\pm$ 0.98 ^a	9.50 $\pm$ 1.10 ^b	4.89 $\pm$ 0.98 ^a	4.56 $\pm$ 0.98 ^a	< 0.05	NS
	Fib.	0.19 $\pm$ 0.10	0.41 $\pm$ 0.11	0.16 $\pm$ 0.20	0.17 $\pm$ 0.10	NS	NS
<i>DMRTA1</i>	Epi.	1.80 $\pm$ 0.38	1.12 $\pm$ 0.42	1.70 $\pm$ 0.38	1.33 $\pm$ 0.38	NS	NS
	Fib.	1.78 $\pm$ 0.45	1.38 $\pm$ 0.50	1.28 $\pm$ 0.45	1.41 $\pm$ 0.45	NS	NS
<i>ISG15</i>	Epi.	0.50 $\pm$ 5.31 ^a	130.27 $\pm$ 5.94 ^b	0.60 $\pm$ 5.31 ^a	0.55 $\pm$ 5.31 ^a	< 0.001	NS
	Fib.	0.27 $\pm$ 7.70 ^a	58.40 $\pm$ 8.61 ^b	0.23 $\pm$ 7.70 ^a	0.19 $\pm$ 7.70 ^a	< 0.001	NS
<i>LGALS3BP</i>	Epi.	0.98 $\pm$ 0.16 ^a	4.12 $\pm$ 0.18 ^b	1.05 $\pm$ 0.16 ^a	1.02 $\pm$ 0.16 ^a	< 0.001	NS
	Fib.	0.73 $\pm$ 0.21 ^a	1.77 $\pm$ 0.23 ^b	0.58 $\pm$ 0.21 ^a	0.59 $\pm$ 0.21 ^a	< 0.01	NS
<i>LIF</i>	Epi.	4.27 $\pm$ 0.77 ^a	7.11 $\pm$ 0.86 ^b	4.89 $\pm$ 0.77 ^a	4.56 $\pm$ 0.77 ^a	NS	NS
	Fib.	0.27 $\pm$ 0.09	0.27 $\pm$ 0.10	0.24 $\pm$ 0.09	0.23 $\pm$ 0.09	NS	NS
<i>PRRT3</i>	Epi.	0.62 $\pm$ 0.09	0.47 $\pm$ 0.10	0.62 $\pm$ 0.09	0.57 $\pm$ 0.09	NS	NS
	Fib.	2.62 $\pm$ 0.83	2.49 $\pm$ 0.93	2.08 $\pm$ 0.83	1.87 $\pm$ 0.83	NS	NS

IFNT Responsive Endometrial ISGs	
LE	Stroma
<i>BATF2</i>	<i>OAS2</i>
<i>MX2</i>	<i>IFI44L</i>
<i>IFI44</i>	<i>RSAD2</i>
<i>IFIT1</i>	<i>ZBP1</i>
<i>OAS2</i>	<i>MX2</i>
<i>PLAC8</i>	<i>MX1</i>
<i>KIF5C</i>	<i>PLAC8</i>
<i>ZBP1</i>	<i>IFI44</i>
<i>RSAD2</i>	<i>TNFSF10</i>
<i>IFIT3</i>	<i>SAMD9</i>

Figure 3.14. Model for IFNT regulation of classical and non-classical up-regulated ISGs in the bovine endometrium during early pregnancy. The conceptus secretes IFNT which results in the differential expression of endometrial ISGs. The genes listed were the top 10 genes up-regulated in response to oIFNT treatment to epithelial and fibroblast cells and were also up-regulated in endometrial explants in response a conceptus and IFNT [143].

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