# TRANSCRIPTIONAL REGULATION OF GALECTIN 15 (*LGALS15*): AN IMPLANTATION-RELATED GALECTIN UNIQUELY EXPRESSED IN THE UTERI OF SHEEP AND GOATS

A Dissertation

by

SHAYE KAMAL LEWIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Physiology of Reproduction

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# ABSTRACT

Transcriptional Regulation of Galectin 15 (LGALS15): An Implantation-Related Galectin Uniquely Expressed in the Uteri of Sheep and Goats. (August 2009)

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Galectins are a family of secreted animal lectins with a high affinity to beta-galactosides commonly involved in cellular functions such as apoptosis, adhesion and migration. Galectin 15 (LGALS15), a newest member of the galectin superfamily, has a unique C-terminal RGD sequence and participates in integrin-mediated ovine trophectoderm cell attachment and migration. In the ovine uterus, *LGALS15* is expressed only by the endometrial luminal (LE) and superficial glandular (sGE) epithelia, induced by progesterone between Days 10 and 12 of the cycle and pregnancy, and then stimulated by interferon tau (IFNT) from the conceptus after Day 14 of pregnancy. During early pregnancy, the canonical janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is not active in the endometrial LE/sGE. Therefore, IFNT may utilizes a non-canonical signaling pathway to increase transcription of genes, including *CST3, CTSL, HIF2A, LGALS15*, and *WNT7A*, specifically in the endometrial LE/sGE. Alternatively, IFNT and progesterone could

indirectly affect epithelial gene expression by influencing gene expression in the stroma, which then communicates with the epithelium.

Although the *LGALS15* gene is present in ovine, caprine and bovine species, it is only expressed in uteri of sheep and goats. Available data shows a tissue- and species-specific expression pattern for *LGALS15,* likely involving multiple layers of transcription regulation in the ruminant endometrium. Further analysis of the *LGALS15* 5′ promoter/enhancer region revealed similar predicted transcription factor binding sites in all three species, including; PU.1, Ets-1, AP1, Sp1, and GRE or PRE sites. Interestingly, the proximal promoter region of the *LGALS15* gene in all three species exhibited a conserved Sp1 binding site upstream of an AP1 binding site on both sense and antisense strands, and with similar spacing between binding sites.

Sequence analysis revealed key differences in *LGALS15* gene structure between ruminant species including the proximity of repetitive DNA sequences to the transcription start site (+1). Bovine *LGALS15* has repetitive DNA sequences start at -145 whereas in ovine or caprine *LGALS15* it starts at about -300. The length of the repetitive DNA sequence is similar (~1.2 kb) in the 5′ promoter/enhancer region of LGALS15 in all three species. Transient transfection analyses found that repetitive DNA sequences reduced basal promoter activity and responsiveness to treatments. None of the promoter construct showed responsiveness to interferon tau (IFNT). The bovine *LGALS15* gene promoter showed no activity under any experimental conditions. The current studies indicate that uterine *LGALS15* is expressed in ovine and caprine but not bovine species. Additionally, repetitive DNA sequences found in the promoter region

may contribute to modulating the *LGALS15* gene expression. Therefore, the ruminant *LGALS15* gene, like other galectins, is under tight transcriptional control involving hormones, requisite transcription factors and potentially chromatin remodeling complexes working synergistically for *LGALS15* promoter transactivation.

## DEDICATION

To my father, who showed me what it is to be a man, a husband and a father. Our struggles are different but equal. To those who look beyond immediate gratification, even so far as to forgo the basic necessity of shelter, in order to pursue something more significant than personal success. To other members of the negligible ninetieth who have attempted the impossible. To all of you, I stand on your shoulders to uplift and embolden the next generation. They will dream bigger and accomplish more. Our struggles will be different but equal.

## ACKNOWLEDGMENTS

I sincerely thank my mentors, Drs. Fuller Bazer and Thomas Spencer, for the opportunity to earn my Ph.D. in their laboratory. The rigors of their program have engrained in me qualities necessary to do good science. Though difficult at times, I realize they care. For that, I am grateful and stronger. I am also thankful for the instruction of my committee members Drs. Robert Burghardt and Gregory Johnson. In addition to their expertise and professionalism, their patience and kindness are enduring qualities I hope to show my students and colleagues in the near future. I am thankful for the help and guidance by Gary Newton, who has been a friend, mentor and collaborator over the years. Throughout the years, many members of the Laboratory for Uterine Biology and Pregnancy have also helped me greatly, but none greater than Dr. Gwonhwa Song. Dr. Song has been a tremendous friend and colleague whose shrewd advice has served me well. For that I am deeply appreciative. To the faculty in the Department of Biology at Prairie View A&M University who encouraged my interest in research; your commitment to the improvement of passionate young adults will never be forgotten. These individuals gave me hope when I was near defeat, guided me when I lost direction, and opened doors when I was rejected by others. I show my gratitude every time I return those acts of kindness to others.

Finally, the support and love given to me by my family has sustained me throughout this process. The enduring strength, wisdom and affection of my wife Annisa and the joy and excitement of my son Micah has been a special balance.

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# CHAPTER I

## INTRODUCTION

Fundamentally, successful conceptus survival and development depends on the establishment and maintenance of pregnancy. In mammalian species, this involves a synchronized assortment of conceptus- and maternally–derived hormones and cytokines acting in concert. In sheep, morula embryos enter the uterus on Days 4 to 5 post-mating to a uterine environment primed by the ovarian hormones estrogen and progesterone. Circulating estrogen declines following estrus while the newly formed corpus luteum (CL) results in increased concentrations of circulating progesterone. The main source of progesterone during early pregnancy is the CL, and it is required for successful pregnancy in mammalian species. Acting through its endometrial receptor (PGR), progesterone stimulates early conceptus growth and development in the uterus as well as endometrial differentiation and functions. Coordinate with increases in circulating concentrations of progesterone, conceptus growth proceeds from a spherical to tubular form by Day 11. After about 8 to 10 days of continuous progesterone exposure, endometrial PGR expression is lost first in the luminal epithelium and then in the glandular epithelium [1]. The negative autoregulation of PGR by progesterone is required for exposure and/or expression of adhesion molecules involved in the attachment of conceptus trophectoderm to the endometrial luminal and superficial glandular epithelia [2]. The conceptus elongates to a filamentous form between Days 12

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This dissertation follows the style of Biology of Reproduction.

and 16 of pregnancy. This elongation is coincident with the production of interferon tau (IFNT), the pregnancy recognition signal in ruminant species. IFNT is synthesized and secreted by mononuclear trophectoderm cells of the conceptus and induces interferon-stimulated genes (ISG) by binding to Type I IFN receptors (IFNAR). Type I and type II interferons induce the expression of a subset of gene designated as ISGs. The products of these genes are responsible for the antiviral, antiproliferative, and immunomodulatory properties of interferons. Functionally, progesterone and IFNT cooperate in a permissive relationship culminating in a uterine environment receptive to conceptus implantation. The antiluteolytic effects of IFNT are critical for the protracted synthesis and secretion of progesterone from the CL. Additionally, progesterone downregulation of PGR in the endometrial epithelia is required for expression of some non-classical ISGs such as galectin 15 (*LGALS15*) and cystatin C (*CST3*) [3] [4]. These subsets of ISGs are classified as progesterone-induced and IFNT-stimulated, and many exhibit epithelial-specific expression during the peri-implantation period in sheep.

Actions of both estrogen and progesterone, acting through their respective endometrial receptors [1] on the uterus, results in expression of a well-synchronized array of adhesion molecules during the peri-implantation period [5, 6]. Subsequent implantation events are regulated by the constitutive expression of adhesion molecules on the apical surfaces of conceptus trophectoderm and endometrial epithelia in domestic species. Expression of cell surface glycoproteins involved in the implantation cascade coincides with uterine receptivity to implantation by the developing conceptus.

The H-type 1 (HT1) carbohydrate antigen is thought to be involved in the initial adhesive forces between conceptus trophectoderm and endometrial epithelia. Endometrial expression of the fucosylated HT1 carbohydrate antigen during the peri-implantation period when cell adhesions occur between the trophectoderm of the developing conceptus and endometrial luminal epithelium was characterized *in situ* and in polarized uterine luminal and glandular epithelial cells of goats [7] and sheep [8]. The expression of the HT1 by ovine and caprine uterine epithelia and receptors for HT1 on goat conceptus tissues [9] suggests its putative role in facilitating initial attachment of conceptus trophectoderm to endometrial luminal epithelium during implantation.

Firm adhesions between conceptus trophectoderm and maternal epithelia are mediated by integrin receptors and their extracellular matrix (ECM) or cell surface ligands. Integrins are type I integral transmembrane proteins commonly associated with firm adhesion to epithelial cells. The short cytoplasmic tail of an integrin can bind various cytosolic ligands and coordinate the assembly of cytoskeletal molecules and signaling complexes. On the extracellular surface, integrins either engage ECM molecules or counter receptors on adjacent cell surfaces. They can bind a wide variety of ECM or soluble protein ligands. The cellular adhesive property attributed to some cell surface glycoproteins is mediated by carbohydrate binding proteins. These carbohydrate binding proteins, called lectins, commonly crosslink adjacent cells by binding to cell surface glycoproteins [10] or ECM glycoproteins [11], [12].

Galectins are a family of mammalian lectins characterized by their affinity for beta-galactoside containing carbohydrate residues. Galectins can be divided into three

groups based on their structural characteristics: prototype, tandem repeat and chimera. Prototype galectins consist of a peptide chain containing a single carbohydrate recognition domain (CRD) and are found either as monomers (LGALS5, LGALS8, LGALS10, LGALS13, LGALS14 and LGALS15) or as either a monomer or a dimer (LGALS1, LGALS2, LGALS7 and LGALS11). Tandem repeat galectins are characterized by the presence of two CRDs on the same chain connected by a short linker peptide (LGALS4, LGALS6, LGALS8, LGALS9 and LGALS12). The only known chimeric galectin is LGALS3 that is composed of a single CRD attached to a domain possessing different functions. Galectins exhibit diverse biological roles in apoptosis [13], tumor progression [14], and cell adhesion [15]. Galectins may contribute to successful reproduction in mammalian species. They are expressed by the pre-implantation conceptus trophectoderm and in various organs during embryogenesis in mice and humans [16] [17]. Additionally, galectins are expressed in the endometrium during the peri-implantation period of conceptus (embryo and associated extra-embryonic membranes) development in mice, humans and cattle [18]. Based on galectin fingerprinting studies in human endometrial tissue, *LGALS1* and *LGALS3* transcripts are abundant and their expression is dependent on the phase of the menstrual cycle and cell type [19]. *LGALS1* is mainly expressed in the stroma and its expression significantly increases in the endometrium and decidua during the late secretory phase of the menstrual cycle. *LGALS3* is localized to the epithelium, and its expression significantly increases during the secretory phase of the menstrual cycle. Additionally, *LGALS9* is specifically expressed in endometrial epithelial cells and is considered a marker of the

middle and late secretory phases of the menstrual cycle or pregnancy in humans [20]. In the bovine endometrium, transcriptional profiling studies revealed that *LGALS3* binding protein (*LGALS3BP*) and *LGALS9* are up-regulated in pregnant animals [21]. *In situ* hybridization analysis of endometrial tissue suggested that expression of either *LGALS3* or *LGALS9* was moderate to strong in luminal epithelial (LE) cells, while weak to no expression was observed in superficial (sGE) or deep (dGE) glandular epithelial cells or stromal cells.

The developing conceptus is actively involved in the process of implantation at the conceptus-maternal interface. This is evident by the array of adhesion molecules and cognate receptors expressed by the conceptus trophectoderm. For example, galectins expressed by the conceptus trophectoderm could bind to carbohydrate moieties of glycoproteins associated with the endometrium. Caprine conceptuses express *LGALS3*, a putative receptor for endometrial HT1, during the peri-implantation period [9]. Murine conceptuses express both *Lgals1* and *Lgals3*; however, successful reproduction in single or double null mutants suggests that they are functionally unnecessary during the peri-implantation period [22, 23]. Alternatively, functional redundancy may explain the successful reproduction phenotype in *Lgals1/Lgals3* double mutants as other galectins like *Lgals5* are also expressed by the developing conceptus [23]. Similar to other galectins, *LGALS15* is expressed in the female reproductive tract. Specifically, *LGALS15* is expressed in the endometrium during the peri-implantation period in sheep [3]. It is a new member of the galectin superfamily and was initially discovered in sheep abomasal tissue infected with the nematode parasite, *Haemonchus contortus* [24]. In the

ovine uterus, LGALS15 is an abundant component of endometrial secretions collectively termed uterine histotroph [3].

Uterine glands are critical for peri-implantation conceptus growth and development [25], [26], as the uterine gland knockout ewe model (UGKO) was used to show that uterine glands are required for conceptus development and survival. In the absence of uterine glands, conceptus growth is severely retarded and conceptuses fail to survive past Day 14 of pregnancy. Since LGALS15 is an abundant secretory product in uterine histotroph [3] and galectins are known to affect cell migration, adhesion, and proliferation, we hypothesized that LGALS15 supports conceptus attachment and outgrowth during the peri-implantation period in sheep. Defining clear biological functions of galectins must also assess mechanisms of gene regulation while respecting physiological relevance. Mechanisms of galectin gene regulation are poorly understood. Ovine endometrial *LGALS15* expression is induced by progesterone and further stimulated by IFNT [3]. The *LGALS15* expression pattern is coordinate with early conceptus elongation, growth and development during the peri-implantation period in the ovine uterus. Spatially, *LGALS15* mRNA is limited to endometrial LE and sGE and represents one of a growing list of non-classical interferon-stimulated genes (ISG) expressed by LE and sGE regulated by a novel JAK/STAT-independent cell-signaling pathway [4].

It is unknown if progesterone and IFNT regulate *LGALS15* transcription in the ovine uterus directly or indirectly, but the temporal and spatial expression of *LGALS15* mRNA requires loss of PGR in uterine epithelia. Previous results from our laboratory

indicated that expression of some ISGs containing interferon stimulated response elements (ISRE) in their promoters show uterine-specific expression [27]. Transcriptional control of other galectin family member genes is regulated by epigenetic modification of gene promoters. Transcriptional repression of the *LGALS3* gene *in vitro* in malignant prostate epithelial cells is regulated by DNA methylation status of the 5′ proximal promoter [28]. Epigenetic modifications such as DNA methylation of cytosine residues at CpG (cytosine paired guanine) dinucleotides are common in the promoter/enhancer region of genes. Methylations at cytosine residues within promoters are considered stable modifications even if other repressive modifications are reversed [29]. These DNA modifications can thus be retained from one generation to the next in similar patterns within the promoter/enhancer region. In addition to transcriptional repression and/or silencing, DNA hypermethylation of gene promoters is responsible for tissue specific patterns of gene expression [30], [31]. To date, most galectins studied exhibit tight regulation, restricting expression to specific organs and even specific cell types within an organ. There are not many studies showing a direct relationship between galectin gene expression and promoter methylation; however, many galectin family members have a CpG dinucleotide density indicative of DNA hypermethylation in the 5′promoter and gene coding/noncoding regions [32].

From a holistic view, *LGALS15* expression in the uterus of domestic species may be the result of multiple layers of tight regulation. These include, but are not limited to, chromatin accessibility, the sequential effects of progesterone and IFNT, and putative cis/trans interactions at the level of the promoter. Steps toward a better undstanding of

the mechanisms by which progesterone and IFNT regulate the expression of genes during the implantation period will further our understanding of similar mechanisms in humans and other domestic animals to improve fertility. Thus the studies conducted herin will ascertain the transcriptional control mechanisms of *LGALS15*, an implantation-related gene expressed in the ovine endometrium.

# CHAPTER II

# LITERATURE REVIEW

## Early Pregnancy in Ruminant Species

### *Conceptus Development in Sheep*

Successful conceptus development and survival in mammalian species depends on the establishment and maintenance of pregnancy. This requires concerted interactions between the endometrium and the developing conceptus. In sheep, morula embryos enter the uterus on Days 4 to 5 after onset of estrus and a uterine environment primed by the ovarian hormones estrogen and progesterone. The endometrium is transiently exposed to high levels of estrogen during estrus (Day 0 = mating). Estrogen is derived from follicles on the ovary during proestrus (~ two days prior to estrus). As circulating estrogen declines following estrus and ovulation of the dominant follicle(s), the newly formed corpus luteum (CL) increases concentrations of circulating progesterone during metestrus (Days 1 to 4) that reach maximum levels during diestrus (Days 5 to 15) (Figure 2.1). As the morula embryo develops, the blastomeres compact and the cells on the outside begin to form tight junctions and become polarized. This is the initial formation of the trophectoderm (TE). The inner cells of the morula begin to form gap junctions and the beginning of the inner cell mass (ICM).

Blastocyst formation marks the segregation of the first two cell lineages in the mammalian pre-implantation embryo: the ICM will form the embryo proper and the TE will give rise to the placenta. In sheep, blastocyst formation occurs by Day 6, and is distinguished by the formation of a fluid filled cavity or blastocoel surrounded by a

single layer of trophoblast cells [33]. Day 8 marks initiation of hatching of the blastocyst from the zona pellucida (Figure 2.1). Already in the uterus, the developing blastocyst no longer needs the zona pellucida to prevent premature attachment of the embryo. Hatching is followed by rapid development of the blastocyst from a spherical to tubular form on Day 11 to a filamentous structure between Days 12 and 17 of pregnancy (Figure 2.1). The conceptus is located in the uterine horn ipsilateral to the CL and elongates therein between Days 13 and 16, before extending into the contralateral uterine horn thereafter [34]. Interestingly, the uterus appears to be required for elongation and trophectoderm outgrowth of sheep conceptuses [34, 35]. *In vitro*, hatched blastocysts and trophoblastic vesicles do not elongate; however, similar blastocysts or trophoblastic vesicles can elongate when transferred into the uterus [34, 35]. Thus, the early developmental program of the conceptus requires substances synthesized and/or secreted and/or transported into the uterine lumen to support continued growth and survival of the conceptus.

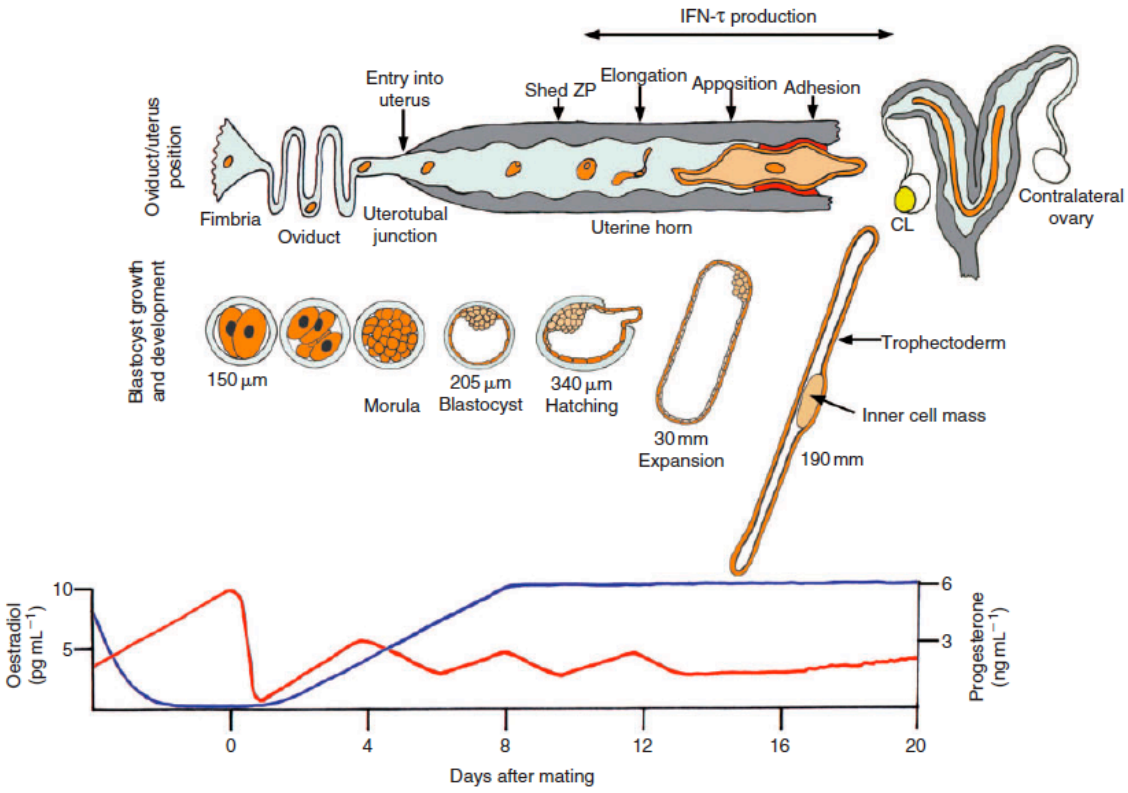


Figure 2.1. Early pregnancy events in sheep. Fertilization occurs in the oviduct and morula-stage embryos enters the uterus on Day 4 after mating. The embryo develops into a blastocyst by Day 6 and hatches from the zona pellucida (ZP) between Days 8 and 9 by the actions of proteases. After Day 10, the spherical blastocyst assumes a tubular form by Day 11, and then becomes a filamentous conceptus between Days 12 and 16 of pregnancy. The beginning of implantation involves apposition and transient attachment (Days 12 to 15) and firm adhesion by Day 16. By Day 17, the filamentous conceptus occupies the entire ipsilateral uterine horn and elongated through the uterine body into the contralateral uterine horn between Days 19 and 21 of pregnancy. Adapted from [36] and originally drawn by Dr. Greg A. Johnson.

***Implantation in Sheep***

As a result of this rapid growth and expansion of the developing conceptus, implantation competent conceptuses become apposed and transiently attached to the endometrial LE and sGE epithelia. Specifically, this involves the conceptus trophectoderm contacting the LE of caruncular areas and, in intercaruncular areas, both LE and sGE as conceptus trophectoderm has specialized multicellular protrusions or papillae that project into the mouths of the endometrial glands, acting to transiently anchor the trophectoderm to the maternal uterine LE/sGE [37], [38].

Implantation in domestic ruminants can be divided into distinct phases [33], [39] including: shedding of the zona pellucida, pre-contact and blastocyst orientation, apposition, adhesion, and endometrial invasion (Figure 2.2). Unlike primates which have a more invasive type of implantation, conceptuses of domestic ruminants undergo an extended period of growth and development prior to implantation. During this period, conceptus development is supported by uterine secretions. These secretions, which are produced under the influence of progesterone, include growth factors, cytokines, adhesion molecules and other substances collectively termed histrotroph [40], [41]. In sheep, apposition of the conceptus trophectoderm and LE/sGE is initiated on Day 12, and this is quickly followed by transient attachments and then firm adhesion by Day 16 [42]. The process of apposition involves transient contacts between the conceptus and the LE/sGE, which are believed to be important for elongation of the conceptus trophectoderm. Modulation of anti-adhesive mucins such as decreases in MUC1 [43] during the attachment phase may promote or allow intermolecular contacts between cell

surface integrins and other glycoconjugates with secreted adhesion proteins such as glycosylated cell adhesion molecule 1 (GLYCAM1) [44], galectin 15 (LGALS15) [3], and secreted phosphoprotein 1 (SPP1) [45] (Figure 2.2).

These interactions facilitate firm adhesions involving interdigitation of microvilli on maternal uterine LE/sGE and trophectoderm. In contrast to primates and rodents, invasion of conceptuses into the uterine endometrium in domestic species such as sheep, goats and cattle is absent or very limited. However, there is evidence for inter-species conservation of adhesive molecules used to accomplish conceptus apposition, attachment and adhesion to the maternal endometrium. During synepitheliochorial placentation in sheep, mononuclear trophectoderm cells differentiate into giant binucleate cells between Days 14 and 16 and then fuse apically with the endometrial LE to form multinucleated syncytial plaques (Figure 2.2) [46].

Integrins are widely expressed in endometrial LE/sGE during implantation and are commonly accepted as cell surface molecules that support firm trophectoderm adhesions to the LE/sGE in many species [6], [47]. In fact, during the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on the conceptus trophectoderm and apical surface of the endometrial LE/sGE [43]. Additionally, adhesive molecules such as SPP1, a secreted ECM protein that associates with integrin molecules, are also expressed in the endometrium during the peri-implantation period [43], [48]. Therefore, in sheep, implantation may not involve changes in temporal or spatial expression patterns of

integrins, but may depend on expression of ECM or secretory proteins, such as SPP1, which is a ligand for αvβ3 and several other integrin heterodimers [49], [45].

***Maternal Recognition of Pregnancy***

If the oöcyte is fertilized, progesterone secretion from the CL must be maintained to establish pregnancy. During early pregnancy, the blastocyst must signal its presence to the maternal system to ensure CL maintenance prior to the time that normal luteal regression would occur. As a phrase, "maternal recognition of pregnancy" was coined by Roger Short in 1969 and generally refers to a secreted-conceptus derived signal that prevents CL regression and extends its lifespan for secretion of progesterone. The process involves secretion of a luteotropic hormone that acts directly on the CL or prevention or redirection of the secretion of luteolytic hormones by an antiluteolytic hormone. There exists a well-designed relationship between the uterus, the CL, and the conceptus that requires hormonal crosstalk between these tissues resulting in CL maintenance and protracted secretion of luteal progesterone. IFNT is the signal produced by the conceptus trophectoderm in ruminant species that acts on the maternal endometrium to elicit pregnancy recognition. Indeed, the developing ovine conceptus is required for maintaining a functional CL [50]. Rowson and Moor originally proposed a conceptus derived pregnancy recognition signal for sheep [51]. IFNT was originally named trophoblastin [52] and then ovine trophoblast protein-1 [53].

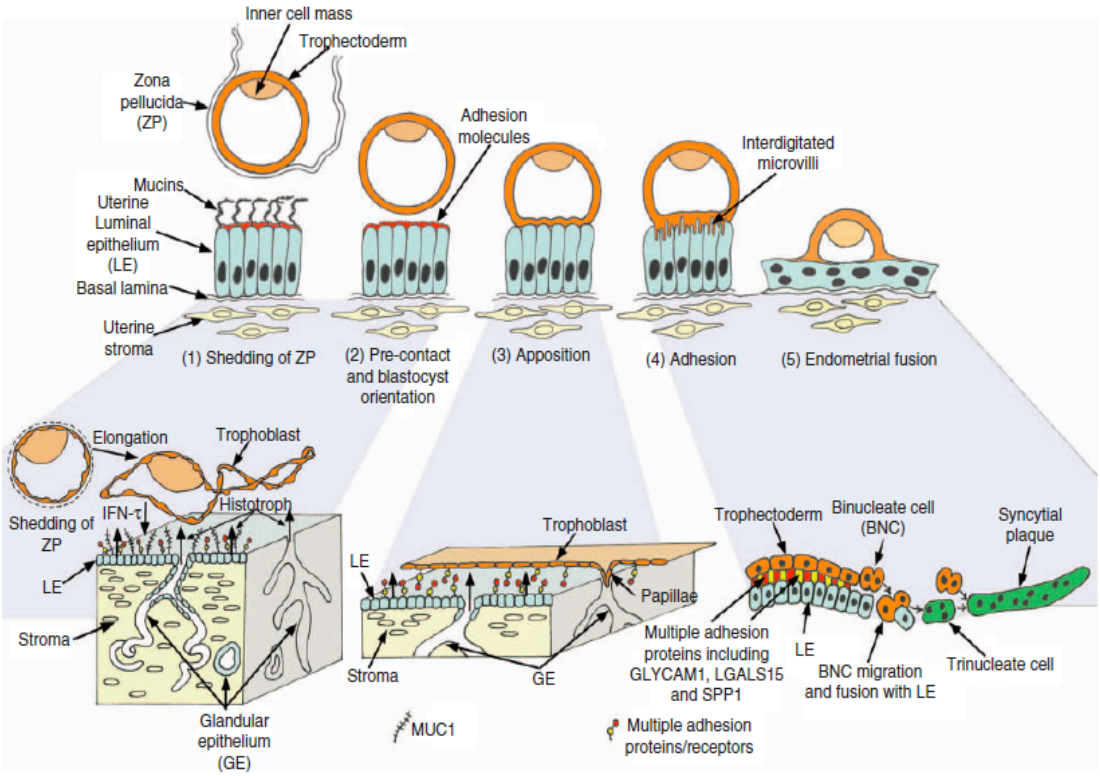


Figure. 2.2. The phases of blastocyst implantation in sheep. Shedding of the zona pellucida (Phase1): The embryo enters the uterus on Day 4. The blastocyst is formed on Day 6 and the zona pellucida is shed on Day 8 or 9 due to blastocyst growth and uterine and/or embryonic proteases. After Day 10, the blastocyst elongates and develops into a tubular and then into a filamentous conceptus. Precontact and blastocyst orientation (Phase 2): Between Days 9 and 14, there is no definitive cellular contact between the conceptus trophectoderm and the endometrial epithelia, but the conceptus appears to be positioned and immobilized in the uterus. During this time, the elongating conceptus produces IFNT for pregnancy recognition. Apposition (Phase 3): The conceptus trophectoderm associates closely with the endometrial LE followed by unstable adhesion. In ruminants, the trophoblast develops finger-like villi or papillae that extend into the superficial ducts of the uterine glands where it is hypothesized to anchor the peri-attachment conceptus and absorb histotroph. Adhesion (Phase 4): On Day 16, the trophoblast begins to adhere firmly to endometrial LE. The interdigitation of the trophectoderm and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. During this time, the mononuclear trophectoderm cells differentiate into trophoblast giant binucleate cells. Adapted from [36] and originally drawn by Dr. Greg A. Johnson.

IFNT is a type I interferon with potent antiviral, antiproliferative and immunosuppressive activities [54] produced by conceptuses of ruminants, including ovine, bovine and caprine species [52], [55], [56]. Zoo blot analysis revealed that IFNT genes are restricted to ruminant species within the Artiodactyla order [57]. IFNT is produced by the mononuclear trophectoderm cells of the conceptus between Days 10 to 21 in sheep (maximally on Days 13 to 16) and acts in a paracrine manner on the endometrium [53]. IFNT maintains the functional CL by inhibiting transcription of estrogen receptor (ESR1) and thus the oxytocin receptor (*OXTR*) gene, resulting in abrogation of the endometrial production of luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [58] (Figure 2.3).

*Anti-luteolytic Mechanisms*

Although IFNT prevents the luteolytic mechanism from occurring, it is not luteotrophic [53]. In sheep, IFNT acts in a paracrine manner on the endometrium to suppress transcription of ESR1 and OXTR genes [59], [60], [61], thereby abrogating development of the endometrial luteolytic mechanism by preventing production of luteolytic pulses of PGF2α (Figure 2.3). Indeed, increased endometrial

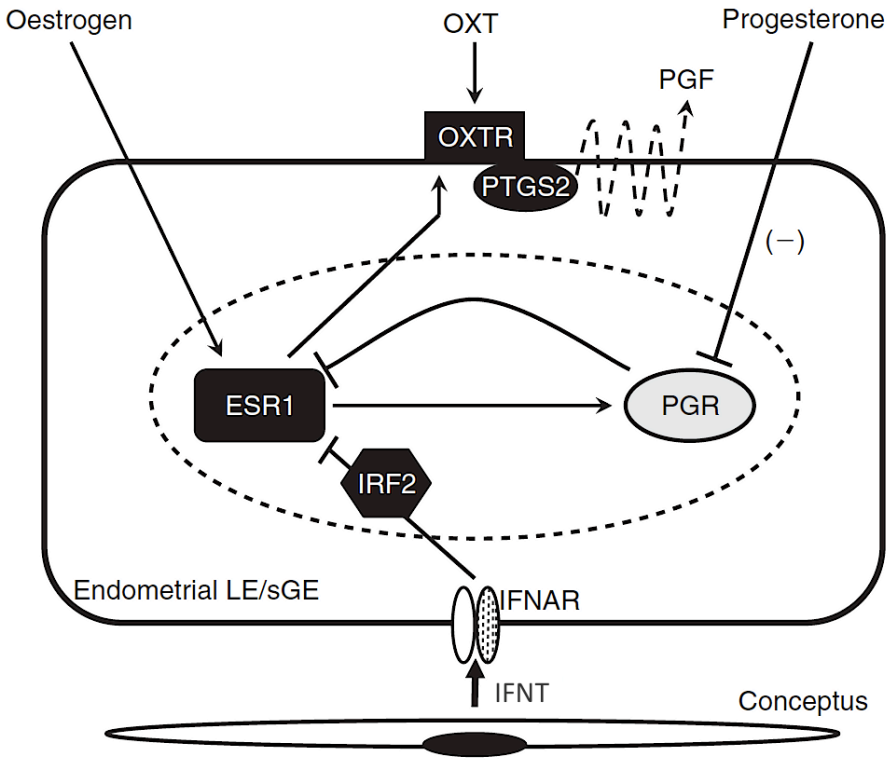


Fig. 2.3. Current theory for antiluteolytic mechanisms in sheep. Schematic illustrating the current theory for hormonal regulation of the endometrial antiluteolytic mechanism and cross talk between the conceptus and the maternal endometrium for pregnancy recognition. During the peri-implantation period, ovine IFNT, synthesized and secreted by the mononuclear trophectodermal cells between Days 10 and 21-25 (maximally on Days 14-16), acts directly on endometrial LE and sGE to suppress transcription of *ESR1* which precludes expression of the *OXTR* gene, thereby preventing production of oxytocin-induced luteolytic pulses of PGF. During the estrous cycle, *ESR1* expression increases as *PGR* expression decreases on Days 11 to 13 and then E2 induces *OXTR* expression on Days 13 to 14, thereby allowing oxytocin from the posterior pituitary and/or CL to induce release of luteolytic pulses of *PGF* on Days 15 to 16. In contrast, during early pregnancy, secreted IFNT from fully elongated conceptus silences *ESR1* expression, which prevents E2-induced *OXTR* expression. However, IFNT does not stabilize *PGR* expression in endometrial epithelia during pregnancy. Adapted from [36].

production of luteolytic pulses of PGF2α parallel increases in endometrial expression of oxytocin receptors in cyclic ewes [62] [63]. Progesterone and estrogen control OXTR expression in the endometrial epithelia resulting in control of luteolytic PGF2α [64], [65] (Figure 2.3). In sheep, progesterone exerts both positive and negative effects in the regulation of appropriate PGF2α secretion [64]. First, extended progesterone exposure promotes increased uterine arachidonic acid, prostaglandin endoperoxide synthase (PTGS1, PTGS2), and other substances needed for synthesis of PGF2α. Second, progesterone acts through PGR to block expression of ESR1 and OXTR in the endometrial epithelium, but PGR is negatively autoregulated by progesterone first in the LE and then in the GE on Days 11 and 13, respectively. However, PGR expression in uterine stromal cells is maintained throughout diestrus and pregnancy [1]. Therefore, progesterone loses its ability to negatively regulate ESR1 and OXTR in the endometrial epithelia after about Day 11 of diestrus and this results in increased ESR1 and OXTR expression in the LE and sGE. The antiluteolytic actions of IFNT are to silence expression of epithelial ESR1 [59] which, in turn, prevents the requisite ESR1/Sp1-mediated transcriptional activation of the OXTR gene [66].

*IFNT Signaling*

Type I IFN receptors are present in all endometrial cell types [60], and are known to classically activate the janus kinase-signal transducers and activators of transcription (JAK-STAT) cell signaling pathway to stimulate or repress gene transcription. Genes activated by interferons are collectively refered to as interferon stimulated genes (ISG). IFNT induces stromal and/or deeper GE to express a number of classic ISG such as

inerferon regulatory factor-1 (IRF1) [67] [27], β2 microglobulin (B2M) [68], interferon stimulated gene 15 (ISG15) [69], and 2′5′-oligoadenylate synthase (OAS) [70] (Figure 2.4). Interestingly, components of the JAK-STAT pathway such as STAT1, STAT2, and IRF9 are expressed in the endometrial stroma and GE [27], but this cell signaling pathway is likely inactive in the endometrial LE and sGE (Figure 2.5), because the LE/sGE express IRF2, a transcriptional repressor of classical ISG [27] (Figure 2.5). Reports of non-classical ISGs specifically expressed in the endomtrial LE/sGE suggest a novel, cell specific JAK-STAT independent pathway [3], [4], [71], [72] (Figure 2.5). These novel ISGs may be activated by IFNT through a non-traditional signaling pathway such as the p38 MAP kinase [73, 74] or nuclear factor-kappa B pathways [75].

Proper conceptus growth and survival is also under the influence of endometrial secretions. Specifically, these secretions provide nutrients for the conceptus to undergo cellular reorganization during elongation, differentiation prior to placentation, as well as production of the signal for maternal recognition of pregnancy. In fact, conceptus elongation is critical for developmentally regulated synthesis and secretion of IFNT [76], [77]. In many mammals, the placenta eventually provides sufficient progestational support for maintenance of pregnancy until parturition. However this luteal–placental shift occurs later in pregnancy. Thus, the developing conceptus actively participates in its own growth and survival by secreting paracrine factors that influence both the functional lifespan of the CL during early pregnancy and endometrial gene expression.

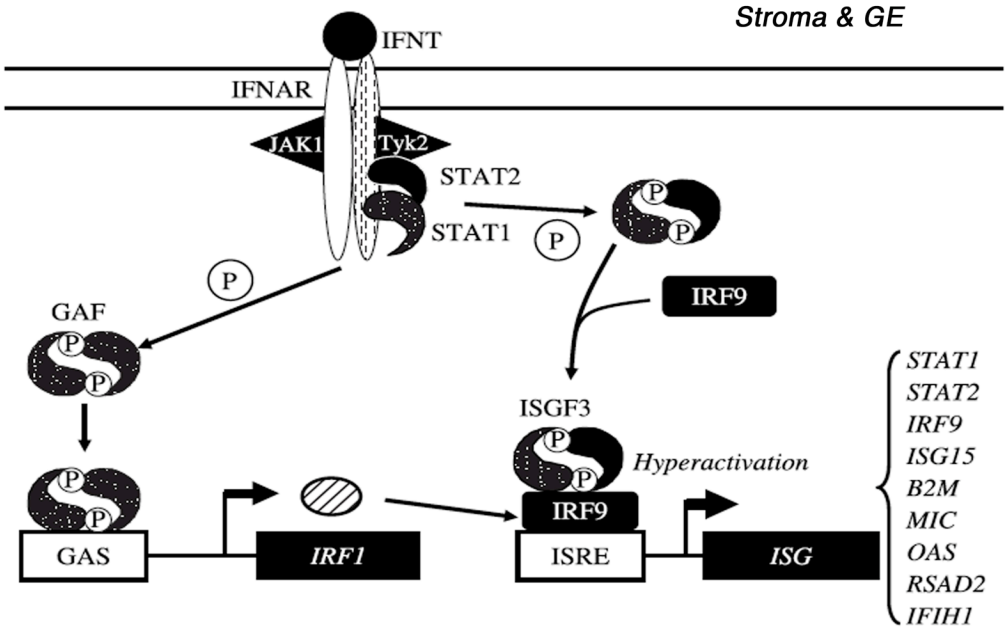


Fig. 2.4. Current theory for IFNT signaling in endometrial stroma and middle to deep glandular epithelium. The stromal cells and GE do not express IRF2, a potent repressor of gene transcription. Thus, IFNT-mediated association of IFNAR subunits facilitates cross-phosphorylation and activation of JAK1 and Tyk2, which in turn phosphorylates the receptor and create a docking site for STAT2. STAT2 is then phosphorylated, thus creating a docking site for STAT1, which is then phosphorylated. STAT1 and STAT2 are then released from the receptor and can form two transcription factor complexes, γ activated factor (GAF) and ISGF3. Association of a STAT1-2 heterodimer forms ISGF3 and IRF9 in the cytoplasm, translocates to the nucleus, and transactivates genes containing an ISRE(s), such as STAT1, STAT2, IRF9, B2M, ISG15, MHC class I polypeptide-related sequence (MIC), and OAS. Formation of GAF by STAT1 homodimers, which translocates to the nucleus and transactivates genes containing a γ activation sequence (GAS) element(s) such as IRF1. IRF1 can also bind and transactivate IFN-stimulated response element (ISRE)-containing genes as well as IRF-response element (IRFE)-containing genes. The simultaneous induction of STAT2 and IRF9 by IFNT appears to shift transcription factor formation from GAF towards predominantly ISGF3. Therefore, IFNT activation of the JAK-STAT-IRF signal transduction pathway allows for constant formation of ISGF3 and GAF transcription factor complexes and hyperactivation of ISG expression in the stroma and GE. Adapted from [78].

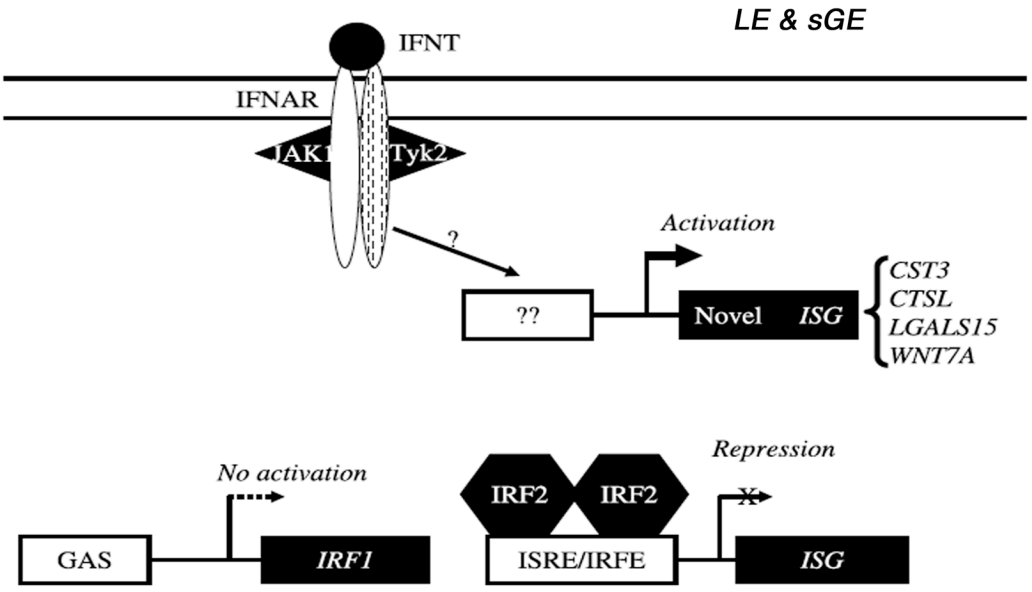


Fig. 2.5. Current theory for IFNT signaling in endometrial luminal and superficial glandular epithelium. In LE and sGE, expression of IRF2, a potent and stable transcriptional repressor, increases during early pregnancy to inhibit expression of classical ISGs (STAT1, STAT2, IRF9, B2M, ISG15, MHC, and OAS) through direct ISRE and IRFE binding and coactivator repulsion. Thus, critical factors in the classical JAK-STAT-IRF pathway (STAT1, STAT2, and IRF9) are not present, resulting in the absence of ISGF3 or IRF1 transcription factors necessary to transactivate ISG. However, IFNT activates an unknown cell-signaling pathway that results in induction of wingless-type MMTV integration site family, member 7A (WNT7A) in the LE and stimulation of non-classical IFNT-stimulated genes, e.g., CST3, CTSL and LGALS15, specifically in LE and sGE. Adapted from [78].

## Lectins

### *Historical Perspective of Lectins*

Lectins are a family of soluble or membrane bound proteins highly conserved in many living organisms from viruses and bacteria to humans and sponges. They are varied in structure and localization resulting in ubiquity of function. They can be broadly defined as carbohydrate binding proteins that do not either catalyze reactions with or structurally modify the ligands to which they bind. Additionally, they are capable of specific and reversible ligand interactions [79]. The study of lectins dates to the late 19$^{th}$ century. It is widely accepted that Peter Hermann Stillmark who, for his doctoral dissertation published in 1888, isolated ricin from castor tree seeds (*Ricinus communis*) and first observed lectin hemagglutination, a defining characteristic of lectins at that time. However, ealier reports indicated that animal lectins were discovered first, although not in the context of hemagglutinins. The first animal lectins were likely discovered by J.M. Charcot and C. Robin in 1853 [80] and were called Charcot–Leyden crystals. There hemagglutination properties were observed by S. Weir Mitchell and Edward T. Reichert in 1886 prior to reports by Stillmark according to Kilpatrick [81] and Cervetti [82]. Additionally, Flexner and Noguchi [83] cited an earlier study showing a relationship between snake venom and hemagglutination. The isolation of concanavalin A (Con A) from jack bean (*Canavalia ensiformis*) by James Sumner was a seminal lectin discovery [84]. Con A could agglutinate erythrocytes from select species such as horse, dog, cat, rabbit, guinea pig, and rat [85]. The Sumner group first demonstrated sugar specificity of lectins by showing that the hemagglutination activities

of Con A could be inhibited by sucrose. Remarkably, analysis of their data led them to conclude that a possible mechanism by which Con A agglutinates erythrocytes is by association with the carbohydrate moiety of cell membrane glycoproteins. Scientists continued to discover new plant lectins and in the early to mid 20$^{th}$ century began ascribing lectin hemagglutination utility to selective blood type erythrocytes. The ability of plant agglutinins to distinguish between erythrocytes of different blood types led to a nomenclature shift from plant agglutinins to lectins [86]. The name lectin was taken from the Latin "*legere*", to pick out or choose. The term lectin is now used broadly to describe all carbohydrate-binding proteins of non-immune origin regardless of source (microbial, plant or animal).

The steady increase in the number of studies focusing on understanding lectin properties and functions are the result of some seminal discoveries about these carbohydrate-binding proteins. First, Peter Nowell serendipitously observed that the lectin of the red kidney bean (*Phaseolus vulgaris*), also known as phytohemagglutinin (PHA) is mitogenic, possessing the ability to stimulate lymphocytes to undergo mitosis [87]. Later, mitogenic actions were observed for other lectins including Con A [88]. Interestingly, the mitogenic stimulation of lymphocytes induced by Con A could be inhibited by low concentrations of monosaccharides. This represents one of the earliest examples of a biological role for cell surface lectin-carbohydrate interactions. Second, the discovery that wheat germ agglutinin (WGA) was capable of selective agglutination of malignant cells [89, 90] was very important to lectinology. These studies provided early evidence that changes in cell surface sugars are associated with the development of

cancer. The common era of lectinology was met with fervent scientific endeavors initiated from many different disciplines due to their utility in studying the structure and function of complex carbohydrates, especially glycoproteins, and for studying changes that occur on cell surfaces during physiological and pathological processes.

***Plant Lectins***

As alluded to previously, early work in lectin biology typically involved plant lectin isolation, characterization, and determination of erythrocyte agglutination or carbohydrate binding capabilities. To date, a vast number of lectins have been identified and isolated from many different plant species. They can be classified based on protein structure and degree of evolutionary conservation [91]. Two main families of plant lectins are the legume family and the cereal family of lectins. Historically, lectins, such as; Con A from jack bean [92], soybean agglutinin [93], and wheat germ agglutinin, have been studied extensively. In practice, affinity chromatography proved invaluable in isolating and characterizing novel plant lectins. More recently, lectinology has been advanced by molecular cloning techniques used in combination with affinity chromatography. Recently, a new galactose-specific plant lectin was purified from seeds of a Caesalpinoideae plant, *Bauhinia variegata* [94]. These novel lectins may be used as biological sorters such as in the identification of various bacterial species [95] because the lectin-carbohydrate interactions are specific even to bacterial species. This specificity is aided by the ability of lectins to recognize fine differences in terminal carbohydrate motifs depending on the bacterial strain.

Plant lectins such as the mistletoe lectin I (MLI) are widely used for adjuvant tumor therapy. It is a glycoprotein classified as a type II ribosome-inactivating protein (RIP) due to the rRNA-cleaving enzymatic activity of the A-subunit, also referred to as toxic entity. Production of biochemically defined recombinant mistletoe lectin was achieved by cloning and expressing the catalytically active A-chain and the carbohydrate binding B-chain in *Escherichia coli*, yielding an active heterodimeric protein named rViscumin [96, 97]. The anti-cancer drug rViscumin is preferentially cytotoxic to tumor cells harboring terminal α2-6-sialylated neolacto-series gangliosides [98]. Thus the cytotoxic nature of some lectins lends to their utility in cell-targeted treatments.

Conversely, lectins are also involved in cell survival as studies show that treatment with the plant lectin PHA significantly improved the fusion efficiency of somatic cells with oöcytes during somatic cell nuclear transfer cloning in cattle [99] thus increasing the development of cloned embryos. Additionally, the effiency of pig embryos developed parthenogenetically or by somatic cell nuclear transfer was increased by treatment with PHA [100] and is believed to support peri-implantation development in pigs by enhancing blastocyst hatching, expansion, and decreasing apoptosis by positively modulating the expression of embryonic survival related genes [101]. It is unknown if the previously mentioned functions of plant lectins are mediated merely by the carbohydrate binding domains, but lectin binding specificity is enhanced by subsite binding and/or subunit multivalency [102]. Thus plant lectins exhibit a high degree of intra-family conservation. Nevertheless subtle differences in their primary sequence and/or tertiary structure facilitate highly selective carbohydrate binding characteristics.

This validates their efficacy in various biological systems and as cell-specific therapeutic drug targets.

***Animal Lectins***

Animal lectins have been known for as long or longer than plant lectins, although not as agglutinin or carbohydrate binding proteins. In 1853, Charcot and Robin [81] observed crystal-like structures in abnormal tissues and the presence of these crystals was thought to be mediated by infiltration of tissues by eosinophils at sites of inflammation [81]. These stuctures subsequently became known as Charcot–Leyden crystals (CLC) and are now characterized as the carbohydrate binding protein LGALS10 [103]. Animal lectins comprise a superfamily of multifunctional carbohydrate binding proteins with highly conserved functional domains, but divergent specificities for carbohydrate moieties of glycoproteins and/or glycolipids [104]. Early studies of animal lectins proved important to our understanding of the carbohydrate nature of blood group antigens, especially lectins initially discovered in the electric eel. Similar to plant lectins, animal lectins have contributed greatly to our understanding of biochemical structures and functions of cell surface and ECM molecules involved in cell adhesion [105]. Given the enormous complexity of carbohydrates, and the huge array of glycoproteins and glycolipids that present them, it is not surprising that lectins are involved in remarkably diverse functions.

Animal lectins have been characterized in diverse species such as the nematode (*Caenorhabditis elegans*) [106], electric eel (*Electrophorus electrucus*) [107], frog (*Xenapus laevous*) [108], sheep (*Ovis aries*) [24], humans (*Homo sapiens*) [109], and

rainbow trout (*Oncorhynchus mykiss*) [110]. They can be divided into structural families based on the primary amino acid sequence. Five of the recognized families include C-type, Galectins, I-type (siglecs), P-type (phosphomannosyl receptors), and pentraxins. Research in lectinology has revealed the presence of other structurally distinct animal lectins such as calreticulin/calnexin [111] and ERGIC-53/VIP-36 [112]. As noted, lectins are involved in diverse biological functions exemplified by the galectins. They mediate specific functions in the nucleus, cytoplasm, cell surface and in the extracellular milieu. A brief discription of the primary animal lectins will emphasize the galectin family of animal lectins.

*C-Type Lectins*

C-type lectins can be further divided into subfamilies based on primary amino acid sequence especially in the non-lectin domains in addition to gene structure. These include; endocytic lectins, collectins, selectins and lecticans which are a minor group. Their dependency on calcium ($Ca^{++}$) for carbohydrate binding is reflected in the name C-type lectin; however, there is some evidence for $Ca^{++}$-independent carbohydrate binding [113]. Crystallographic studies showed that the CRD of the C-type lectins has a compact globular structure [114]. Functionally, $Ca^{++}$ ions associated with CRDs of most C-type lectins are directly involved in carbohydrate binding, as well as in maintaining the structural integrity of the CRD necessary for the lectin activity [113]. Depending on the amino acid sequence, the CRD is specific for mannose, galactose or fucose. Additionally, the interaction of these carbohydrates with different C-type lectins is further regulated by distinct carbohydrate branching, spacing and multivalency [102].

Similar to plant lectins, the cell specific, multifunctional nature of animal lectins is influenced not only by specific terminal carbohydrates, but also by the conformation and branched organization of the associated oligosaccharides in determining precise biological responses. As versatile molecules entrusted with deciphering the complex glycode, it is appropriate that lectin proteins are found in multiple cellular compartments and C-type lectins are no exception. They are found either as secreted soluble proteins or as transmembrane proteins, and can act both as adhesion and as pathogen recognition receptors [115].

A well studied, secreted, and soluble C-type lectin is the collectin family of lectins [116] [117] present in serum and on mucosal surfaces. Collectins represent the first line of host defense in their ability to recognize pathogen-associated molecular patterns (PAMPs) [116] [117]. Upon recognition of a pathogen, collectins initiate effector mechanisms like opsonization, agglutination, complement activation and phagocytosis to curtail pathogen proliferation within the host [118]. There have been many collectins characterized to date, but the mannose-binding lectin (MBL) present in serum [119] and surfactant proteins-A (SP-A) and -D (SP-D) secreted at the luminal surface of pulmonary epithelial cells [120] are the best characterized.

Transmembrane C-type lectins can be divided into two groups, depending on the orientation of their N-terminus, as type I and type II C-type lectins. Type I C-type lectins have an N-terminus in the extracellular compartment while type II C-type lectins have a N-terminus in the intracellular compartment. Examples of transmembrane C-type lectins are the selectins [121], the mannose receptor (MR) family [122], and the dendritic

cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) [123] which is also a receptor for HIV-1 and is expressed in placentae [124].

*I-Type Lectins*

I-type lectins (siglecs and others) is a common term to describe any carbohydrate-binding proteins belonging to the immunoglobulin (Ig) superfamily [125]. Most, but not all I-type lectins recognize sialic acids which are acidic monosaccharides frequently found at the outer end of secreted and cell surface glycoconjugates. There are over 40 different forms of sialic acids attached in a variety of linkages to underlying carbohydrates. This follows a common theme in lectinology that results in a large degree of molecular diversity and thus biological functions attributed to lectin proteins. The Siglecs (Sialic acid-binding immunoglobulin superfamily lectins) are a structurally distinct subfamily of I-type lectins that recognize sialic acids [126]. Other I-type lectins, structurally different from Siglecs but recognizing sialic acids, include CD83 [127, 128] and the neural cell adhesion molecule L1 [129].

*P-Type Lectins*

P-type lectins (phosphomannosyl receptors) consist of two members, which include the ~ 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and the ~ 300-kDa insulin-like growth factor II/mannose 6-phosphate receptor (IGF-II/MPR) that bind phosphorylated mannose residues. They are essential for the survival of organisms due to their role in the generation of functional lysosomes which degrade internalized and endogenous macromolecules [130]. The MPRs target newly

synthesized lysosomal enzymes bearing mannose 6-phosphate residues in the Golgi and deliver these ligands to a late endosome.

*Galectins*

Galectins were previously termed S-type lectins due to their requirement for reducing conditions to maintain carbohydrate binding activity [131]. It was widely accepted that the sulfhydryl dependency of the thiol groups of cysteine residues was a distinguishing characteristic of all galectins similar to the $Ca^{++}$ dependency of C-type lectins. The earliest discovery of an animal galectin in the electric organ of *Electrophorus electrucus* was called electrolectin [107]. Galectins are commonly divided into three groups based on their structural characteristics: prototype, tandem repeat and chimera. Prototype galectins consist of a peptide chain containing a single carbohydrate recognition domain (CRD) and are found either as monomers (LGALS5, LGALS8, LGALS10, LGALS13, LGALS14 and LGALS15) or as either a monomer or dimer (LGALS1, LGALS2, LGALS7 and LGALS11). Tandem repeat galectins are characterized by the presence of two non-identical CRDs separated by a short linker sequence on the same chain (LGALS4, LGALS6, LGALS8, LGALS9 and LGALS12). The only known chimeric galectin is LGALS3 composed of a single CRD attached to a N-terminus domain characterized by a proline and glycine-rich repetive sequence. Galectins exhibit diverse biological roles in pre-mRNA splicing [132], cell growth [133], cell adhesion [15], regulation of cell cycle [134], and apoptosis [13]. Galectins are involved in interactions of cells with the ECM to regulate cell adhesion, motility, growth, survival and differentiation partly through integrin-mediated signal transduction.

Galectins show a broad range of tissue distribution from early stages of embryonic development to adults. Additionally, changes in their expression patterns occur during pathologies such as tumor metastasis [135], gastrointestinal disease [136] and preeclampsia [137]. Fingerprinting studies have shown that multiple galectin family members are concurrently expressed in a single tissue [19] [21]. In additon, galectins commonly exhibit a cell-type specific pattern of expression within a given organ. While overlapping expression of galectins is common, affinities to specific carbohydrate ligands are robust. Therefore, redundancy of expression may not translate into redundancy of function in the initiation and maintenance of biological processes.

Circumvention of the immune system, specifically Th1 derived cytokines, is critical to biological processes such as pregnancy in eutherian mammals [138, 139] and tumor metastasis [14]. During pregnancy, galectins are important in establishing an immune protected environment for intimate contact between the maternal and fetal cells during implantation and placentation. Fundamental to the process of tumor metastis is evasion of the host immune system. Evidence suggest that tumor evasion of host defense mechanisms is mediated by tumor-derived secretory factors (LGALS1 and LGALS9) that induce apoptosis of activated T lymphocytes [14]. In establishment and maintenance of pregnancy or tumor progression, multiple galectin proteins are expressesed in a temporal and cell-specific manner suggestive of redundancy of function, but this may actually “fine tune” biological processes. As a consequence, there is strong evidence for a role for galectins in conceptus survival and tumor

metastasis and a remarkable ability of these highly conserved proteins to use fairly divergent carbohydrate ligands to mediate biological processes.

***Classifications of Galectins***

*Prototype Galectins*

The majority of the 15 known members of the galectin family belong to the prototype subgroup. *LGALS1*, a monomeric galectin, is the original member of the galectin family discovered by screening a human hepatoma cDNA library with an antibody specific to a human soluble beta-galactoside-binding lectin [140]. At least three variants encoding this lectin are expressed in human tissue. The protein exhibits affinitiy for oligosaccharides containing multiple repeating units of disaccharide (3GalB1-4GlcNAcB1)n or poly-N-acetyllactosamine while terminal B-galactosyl residues are not necessary for high affinity binding of poly-n-acetyllactosamine to LGALS1 [11]. Additionally, LGALS1 can bind laminin and could promote cell adhesion to the ECM [11]. An effective approach to determine glycan specificity of galectins is the use of a glycan microarray containing many structurally different glycans and then validating these results with binding determinants on cells [141]. These studies have validated previous work showing LGALS1 binding affinity to poly-N-acetyllactosamine in addition to ellucidating new glycan binding affinities such as its specific binding to $\alpha$2-3, but not $\alpha$2-6 sialylated glycans. In this way, glycan binding specificity of each galectin underscores the basis for differences in biological activities of individual galectins as influenced by their respective cellular mileu.

Temporal expression of LGALS1 in the reproductive tracts of mice [18], humans [19], and cattle [142] are similar, with abundant expression early in the estrous or menstrual cycle. In cattle, LGALS1 appears to be only expressed in uteri of cyclic animals. Spatially divergent patterns exist when comparing mice and humans to cattle. In cattle LGALS1 is localized to uterine LE and GE, whereas in mice and humans it is mainly expressed in the stromal and decidual compartments with increased expression in the late secretory phase and in decidual tissue of human uteri [19]. These different spatial and temporal patterns of expression among humans, mice and cattle may reflect species-specific differences in placentation and may be important in the regulation of implantation. However, the argument for the utility LGALS1 alone or in concert with LGALS3 to implantation is weakened by the fact that implantation and reproduction is normal in *Lgals1*$^{-/-}$ mutant, *Lgals3*$^{-/-}$ mutant, and *Lgals1*$^{-/-}$/*Lgals3*$^{-/-}$ double mutant mice [22, 23]. These findings are tempered by the existence of other galectins, such as LGALS5, concomitantly expressed by conceptus trophectoderm during the peri-implantation period of pregnancy in mice [23].

*LGALS2* was discovered along with LGALS1 by screening a human hepatoma cDNA library with an antibody specific to a human soluble beta-galactoside-binding lectin [140]. The LGALS2 protein is a homodimer and glycan array studies indicate a high affinity for fucose-containing A and B blood group antigens and low affinity for all sialyated glycans [141]. Similar to LGALS1, LGALS2 has a high affinity for glycans with poly-N-acetyllactosamine sequences (GalB1-4GlcNAc)n when compared with N-acetyllactosamine glycans (GalB1-4GlcNAc) [141]. LGALS2 is presumed to play a

regulatory role in the intracellular trafficking of lymphotoxin-α (LTA) to which it binds. LTA is a pro-inflammatory cytokine and expression of some LTA SNP variants has been implicated as a risk factor for arteriosclerosis and cardiovascular diseases [143, 144]. Additionally, a SNP in the *LGALS2* gene encoding a variant of the galectin 2 protein results in increased transcription of *LGALS2*, a subsequent increased secretion of LTA and a degree of endothelial inflammation in establishing susceptiblility to myocardial infarction [145]. The efficacy of these findings in clinical practice is debatable because the positive correlation of LTA and LGALS2 polymorphisms on the incidence of myocardial infarction seems to be dependent on ethnicity of the patient, as similar studies in Japanese patients were inconsistent with previous reports [146, 147].

*LGALS5*, a monomeric prototype galectin with one CRD, was first detected in rat lung and kidney and initially identified as RL18 [148]. Early studies characterized RL18 carbohydrate binding to Galβ1-3GalNAc as well as lactose [149]. Following isolation and characterization of cDNA derived from rat erythrocytes, RL18 was designated LGALS5 [150]. Additionally, expression has been observed in mice peri-implantation conceptus trophectoderm cells suggesting a role in development [23].

*LGALS7* is another monomeric galectin initially discovered in a search for keratinocyte proteins differentially regulated in transformed cells that might play a role in maintenance of a normal phenotype [151]. Specifically, abundant proteins down-regulated in transformed cells are expected to play a role in cytoskeletal organization and cell-cell interactions because transformed cells often show altered morphology, are less adherent to neighboring cells, and usually reorganize the ECM. Similar to other

galectins, LGALS7 binds lactose and is secreted, but lacks a typical signal peptide. Its expression is tightly regulated at the protein level mainly in stratified squamous epithelial cells [151]. Intracellular location of the protein is primarily in the suprabasal compartment of epithelial cells in areas of cell-to-cell contact. LGALS7 cellular localization and its conspicuous down-regulation in cultured keratinocytes incapable of anchorage dependent growth imply a role in cell-cell and/or cell-matrix interactions necessary for normal growth control.

*LGALS10*, commonly refered to as Charcot-Leyden crystals (CLC), is a unique autocrystallizing component of esosinophils that form in bipyramidal crystals found in human tissues and secretions associated with increased numbers of peripheral blood or tissue eosinophils and basophils in parasitic and allergic processes. It was first observed by Charcot and Robin [80] in postmortem blood and spleen of a patient with leukemia and also by Leyden [152] in sputum of an asthmatic patient [81]. Historically, CLC were not studied as a lectin and are often ignored as seminal discoveries in lectinology. Initially, the crystals were characterized as single proteins possessing lysophospholipase activity [153] [154]. Cloning of the CLC cDNA revealed a ~ 16.5 kDa predicted protein with no sequence homology to any known sequences of lysophospholipase, phospholipases or other lipolytic enzymes, but with some similarities to members of the galectin superfamily of lactose-binding animal lectins [155]. Ambiguity in the role of CLC as bifunctional lysophospholipases and lectins was clarified with elegant experiments that tested the lysophospholipase activity of CLC depleted eosinophil lysates [156]. Indeed, eosinophil lysates depleted of CLC proteins retained their

lysophospholipase activities while purified CLC proteins from depleted eosinophils did not. X-ray crystallographic studies of the structure of CLC protein showed that its overall tertiary fold was highly similar to the fold found in prototype galectins [157]. Similar studies revealed that CLC protein has a high affinity for mannose, but not β-galactosides, and binds mannose via its CRD in a unique manner that differs from carbohydrate binding by other galectins [103].

CLC is genetically related to members of the galectin gene family. The CLC protein is encoded on four exons with the third exon encoding the carbohydrate binding domain [158]. This is identical to that for LGALS1, LGALS2, LGALS15 (SKL unpublished data) and LGALS3 in which the carbohydrate binding domain is encoded only by the fifth exon [109, 159]. Eosinophils express several proteins that are restricted to these lineages, including the IL-5 receptor alpha subunit (IL-5Ra), and CLC protein. This presents an interesting phenomenon at the transcriptional level because the CLC promoter should exhibit unique cis-regulatory elements that confer some specificity for expression in eosinophil lineages. Indeed a 562 bp region 5′ of the transcription start site has promoter activity and consensus sites for the eosinophil transcription factor EoTF (GGAGA[G/A]) and GATA-1 that when mutated, disrupted promoter activity [160]. Analysis of the minimal promoter revealed nine consensus-binding sites for transcription factors, including several also found in minimal promoters of LGALS1, LGALS2, and LGALS3 [161].

*GRIFIN* or galectin-related inter-fiber protein is a novel, highly abundant soluble lens specific protein believed to be a member of the crystallin family of proteins that

assist the lens in focusing light on the retina [162]. Localization of GRIFIN protein is developmentally regulated only in the lens, localized intracellularly to the lateral compartment of lens fiber cell in adults, but more centrally within lens fiber cells in neonates [162]. Interestingly, the GRIFIN gene has two sets of large repetitive DNA segments, one in the 5′promoter region and another that encompasses the 3′splice site of exon IV [162]. Furthermore, the repetitive DNA segments contain potential binding sites for the transcription factor δEF1 [162] believed to confer lens-specific expression of some genes.

Previously, GRIFIN was considered a galectin-related protein because it lacked affinity for lactose and there was little evidence for a functional carbohydrate binding domain. Despite this, mammalian GRIFIN is commonly accepted as LGALS11 [163] [32], seemingly a divergence in the definition of lectin proteins based fundamentally on their glycan binding affinity. Consideration of non-mammalian homologues of GRIFIN reveal that it does bind to lactose glycans [164]. In fact, a homologue identified in zebrafish (*Danio rerio*) and designated DrGRIFIN, is also expressed in the lens, particularly in fiber cells. In adult zebrafish, however, DrGRIFIN is also expressed in oöcytes, brain, and intestine and unlike the mammalian equivalent (lacking two out of seven amino acids), DrGRIFIN contains all amino acids critical for ligand-binding and binds to β-galactosides [164].

*Placental Protein 13 (PP13)* was first isolated and purified from human term placenta [165]. Immunoscreening of a term placenta cDNA library with antibodies derived from anti-PP13 serum resulted in the PP13 coding sequence being cloned [166].

The predicted protein conatains 139 amino acid residues, a molecular mass of 16.1 kDa and it contains a putative N-glycosylation site in its N-terminus and several potential phosphorylation sites [166]. Sequence analyses, alignments and computational modelling identified its conserved structural and functional homology to members of the galectin family and it was designated LGALS13 [167]. The protein was found to be a homodimer of 16 kDa subunits linked by disulphide bonds, a phenomenon differing from noncovalent dimerization of known prototype galectins, and reducing agents decrease its sugar binding activity [167]. Phosphorylation of the purified protein was confirmed and proteins such as; annexin II and beta/gamma actin were identified as proteins bound to PP13 in placental and fetal hepatic cells [167]. LGALS13 shares 69% amino acid identity with CLC protein and exhibits lysophospholipase activity [168]. LGALS13 protein has been detected in placenta, fetal and adult spleen, fetal kidney, adult bladder and some tumor tissues [166]. LGALS13 exhibits carbohydrate binding affinity to N-acetyl-lactosamine, mannose and N-acetyl-glucosamine residues [168], all of which are abundant in the placenta. Recently, PP13 in maternal serum during the first trimester has been used as a diagnostic indicator of preeclampsia in women [137]. Low levels of PP13 in early pregnancy identify at-risk pregnancies, whereas high levels precede the syndrome in late pregnancy and suggest necrosis of the syncytiotrophoblast [169, 170].

*LGALS14*, previously called Charcot-Leyden Crystal protein 2 (CLC2) and placental protein 13-like protein (PPL13), was initially discovered by screening a human 18-week fetal brain library [171]. However, *LGALS14* expression is most abundant in

placentae as two isoforms due to alternatively spliced transcript variants. LGALS14 protein can be sequestered in nuclei even though it lacks a known nuclear localization signal. Human LGALS14 shows 78%, 67% and 54% identity with prototype galectins PP13, LGALS10, and LGALS15 in amino acid sequence, respectively, and all four of these galectins contains 7 of the 8 conserved amino acids (H.N.R.N.W.E.R) of the CRD thought to be important for carbohydrate binding by all galectins. In the same year that human LGALS14 was characterized, ovine LGALS14 was discovered and shown to also be an eosinophil specific galectin secreted in response to an allergic reaction [172]. Ovine LGALS14 shows 57% identity with tandem repeat galectin, human LGALS9/ecalectin, in amino acid sequence. LGALS14 is clearly a prototype galectin with only one C-terminus CRD. However LGALS14 has an extended N-terminus uncharacteristic of other known prototype or tandem repeat galectins. In fact, only chimeric LGALS3 has such an extended N-terminus. However LGALS14 N-terminus is much shorter than that of LGALS3 and does not contain a proline and glycine-rich repetitive sequence characteristic of chimeric galectins. Validation of the eosinophil specific expression and a functional analysis of LGALS14 was conducted by this same group.

Young et. al [173] proved that LGALS14, similar to LGALS10, is uniquely expressed and secreted by eosinophils especially following an allergic reaction or infections caused by helminth parasites. In fact, they showed that LGALS14 is spontaneously released by eosinophils derived from allergen challenged mammary gland lavage fluid, but not from resting peripheral blood eosinophils. Functionally, LGALS14

exhibits carbohydrate binding activity as glycan array screening revealed affinity for type 2 polylactosamine glycans Galβ1-4GlcNAc, α2-6-sialylated glycans and highest affinity to lacto-*N*-neotetraose (LNnT) oligosaccharides expressed by helminth parasites. This LNnT oligosaccharide skews the immune response toward a Th2-type mediated response and suppresses Th1-type and inflammatory responses [174]. LGALS14 is believed to function in cell adhesion because it is secreted especially at basolateral epithelial surfaces following eosinophil infiltration where it specifically binds laminin, epithelial cells lining the gastrointestinal tract, eosinophils and other inflammatory cells in the local tissue environment such as neutrophils and lymphocytes. Interestingly, *LGALS14* is constitutively expressed by ovine eosinophils and is only secreted by eosinophils that have infiltrated a tissue in response to an allergic or parasitic stimuli [174].

*LGALS15*, the newest member of the galectin super-family, was initially discovered in sheep abomasal tissue infected with the nematode parasite, *Haemonchus contortus,* and designated OVGAL11 [24]. The tissue in which the galectin was upregulated was subject to inflammation and eosinophil infliltration. Immunohistochemistry revealed that the protein was localized in the cytoplasm and nucleus of the upper epithelial cell layer of the gastrointestinal tract and in mucus collected from infected abomasal tissue suggesting that it was secreted. Similar to other galectins, it lacks a typical signal peptide.

Recently, LGALS15 was discovered in the endometrium of sheep by gene expression profiling to understand recurrent early pregnancy loss in UGKO ewes [3]. In

the ovine uterus, *LGALS15* mRNA was detected only in the endometrial LE and sGE [3]. At these intercaruncular areas, finger-like villous projections of trophoblast extend into the lumen of the mouths of uterine glands to establish contact with sGE which effectively anchoring the pre-attachment conceptus and absorb histotroph from uterine glands [42]. Furthermore, in the ovine uterus, LGALS15 is an abundant component of uterine histotroph [3]. Ovine endometrial expression of *LGALS15* is induced by progesterone and further stimulated by IFNT [3] coordinate with early conceptus elongation, growth and development during the peri-implantation period. In line with its spatial and temporal expression patterns in the ovine uterus, *LGALS15* represents one of a growing list of non-classical interferon-stimulated genes (ISG) expressed by LE and sGE which may regulated by a novel JAK/STAT-independent cell signaling pathway [175-177].

Ovine endometrial LGALS15 contains a conserved CRD that binds β-galactosides [3] and predicted cell attachment sequences (LDV and RGD) that could mediate binding to integrins [178] [179]. Results of recent *in vitro* studies suggest a role for LGALS15 in cell migration and attachment which are integrin-mediated and involve formation of focal adhesions which transmit force at adhesion sites and serve as signaling centers from which intracellular signaling pathways emanate [180]. Interestingly, these adhesive and migration functions of LGALS15 are independent of the CRD, but dependent on the C-terminal RGD integrin recognition sequence. Other galectins bind fibronectin and laminin as these proteins are modified with carbohydrate ligands decoded by lectins [181]. Recently, animal and plant lectin interactions with

non-carbohydrate ligands (lectin-protein interactions) were proposed to mediate many of their divergent functions [182, 183] [184]. So, it is not surprising that LGALS15 mediates it adhesive and migratory roles in the ovine uterus independent of its prototypical CRD.

LGALS15 may be the 14K protein from sheep endometrium initially characterized as a progesterone-modulated protein associated with crystalline inclusion bodies in uterine epithelia and conceptus trophoblast [185]. Immunogold electron microscopy revealed that within trophoblast, the 14K protein was localized to large, membrane-bound rhomboidal or needle-shaped crystal structures. Thus, it was suggested that the protein was secreted by the endometrial epithelia, taken up by the conceptus from uterine histotroph, and assembled into crystals [185]. These crystals are first observed in the sheep trophoblast on Day 10 and then increase in number and size between Days 10 and 18 of pregnancy [186]. These crystalline inclusion bodies are observed in endometria and conceptuses in other animals such as mice, but they are more prominent in the ovine species [186], [187]. There is no evidence to suggest the presence of these progesterone-induced crystalline inclusions in endometria or conceptuses of cattle. Interestingly, development in the sheep uterus of *in vitro* produced bovine blastocysts resulted in the presence of crystalloid bodies in trophectoderm cells of elongated blastocysts [188]. The association of intracellular LGALS15 protein with crystalloid bodies, is reminescent of CLC proteins. One can only speculate on the functions of intracellular LGALS15 crystals within the scope of other galectins.

*Tandem Repeat Galectins*

*LGALS4* was characterized by differential display analysis showing that its expression is significantly decreased during colorectal carcinogenesis [189]. The LGALS4 protein contains approximately 150-amino acids, a CRD and all amino acids typically conserved in galectins. Its expression is restricted to the small intestine, colon, and rectum. A cDNA encoding LGALS4 was cloned from a human colon adenocarcinoma cell line [190]. *In vitro*, the cellular adhesive properties of recombinant LGALS4 in addition to its differential intracellular localization in confluent (cytosolic near basal membrane) versus subconfluent (leading edge of lamellipodia) cells, suggest that it functions in cell adhesion [190]. *LGALS4* is expressed in spermatozoa and oöcytes and in 8-cell embryos and later stages of embryonic development [191] suggesting a role in development and cell differentiation. The LGALS4 holoprotein has a high affinity for blood group A and B structures based on a glycan array study [191]. Additionaly, it bound to sulfated lactose and, with high affinity, GalNAcα1-3GalNAc. The two CRD of LGALS4 demonstrate differential ligand binding between blood group antigens A and B with CRD1 specific for type-2 blood group B structures and CRD2 specific for type-2 blood group A structures [191].

*LGALS8* is unique as it has members in both the prototype and tandem repeat subfamilies of galectins. LGALS8 was initially discovered as a tandem repeat galectin encoded by the *LGALS8* gene that encodes many mRNAs by alternate splicing and contains three unusual polyadenylation signals [192]. These mRNAs encode six different isoforms of LGALS8: three are tandem-repeat and three are prototype galectins [192].

Unlike LGALS1 and LGALS3, LGALS8 can inhibit adhesion of human cells [193]. When bound to the α3β1 integrin in a carbohydrate dependent manner at the cell surface, LGALS8 not only inhibits integrin-mediated carcinoma cell adhesion, but also induces apoptosis [193]. In fact, edogenous LGALS8 may have a negative effect on tumor progression in the early stages of tumor metastasis because cells transfected with LGALS8 cDNA showed significantly reduced colony formation [193]. Characteristic differnces in carbohydrate binding specificity in comparison to LGALS1 and LGALS3, likely explain the specific interactions of LGALS8 with human carcinoma cells. Divergent carbohydrate binding affinities are characteristic among members of the galectin family with overlapping patterns of expression. Conversly, immobilized LGALS8 can induce cell adhesion that is carbohydrate dependent [194]. In Trabecular Meshwork (TM) cells, LGALS8 stimulates adhesion and spreading by interacting with α2-3-sialylated, but not α2-6-sialylated glycans on β1 integrins [195]. The α2-3-sialylated glycan has a high affinity for LGALS8, but not LGALS1 or LGALS3. Additionally, α3β1, α5β1 and αvβ1 integrins proved to be major receptors for LGALS8 in TM cells [195]. Characteristically, the functions of galectins are fine-tuned by the glycan complement that can orchestrate a medley of specific functions in a given tissue.

*LGALS9*, isolated from mouse embryonic kidney cells [196], is a 36-kDa β-galactoside binding protein with two distinct N- and C-terminal CRD connected by a link peptide. Structurally, a 31-amino acid insertion between the N-terminus and the linker peptide results in an isoform of LGALS9 expressed exclusively in the small intestine [196]. Similar to LGALS1 and LGALS3, expression of LGALS9 is

developmentally regulated [196] with increased expression specifically in the thymus and liver of Day 13 mouse embryos. It is believed to regulate thymocyte-epithelial interactions via its role in selective induction of apoptosis in thymocytes during positive or negative selection. The human homolog of LGALS9 was first detected and isolated by immunoscreening a cDNA expression library derived from tissue involved in Hodgkin's disease [197]. Similar to mouse LGALS9, the the C-terminal CRD of human LGALS9, is highly homologous to rat LGALS5 with 70% amino acid sequence identity. Additionally, human LGALS9 has an allelic variant designated ecalectin, that is believed to be an important T lymphocyte-derived regulator of eosinophil recruitment to tissues during inflammatory reactions [198]. LGALS9 is the first identified human urate transporter (hUAT) [199] and it has high sequence homology and cellular distribution with rat UAT [200]. Human UAT was localized to plasma membrane in multiple epithelium-derived cell lines and, in polarized cells, it was targeted to both apical and basolateral membranes [200]. The amino- and carboxy-termini of hUAT were both detected on the cytoplasmic side of plasma membranes, but the protein does contain at least one extracellular domain. These results are convincing; however, they have not been linked to any inherited defects leading to high levels of uric acid excretion in urine [201].

In adult tissues, *LGALS9* is expressed in endometria of diverse species such as humans and cattle [19-21] and its temporal and spatial expression during the peri-implantation period in these species indicate that it may be involved in establishing a uterine environment receptive to a developing conceptus. In ruminants, maternal

recognition of pregnancy is mediated by IFNT which is associated with activation of many ISG in the endometrium required for successful pregnancy. Indeed LGALS9 is a novel type I ISG [202]. The peri-implantation period in many species is characterized by the typical expression profile of multiple galectin family members by the endometrium and conceptus trophectoderm [9, 19, 21, 142]. Interestingly, LGALS9 exhibits intermolecular interactions with itself or carbohydrate ligands, as well as other galectin family members such as LGALS3 and LGALS8 [203], adding another level of complexity in understanding the many functions of galectins.

*LGALS12* was initially discovered by sequencing a randomly selected expression sequence tag clone from a G1-phase Jurkat T-cell cDNA library [133]. The deduced 314-amino acid protein lacks a signal sequence and transmembrane domain, like other galectins. LGALS12 contains two CRDs separated by a linker sequence and it exhibits affinity for lactose. LGALS12 is expressed in heart, pancreas, spleen, thymus, and peripheral blood leukocytes and at lower levels in lung, skeletal muscle, kidney, prostate, testis, ovary, and colon, but there is little to no expression in brain, placentae and liver [133]. Additionally, its expression is detectable in hematopoietic and immune cell lines, but not other cell lines tested. *LGALS12* expression is up-reglated in cells blocked in G1, but not in synchronized cells in the mitosis phase of the cell cycle. In fact, overexpression of LGALS12 but not LGALS9, resulted in G1 cell cycle arrest and abrogation of proliferation in cancer cell lines [133]. Thus *LGALS12* is believed to be a tumor suppressor gene. Notably, LGALS12 is abundantly expressed in nuclei of human and mouse adipocytes and increasing its expression reduced the size of adipocytes and

increased apoptosis [204]. Further, expression of LGALS12 was increased in mouse preadipocytes undergoing cell cycle arrest, which is concomitant with differentiation in response to adipogenic hormone stimulation [205]. Thus, LGALS12 is believed to be a major regulator of adipose tissue development.

*Chimeric Galectin*

The only know chimeric galectin is LGALS3 previously known as Galactoside-Binding Protein (GALBP) and Macrophage Galactose-Specific Lectin (Mac-2). Mouse LGALS3 protein binds galactose and IgE secreted by inflammatory macrophages and it interacts with laminin suggesting involvement in cell-ECM interactions [206]. LGALS3 is the most studied of the galectin family members due to its broad distribution in both normal and abnormal tissues and its relevance to both immune responses and tumor progression. At the protein level, LGALS3 is highly conserved. In fact the human homolog of LGALS3 was characterized and the deduced protein revealed 85% identity with mouse LGALS3 especially in the functional CRD [206]. The ribonucleoprotein-like N-terminal domain, containing the proline-glycine-alanine-tyrosine repeat motif, is entirely within exon III and the CRD is entirely within exon V [207]. Unique in its structure, which includes a long N-terminus of undefined function, LGALS3 is an intriguing molecule with seemingly opposing functions depending on the cellular milleu. LGALS3 may participate in cell differentiation based on its developmentally, temporally and spatially regulated expression during embryogenesis [9], [17, 208]. LGALS3 protein is present at the interface of intimately apposed maternal and fetal tissues. It is reasonable to propose a function for LGALS3 during early pregnancy in facilitating

adhesion of the maternal epithelium and the conceptus trophectoderm given its established interactions with cell surface proteins such as integrins [209, 210].

***Galectins and Biological Processes***

*Development and Tissue Regeneration*

To investigate the roles of galectins in development and immune regulation, *Lgals1*$^{-/-}$ and *Lgals3*$^{-/-}$ mice were produced [22, 23] and found to have no obvious phenotypes, even in *Lgals1*$^{-/-}$/*Lgals3*$^{-/-}$ or *Lgals1*$^{-/-}$/*Lgals3*$^{-/-}$ double knockout mice. However, *Lgals1*$^{-/-}$ mice exhibited olfactory neurons with altered neurite outgrowth and targeting, demonstrating a role for lgals1 in neural development [211].

Galectins are involved in the initiation and progression of many diseases and natural regenerative processes in humans such as cancer, gastrointestinal disorders and wound healing. In *LGALS3* null mutant mice, corneal wound healing is delayed due to a slower rate of re-epithelialization of the wound when compared to wild type mice [212]. Interestingly, gene expression profiling revealed that the healing corneas of *Lgals3* null mice had reduced levels of *Lgals7*. The delayed wound healing phenotype could be reversed by administration of exogenous Lgals7, but not Lgals3 proteins [212]. Thus, molecular interactions involving Lgals3 and Lgals7 appear to be important for re-epithelialization of corneal wounds.

*Disease Initiation and Progression*

LGALS9 can block lung cancer metastasis when transfected into highly metastatic cancer cell lines [135]. Many adhesive molecules including CD44, integrins $\alpha$1, $\alpha$4, $\alpha$V and $\beta$1 are expressed concurrently in lung cancer cells. Thus LGALS9 may

suppress both attachment and invasion of tumor cells by antagonizing binding of adhesive molecules on tumor cells to ligands on vascular endothelial cells and to ECM. Similar negative effects on cancer progression have been observed for LGALS8 which suppresses tumor growth rate and cell migration [213]. Interestingly, the expression of LGALS8 and LGALS9 is decreased in these cancers once they acquire an aggressive metastatic phenotype.

*Immune Function and Reproduction*

Immune functions in *Lgals1*$^{-/-}$ and wild-type mice were not different; however, *Lgals3*$^{-/-}$ mice exhibited defects in inflammatory responses involving altered inflammatory cell dynamics during acute peritonitis [23, 214]. Results obtained from *in vitro* studies, such as the ability of LGALS1, LGALS2 and LGALS9 to induce T cell apoptosis [14, 215], suggest functional redundancies among galectin family members. The *in vitro* data is supported by gene ablation studies in mice, further supporting functional redundancy of galectins *in vivo*. Eosinophils represent a unique cell type that exhibits restricted expression of at least two galectins, *LGALS10* and *LGALS14*. Preliminary results suggest that lentiviral shRNA knockdown of *LGALS10* expression in developing human eosinophils impairs granulogenesis [216]. To examine functional redundancy *in vivo* and to understand clearly the roles of galectins in various biological processes, mice with mutations in multiple galectins representative of the tissues under investigation must be generated.

Galectins are thought to be necessary during reproduction in eutherian mammals. Expression of the L-14 lectin (LGALS1) is abundant during mouse embryogenesis and

suggests that it has multiple roles during pre- and post-implantation development and cell differentiation [16]. It is initially expressed by trophectoderm of expanded blastocysts immediately prior to implantation suggesting a role in the attachment to uterine LE. Correlative studies of human tissue demonstrated increased expression of LGALS1 and LGALS3 by extravillous trophoblast (EVT) in pre-eclamptic placentae, but no differences between normal control placentae and placentae of fetuses experiencing intra-uterine growth restriction (IUGR) [217]. LGALS1 binds to the Thomsen–Friedenreich (TF) antigen (Galβ1-3GalNAc) [218] expressed apically by the syncytiotrophoblast on extravillous trophoblast cells invading the decidua in the first and second trimesters, and on trophoblastic tumor cells (BeWo) *in vitro* [219]. Expression of the TF antigen is significantly up-regulated in IUGR and preeclamptic extravillous trophoblast cells which correlates with increased expression of LGALS1 decidual tissue of preeclamptic placentae [217]. The binding of LGALS1 to the TF antigen on trophoblast cells could play an important role in successful implantation of the conceptus in endometria of humans.

There is established evidence for the presence of multiple galectins such as LGALS1 and LGALS2 at immune-privileged sites [18, 217, 220]. Therefore, in addition to its suggested adhesive role during implantation, LGALS1 could exert immunosuppressive functions at the conceptus-maternal interface. Preeclampsia is generally attributed to maternal endothelial dysfunction, poor placentation and an increased maternal inflammatory response ultimately resulting in poor trophoblastic invasion into maternal spiral arteries. A high Th1/Th2 cell ratio at the conceptus-

maternal interface may be important in the establishment of preeclampsia [221]. Both LGALS1 and LGALS2 have been implicated in the induction of apoptosis specifically in activated Th1 type lymphocytes [14, 215]. Indeed, an environment favoring Th1 cellular responses is associated with increased inflammation, endothelial dysfunction and poor placentation [222] potentially resulting in decreased tolerance to the fetal semi-allograph and thus poor invasion of the trophoblastic tissue into the endometrium.

**Regulation of Galectin Gene Expression**

Complex mechanisms are involved in the transcriptional control of galectins. Studies of galectins have indicated modulation of their expression during development [223] and under different physiological or pathological conditions [28, 224] in addition to restriction to specific cell lineages. Tissue glycosylation and thus glycan patterns follow similar modulations in expression [225, 226]. As previously noted, the actions of galectins are not exclusively dependent on their CRD. Thus, modulation of expression of different galectins and their glycan and/or non-glycan ligands is finely tuned or even coordinated.

Studying transcriptional regulation of galectins is particularly useful to understanding the pathophysiology of many cancers and disorders of the gastrointestinal tract as expression of galectins is modulated in these tissues. In mammalian species, the digestive tract alone expresses nine members of the galectin family including; *LGALS1, LGALS2, LGALS3, LGALS4, LGALS6, LGALS7, LGALS8, LGALS9,* and *LGALS15* [213, 223]. They are involved in the development and progression of malignancies in the digestive tract, mainly in colorectal cancers [213]. Some galectins are also involved in

inflammatory bowel diseases [136]. So detailed examinations into mechanisms whereby galectins are regulated at the transcriptional level will reveal fundamental characteristic of disease processes.

The upstream regulatory regions of *LGALS1, LGALS2, LGALS3, LGALS4, LGALS6, LGALS9, LGALS10, LGALS11* and *LGALS12* from different species have been cloned [109, 159, 162, 199, 204, 207, 227-231]. The following will detail some of the information regarding transcriptional regulation of galectins, including those believed to be important for successful reproduction in mammalian species such as LGALS1, LGALS3, LGALS9, LGALS15 [9, 18, 19, 21, 142, 208, 232].

The genomic region of *LGALS1* and *LGALS2* were the first to be characterized [109, 159]. In the *LGALS1* gene promoter region, a small segment (−63/+45) spanning the transcription start site (+1) and a Sp1 site (-57/-48) is critical for promoter activation [233]. A consensus initiator Inr sequence (TCCAGTT) located at -34/-28, overlaps the TATA box, and directs RNA initiation from a previously uncharacterized site located at -31. Thus transcriptional initiation can be initiated from both start sites [234]. The LGALS1 gene promoter is under the control of various agents. The region -62/-41, which contains an Sp1 site at -57, is important for the induction of LGALS1 promoter activation by butyrate [231]. Gel shift studies indicate that the Sp1 transcription factor binds an Sp1 site with the proximal promoter region. *In vivo*, glycosylation of mucins is important to their many functions at epithelial surfaces. In the colon, mucin glycosylation can be modified by luminal metabolites of fiber fermentation like butyrate that markedly increase LGALS1 gene expression by 8- to 18-fold [235]. At the

promoter level, the *LGALS1* distal promoter confers responsiveness to retinoic acid (RA) [236]. A strong RA responsive region within the -1578/-1448 region upstream of the transcription start site (+1) is at least in part responsible for the inducible expression of LGALS1. In this system, constitutive expression of LGALS1 was mediated by a sequence (-62/+1) within the proximal promoter region which contains an Sp1 consensus sequence [231, 236]. Again this transcription factor binds the LGALS1 proximal promoter region and the cis-motif is critical for activation of the promoter. In the reproductive tract, LGALS1 transcripts are up-regulated during the peri-implantation period in the uterus and ovary [18]. In the mouse uterus, *LGALS1* expression is regulated by the ovarian steroids progesterone and estrogen and is correlated with blastocyst implantation [237]. These results illustrate the inducible nature of the *LGALS1* gene in the gastrointestinal and reproductive tracts at the level of the promoter.

The murine *LGALS3* gene is composed of six exons and sequence analysis revealed a consensus Inr sequence instead of a TATA box [228]. Functional characterization of the *LGALS3* promoter revealed a small genomic region (-339/+141) important for trascriptional activation that is considered an early immediate gene since its expression is rapidly increased upon serum stimulation [207]. The serum responsive region mapped to -513 /-339 and -339/229, but lacked consensus serum response element (SRE) binding sites [207]. In tissues with limited vascular supply resulting in hypoxia, *LGALS3* expression is modulated. In this context, hypoxia inducible factor-1$\alpha$ (HIF-1$\alpha$) regulates *LGALS3* expression by interacting with hypoxia regulatory elements in the promoter region [238]. Results of studies with human osteosarcoma cell lines

suggest that *LGALS3* has transcripts initiated from a promoter upstream of exon I but also from an internal promoter located within intron II [239]. In fact, a gene embedded within the human *LGALS3* gene named galectin 3 internal gene (*GALIG*), is tightly regulated, with expression limited to activated peripheral blood leukocytes and transcripts producing a secreted protein unrelated to LGALS3 [240]. *GALIG* encodes a protein named mitogaligin which targets the mitochondria and is involved in cytochrome C release [241]. Mitogaligin expression in human cells is associated with morphological changes such as cell shrinkage, cytoplasmic vacuolization, nuclear condensation, and ultimately cell death [241]. Thus, it appears that a novel gene transcribed internally within the LGALS3 gene, may be involved in cell death.

Human *LGALS10* promoter constructs have been functionally analyzed in an attempt to identify DNA elements that regulate gene expression during commitment and differentiation of the eosinophil lineage [227]. The -292/-411 region of the *LGALS10* promoter is responsible for restricting expression to the eosinophil lineage. The *LGALS10* promoter contains two consensus GATA binding sites at -11 (on the sense strand) and -207 (on the antisense strand). A purine-rich element is present at -180/-175 on the antisense strand and -65/-60 on the sense strand that is identical to the binding site of the myeloid- and B-cell-specific ets-related transcriptional activator PU.1, as well as sequences described in other myeloid-specific genes [227]. Functionally, the proximal promoter region of *LGALS10* contains binding sites for transcription factors such as Sp1, Oct, GATA and EoTF that, when mutated, reduce activity observed in wild type promoter constructs [160, 161]. Indeed, Sp1 and Oct transcription factors bind the

*LGALS10* promoter. Furthermore, *LGALS10*, similar to *LGALS1*, is induced by butyrate treatment which requires a functional Sp1 site in the proximal promoter [161].

Interestingly, the *LGALS11* gene promoter contains repetitive DNA segments within the coding and non-coding sequence consisting of consensus sequences sites for C-myb and *δ*EF1 [162]. These sites may restrict expression of LGALS11 to the lens. Similar to *LGALS10*, the human *LGALS12* promoter, contains transcription factor binding sites for Sp1, AP2, and a CCAAT/enhancer-binding protein (C/EBP) commonly found in adipocytes [204]. These transcription factors may be involved in its restricted expression.

A common theme in understanding galectin gene expression is tight transcriptional regulation, resulting in restricted tissue expression. Functionally, galectins present many convoluted interactions, determined by specific cell types and/or pathophysiologic environments resulting in a variety of intracellular responses and biological functions. Given this complexity, it is reasonable to imagine that transcriptional control of galectin gene expression is not the exclusive task of transcription factors available in a cell.

***Epigenetic Control of Gene Expression***

Transient changes in chromatin structure by, mechanisms that alter specific nucleotides and/or proteins are important for the control of mammalian gene expression in adult cells and tissues. This effectively adds another layer of control at the level of the gene promoter. Common manipulations affecting gene transcription include DNA methylation at cytosine paired guanine (CpG) dinucleotides [242] in addition to

methylation and/or acetylation [243] of specific amino acid residues of histone proteins. In fact, DNA methylation and histone deacetylation act as synergistic layers of transcriptional regulation for the silencing of genes especially in cancer, but dense CpG methylation is dominant in conferring stable maintenance of a silent state at these loci [244]. In mammalian cells, DNA methylation is associated with long-term transcriptional silencing and in heterochromatin formation. The methylation of DNA is considered an epigenetic modification and thus is heritable from one mitotic cycle to the next with high fidelity.

DNA methyltransferases (DNMT) methylate DNA with S-adenosylmethionine (SAM) as the methyl group donor (Figure 2.6). There are two types of DNMT: de novo DNMT (DNMT3a and DNMT3b) and maintenance DNMT (DNMT1) [245, 246], although DNMT3b may assist in maintaining the methylation profile in adult tissues [247, 248]. Methyltransferases bind DNA and methylate cytosine residues at the 5′ positions. Commonly, cytosines located 5′ of guanine residues called cytosine-paired-guanine or CpG cytosines, are the target for DNA methyltransferases (Figure 2.6). These CpG dinucleotide sequences are surprisingly sparse within the genome of mammals.

The CpG islands (CGI) classify regions of the genome where CpG dinucleotides occur more frequently. Initially, CGI were defined as regions of the genome longer than 200 bp, containing 50% G+C content, and an observed CpG to expected CpG ration of 0.6 [249]. The fundamental criteria for determining a CGI became more stringent in order to exclude Alu repeats. Currently, regions of 500 bp in length, a G+C content of 55%, and an observed to expected CpG ratio of 0.65 are accepted as basic requirements for a CGI [250]. It is widely accepted that repetitive interspersed DNA sequences and endogenous retroviruses are targets of DNA methylation [251-254]; therefore, these sequences must be screened out of genome-wide analysis of DNA methylation patterns. Other than interspersed repetitive DNA sequences, CGI are often found in promoter regions and about 40% of genes contain CGI that are situated at the end of the 5′ region (promoter, untranslated region, and exon I) [255]. Other regions of the genome have a low density of CpG dinucleotides. With the exception of the inactive X chromosome [242], CpG poor regions of chromosomes in healthy cells are usually methylated while CGI are generally hypomethylated [28].

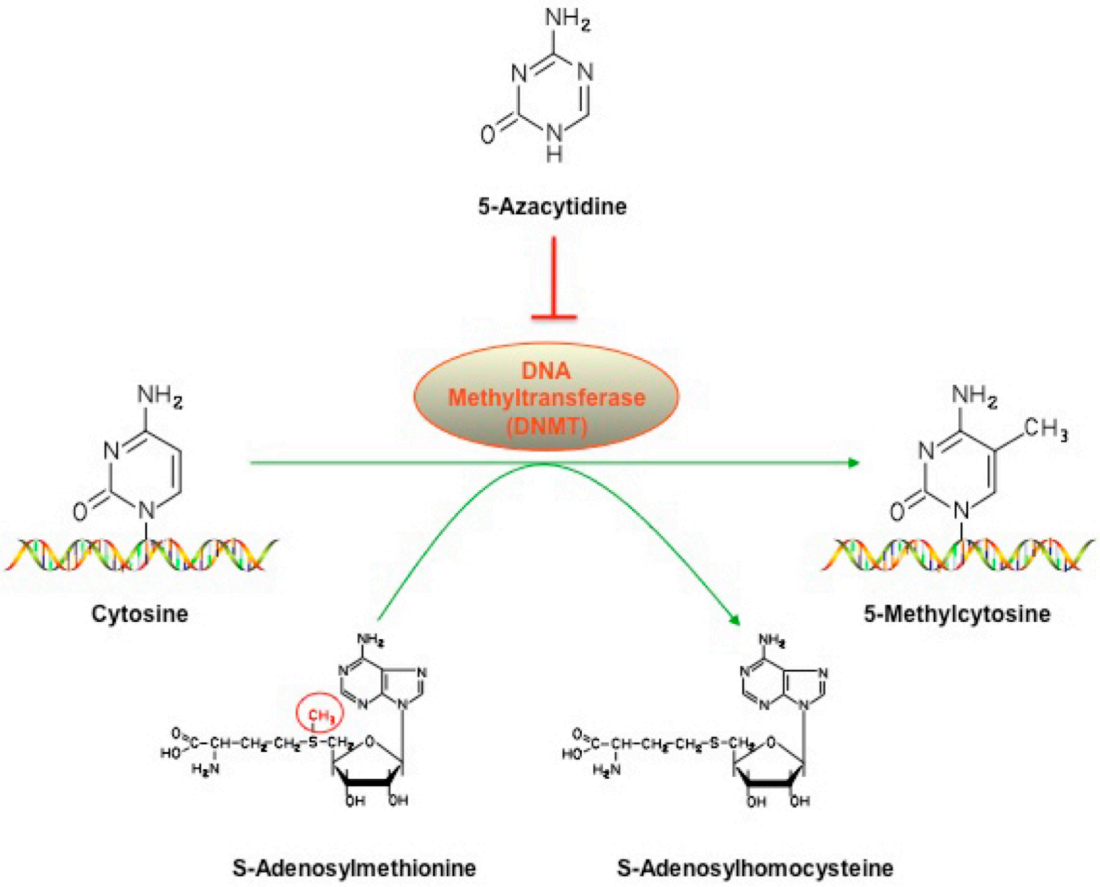


Figure 2.6. Pathways for methylation of cytosine residues in mammalian genome and effects of 5-azacytidine. A family of three active enzymes, the DNA methyltransferases (DNMTs), catalyzes methylation at carbon atom 5 of the cytosine ring, using *S*-adenosylmethionine as the donor molecule for the methyl group ($CH_3$). The drug 5-azacytidine can block this reaction. When this compound is incorporated into the DNA, replacing the natural base cytidine, it acts as a direct and irreversible inhibitor of the DNMTs, since it contains a nitrogen in place of carbon at the 5 position of the cytidine ring. Drawn using ScienceSlides Suite 2008 Fall Edition for Mac from VisiScience.

During the development of cancer, CGI undergo hypermethylation while the CpG poor regions become hypomethylated. This reversal in DNA methylation pattern leads to changes in chromatin structure and accessibility, causing silencing of tumor suppressor genes [255].

The role of CGI methylation in normal development and cell differentiation is highly debated. Accepted dogma states that in normal cells/tissue, CGI associated with gene promoters are typically unmethylated. However, results suggest that in normal cells/tissue, genes with tissue specific expression patterns including placental lactogen (CSH1), prolactin (PRL) and growth hormone (GH1), exhibit tissue-dependent differentially methylated regions (T-DMR) of the promoter that determine their restricted expression patterns [30, 31]. Recently, genome-wide profiling of DNA methylation revealed a class of densely methylated CGI promoters in normal somatic tissues [256, 257]. Apparently these regions escape methylation in germline cells, and DNA methylation is a primary mechanism of tissue-specific gene silencing [256]. These T-DMRs correspond to CGI of moderate to low CpG dinucleotide density (< 10% of the sequence) and methylation of these regions is associated with restricted gene expression.

The effects of DNA methylation on transcription and chromatin structure require that nuclear factors distinguish methylated from unmethylated DNA. Indeed, a methyl-CpG binding protein (MeCP) forms a complex with a variety of unrelated DNA sequences when they are methylated at CpG dinucleotides [258] (Figure 2.7). Strong binding of MeCP to DNA sequences in the formation of a multi-unit complex requires a threshold density of methylated CpG dinucleotides [258]. Interestingly, vertebrate DNAs bind to MeCP, whereas naturally unmethylated genomes or cloned vertebrate genomes do not bind [258]. MeCP-1, a methyl-CpG binding protein, binds strongly to densely methylated gene promoters to repress transcription [259] (Figure 2.7). While sparsely methylated gene promoters form weaker interactions with MeCP-1, the transcriptional repression can be overcome depending on the strength of the promoter [260]. Thus, strong evidence correlates promoter methylation with MeCP mediated transcriptional repression [261], possibly resulting in chromatin modifications in mammalian cells [262].

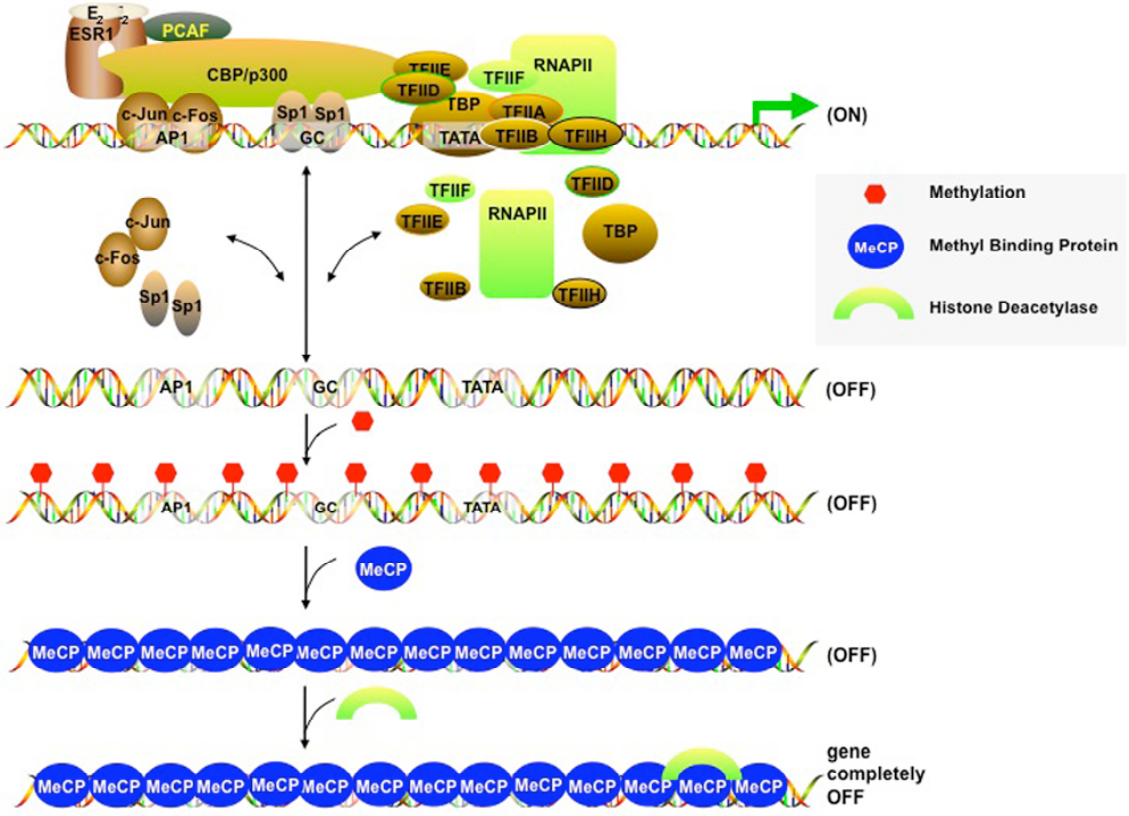


Figure 2.7. Switching genes off through DNA methylation and histone modification. In its unmodified state, the mammalian gene can readily switch between being expressed or not in the presence of activators and the transcriptional machinery (top line). When not associated with basal activators for transcription the gene is off. Methylation of DNA sequences can inhibit binding proteins required for gene transactivation. Promoters with a dense population of methylated CpG dinucleotides in their sequence, promptly associate with methyl CpG binding proteins (MeCP). Hemi-methylated DNA molecules or DNA molecules containing 5-methylcytosine not paired with guanine are not good substrates for MeCP. The MeCP recruit histone deacetylases (and histone methylases), which modify the nearby chromatin. Thus DNA methylation can mark the DNA for subsequent heterochromatin formation, and permanent transcriptional silencing. Drawn using ScienceSlides Suite 2008 Fall Edition for Mac from VisiScience. Adapted from [263].

***Epigenetic Control of Galectin Expression***

Epigenetic control of galectin gene expression is likewise mediated by promoter methylation during cell differentiation or malignant transformation of normal cells. Additionally, epigenetic control of galectin gene expression occurs in healthy differentiated cells as a means to restrict expression temporally to specific cells and tissues in the adult.

The *LGALS1* gene is methylated at every CpG dinucleotide in the proximal promoter region encompassing -50/+50 [264]. This region, which includes consensus Sp1 transcription factor binding sites, is critical for activation of the promoter [265]. The methylation status of CpG dinucleotides within the *LGALS1* promoter is dependent on cell type. In cells not expressing *LGALS1* transcripts, the promoter was fully methylated, whereas in cells expressing transcripts, the promoter was unmethylated [265]. Certainly, hypomethylation of *LGALS1* promoter correlates with differences in expression, and methylation patterns important for establishing the altered expression occur in a small region of the promoter which includes a CpG cluster [266]. Importantly, unlike prolactin and growth hormone where site-specific methylation is important [30], the density of the methyl-CpGs rather than site-specific methylation distinguishes nonexpressing from expressing alleles [266].

The expression of LGALS1, which facilitates tumorogenesis by its induction of apoptosis specifically in activated T lymphocytes [14] and selectively in T cell leukemia, is elevated in differentiating and transformed tumor cell lines [267]. The selective apoptotic actions of LGALS1 in T cell leukemia depend on its endogenous expression

by these cells. Importantly T cell leukemia cell lines in which LGALS1 is transcriptionally silenced are sensitive to apoptotic actions of secreted LGALS1 whereas T cell leukemia cells expressing high levels of LGALS1 transcripts are insensitive to LGALS1-induced apoptosis [268]. Silencing of the LGALS1 gene in sensitive T cell leukemia cells is associated with hypermethylation of the promoter region. The silencing LGALS1 alleles can be reversed by treatment of T leukemia cells with demethylating agensts such as 5-azacytidine [268]. In pituitary tumors, transcriptional activation or repression of LGALS3 is tightly regulated by DNA methylation of the promoter region [269]. LGALS1 and LGALS3 are functionally involved in the initiation and progression of neoplastic as well as inflammatory disorders, thus drawing attention to the importance of galectin research in which individual members of the galectin family and/or their ligands will be used as diagnostic and therapeutic targets.

The pleiotropic functions of galectins are paralleled by the complex regulation of their expression. This exquisite transcriptional control results in their restricted temporal and spatial expression. This is a common theme in galectin biology not lost on the newest member of the family, LGALS15. From a comparative biology perspective, the studies herein will describe the temporal and spatial tissue distribution, transcriptional regulation, and extracellular role of LGALS15 in domestic ruminants. We hypothesize that *LGALS15* nucleotide and amino acid sequence in addition to endometrial expression patterns is highly conserved across domestic ruminants and LGALS15 protein supports conceptus attachment and outgrowth during the peri-implantation period. Furthermore, the transcriptional activation of the *LGALS15* 5′ promoter is dependent on the indirect

actions of liganded PGR and IFNT. Therefore, the objectives of these studies were to: (1) determine if *LGALS15* is expressed in uteri of other domestic ruminants (goat and cattle) and non-ruminants (pigs); (2) investigate the attachment function of LGALS15 using ovine trophectoderm cells; (3) characterize the LGALS15 gene coding and non-gene coding genomic DNA; and (4) determine how progesterone and IFNT regulate *LGALS15* gene transcription at the level of the promoter.

# CHAPTER III

# GALECTIN 15 (*LGALS15*): A GENE UNIQUELY EXPRESSED IN UTERI OF SHEEP AND GOATS THAT FUNCTIONS IN TROPHOBLAST ATTACHMENT

## Introduction

Maternal support of conceptus (embryo/fetus and associated membranes) growth and development is critical for pregnancy recognition signaling and implantation in domestic animals [1, 270-272]. In ruminants, morula-stage embryos enter the uterus on Days 4 to 6 and then form a blastocyst that contains a blastocoele or central cavity surrounded by a monolayer of trophectoderm [273, 274]. After hatching from the zona pellucida, blastocysts develop into a tubular form and then elongate to 10 cm or more in length beginning on Day 12 in sheep and Day 15 in goats and cattle. Peri-implantation blastocyst growth and elongation is crucial for pregnancy recognition signaling, which involves production of interferon tau (IFNT) from mononuclear trophectoderm cells of the elongating blastocyst that inhibits luteolysis [275, 276]. Hatched blastocysts of ruminants will only elongate when transferred to uteri in domestic ruminants [277]. Thus, factors supporting and regulating growth of peri-implantation blastocysts and elongating conceptuses are thought to be derived primarily from secretions of the uterus or histotroph. This hypothesis is supported by studies of asynchronous uterine transfer of embryos and trophoblast vesicles [278, 279], progesterone regulation of blastocyst elongation [280-282], and the phenotype of uterine gland knockout (UGKO) ewes [283, 284].

UGKO ewes display recurrent early pregnancy loss due to inadequate histotroph from the endometrial luminal (LE) and absence of histotroph from glandular (GE) epithelia that is required for peri-implantation blastocyst survival and elongation [283, 284]. In order to better understand the peri-implantation pregnancy defect in UGKO ewes, a gene expression profiling project was conducted using an endometrial cDNA library from Day 14 pregnant ewes [285, 286]. Interestingly, approximately 1.4% of the expressed sequence tags (ESTs) sequenced from that cDNA library were highly similar to *OVGAL11*, a novel member of the galectin family of secreted animal lectins [287]. The sequence of OVGAL11 protein displayed highest similarity to human LGALS10 (also known as Charcot-Leyden Crystal protein) [288, 289] and human LGALS13 (also known as placental tissue protein 13 or PP13) [290]. Since *OVGAL11* did not have a known orthologue, it was proposed to be a new family member and renamed *galectin 15* (*LGALS15*). Galectins are proteins with a conserved carbohydrate recognition domain (CRD) that bind beta-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells and initiating biologic responses [291-293] that include adhesion, chemoattraction, migration, growth, differentiation and apoptosis [294, 295].

Ovine endometrial LGALS15 contains a predicted CRD as well as C-terminal LDV and RGD recognition sequences that allow proteins to interact with integrins and other components of the extracellular matrix [296]. The temporal and spatial alterations in *LGALS15* mRNA and protein in the uterine endometrial LE and sGE and lumen during the peri-implantation period of early pregnancy in sheep, combined with known

biological activities of other galectins, make LGALS15 a strong candidate mediator of conceptus-endometrial interactions during implantation [286, 297]. One proposed extracellular role for LGALS15 in the uterine lumen is to function as a heterotypic adhesion molecule bridging the conceptus trophectoderm and endometrial LE and stimulating biological responses within the trophoblast, such as attachment and migration, that are critical for successful blastocyst elongation [36, 274]. Indeed, advanced growth and elongation of blastocysts in sheep uteri can be elicited by early progesterone treatment that also results in early expression of *LGALS15* in endometrial LE and sGE [280].

Although blastocyst elongation occurs in most domestic animals (sheep, goats, cattle and pigs), LGALS15 has only been investigated in sheep. Therefore, the objectives of this study were to: (1) determine if *LGALS15* is expressed in uteri of other domestic ruminants (goat and cattle) as well as pigs; and (2) investigate the attachment function of LGALS15 using ovine trophectoderm cells. Results indicate that the *LGALS15* gene is present in sheep, goats and cattle, but is uniquely expressed only in endometria of sheep and goats during the peri-implantation period of pregnancy. Both sheep and goat LGALS15 support *in vitro* attachment of ovine trophectoderm cells, thereby supporting a role for LGALS15 in peri-implantation blastocyst elongation in Caprinae, a subfamily of the family Bovidae.

## Materials and Methods

### *Animals and Experimental Design*

All experimental and surgical procedures involving animals complied with Guidelines for the Care and Use of Agricultural Animals in Agricultural Teaching and Research and were approved by the Institutional Animal Care and Use Committees of Texas A&M and Prairie View A&M Universities.

Uterine tissues from sheep, goats, cattle and pigs were obtained during the estrous cycle and/or pregnancy and processed for analysis by *in situ* hybridization and immunohistochemistry. Uteri from Spanish crossbred female goats or does (*Capra hircus*) were obtained (Day 0=estrus/mating) on Days 5, 11, 13, 15, 17 and 19 of the estrous cycle and pregnancy (n=5/day/status) and Day 25 of pregnancy (n=5). Uteri from Angus crossbred cattle (*Bos Taurus*) were obtained at estrus (n=2) and on Days 16, 16.5, 17, 17.5, 18 and 19 of the estrous cycle and pregnancy (n=3/day/status) and Days 22 (n=2) and 23 (n=3) of pregnancy. Uteri from Large White crossbred gilts (*Sus scrofa*) were obtained on Days 5, 9, 12 and 15 of the estrous cycle and Days 9, 10, 12, 13, 14, 15 and 20 of pregnancy (n=3/day/status). Uteri from Suffolk crossbred ewes (*Ovis aries*) were obtained from Days 16 and 18 of pregnancy (n=4/day) as a positive control. Uteri were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO) for histology. Samples of endometria were also frozen in liquid nitrogen and stored at -80ºC for RNA extraction.

***Slot Blot Hybridization Analysis***

Steady-state levels of *LGALS15* mRNA in goat endometria were assessed by slot blot hybridization using methods described previously [298]. Radiolabeled antisense *LGALS15* cRNA probes were generated by *in vitro* transcription with [$\alpha$-$^{32}$P]-UTP using linearized full-length *Ovis aries LGALS15* cDNA as the template [286] and RNA polymerase. Denatured total endometrial RNA (20 μg) from each goat was hybridized with radiolabeled antisense *LGALS15* cRNA. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ).

***In situ Hybridization Analysis***

Location of *LGALS15* mRNAs in uterine tissues was determined by radioactive *in situ* hybridization analysis as described previously [298]. Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized full-length *Ovis aries LGALS15* cDNA [286], RNA polymerases, and [$\alpha$-$^{35}$S]-UTP. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing and ribonuclease A digestion, slides were dipped in Kodak NTB-2 liquid photographic emulsion, and exposed at 4$^{o}$C for 3 days. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed

with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using an Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

***RT-PCR Analysis***

Expression of *LGALS15* mRNA in endometrial samples was determined by RT-PCR as described previously [299]. Total cellular RNA was isolated from endometria of cyclic and pregnant sheep, goats, cattle, and pigs using Trizol (Gibco-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and the integrity of RNA was examined by gel electrophoresis in a denaturing 1% agarose gel. Briefly, cDNA was synthesized from total endometrial RNA (5 μg) using random and oligo-dT primers and SuperScript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl sterile water, and stored at -20ºC. The cDNAs were diluted (1:10) with sterile water prior to use in PCR reactions. The PCR reactions were performed using Ex Taq DNA polymerase (2.5 U) and 10X Ex Taq buffer (Takara Bio, Carlsbad, CA) according to manufacturers' recommendations.

The forward (5'-ACA CAG TTT CAA CAG GGA AG-3') and reverse (5'-CCG CCC CTT ATA ACG TA-3') primers amplified a cDNA of 443 bp that contained the entire coding sequence of the ovine *LGALS15* mRNA. PCR amplifications were conducted as follows: 34 cycles of 95ºC for 30 sec, 47ºC for 1 min, and 72ºC for 1 min. As a positive control, ACTB (beta actin) primers (forward: 5'-ATG AAG ATC CTC ACG GAA CG-3'; reverse: 5'-GAA GGT GGT CTC GTG AAT GC-3') were used to

amplify a cDNA of 270 bp. PCR products were separated on a 1.5% agarose gel, visualized by ethidium bromide staining, cloned into pCR2 (Invitrogen), and sequenced in both directions. A minimum of five clones from five individual sheep and goats were sequenced, and representative clones were deposited in GenBank (Accession Numbers EU009323, EU009324, EU009325, and EU009326).

Multiple alignments of translated protein sequences were carried out using MUSCLE v.3.6 [300, 301] with the –maxiters flag set to 4. Phylogenetic trees were constructed by generating tree files from the MUSCLE alignments using ClustalW [302] and plotted using TreeView X [303].

***Production of Recombinant LGALS15 Proteins***

The entire coding sequence for ovine and caprine endometrial *LGALS15* mRNAs with either the LDVRGD or LVVRGD sequence polymorphism at the C-terminus was used to produce recombinant ovine and caprine LGALS15 in bacteria. PCR reactions (50 µl) were conducted in Optimized Buffer F (Invitrogen, Carlsbad, CA) and contained 10 ng of the appropriate ovine or caprine *LGALS15* cDNA, 0.5 mg/ml forward primer (5'- AGA TGA AGC CAT GGA CTC CTT GCC GAA CCC CTA CC-3'), 0.5 mg/ml reverse primer (5'- AGA GTA AGC TTT GAT AAC GTA TCC ACT GAA GTC AGC-3'), and 1 U ExTaq polymerase (Takara Bio USA) using an Eppendorf Mastercycler thermocycler with conditions of: 1) 95°C for 2 min; 2) 95°C for 30 sec, 54°C for 1 min, and 72°C for 1 min for 35 cycles; and 3) 72°C for 7 min. The amplified *LGALS15* cDNA was restricted with NcoI and HindIII enzymes and then directionally subcloned into the pET-28b (+) vector (Novagen, Madison, WI). This cloning strategy mutated the

stop codon of LGALS15 and placed a His•Tag sequence at the C-terminus. The resulting plasmid was sequenced in both directions to ensure that no mutations were present in the LGALS15 sequence.

Recombinant LGALS15 protein was produced in BL21 Star (DE3) One Shot *E. coli* (Invitrogen) according to the manufacturer's suggestions. Expression of the LGALS15 fusion protein was induced with 5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO). Bacteria were lysed with Bugbuster (Invitrogen) supplemented with recombinant lysozyme and benzonase. Recombinant LGALS15 protein was isolated by affinity chromatography using a Ni-NTA His•Bind Resin purification kit (Invitrogen). Elutions from the column were analyzed by 1D-SDS-PAGE followed by silver staining and Western blot analysis with rabbit anti-ovine LGALS15 IgG. Recombinant protein was dialyzed overnight in PBS (pH 7.2) at 4°C and then concentrated in a spin column with a 3,500 molecular weight cut-off (Vivaspin, Stonehouse, UK). Protein concentrations were determined using a RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

***Production of Rabbit Antibodies to Ovine LGALS15***

Purified recombinant ovine LGALS15 was provided to a commercial service for immunization of rabbits. Serum from high titer rabbits was collected by terminal bleed and anti-ovine LGALS15 IgG was purified from antiserum using an ImmunoPure (A/G) IgG Purification kit (PIERCE, Rockford, IL). The antibody recognized a 15 kDa protein

of the appropriate size in Western blot analysis of ovine uterine flush proteins and recombinant ovine and caprine LGALS15 protein produced in bacteria.

***Immunohistochemistry***

Immunocytochemical localization of LGALS15 protein in the uterus was performed using methods described previously [286]. Immunoreactive LGALS15 protein was detected using purified rabbit anti-ovine LGALS15 IgG at a final dilution of 1:5000 and a Vectastain ABC anti-rabbit kit. Antigen retrieval was performed using boiling citrate buffer as described previously [304]. Negative controls included substitution of the primary antibody with non-immune rabbit IgG. Immunoreactive protein was visualized using diaminobenzidine tetrahydrochloride (Sigma) as the chromagen. Sections were dehydrated and a coverslips affixed with Permount.

***Photomicroscopy***

Photomicrographs of *in situ* hybridization and immunohistochemistry slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY). Digital images were captured using a Nikon DXM 1200 digital camera and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA).

***Trophectoderm Attachment Assays***

Attachment assays were adapted from Liaw and coworkers [305] and Ochieng and coworkers [306]. Greiner Multiwell Tissue Culture Plates (24-well) for suspension cultures (PGC Scientific Co, Monroe, NC) were coated with either BSA Fraction V (Pierce, Rockford, IL) as a negative control, bovine fibronectin (bFN) from bovine plasma (Sigma, St. Louis, MO) as a positive control, and recombinant ovine or caprine

LGALS15 proteins at the indicated amounts in triplicate and allowed to dry overnight in a sterile hood at room temperature. Wells were then blocked with 1 ml per well of BSA (10 mg/ml) in PBS for 1 h and then rinsed three times with 1 ml per well serum and insulin-free medium. Derivation and culture of mononuclear ovine trophectoderm (oTr1) cells have been described previously [307]. The oTr1 cells were seeded into each well, and plates were incubated for 1.5 h. Wells were washed three times with 1 ml per well of serum free and insulin free medium to remove non-attached cells. Cell numbers were then determined using a Janus Green assay [308] as described previously for oTr1 cells [307].

***Statistical Analyses***

All quantitative data were subjected to least-squares ANOVA using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Slot blot data were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Next, least squares regression ANOVA was conducted within pregnancy status. Tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value of 0.05 or less was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

## Results

### *LGALS15 Is Present in Ruminants, but Only Expressed in Uteri of Sheep and Goats*

The coding sequence of ovine *LGALS15* mRNA (GenBank AF252548) and the inferred LGALS15 protein sequence (GenBank AAF64320) were used to interrogate available databases. Multiple BLAST searches found evidence for *LGALS15* mRNA only in sheep and a *LGALS15*-related sequence in bovine (GenBank XM_593263) with 86% and 77% identity to ovine *LGALS15* mRNA and protein, respectively. In sheep, ESTs for LGALS15 were found in several different tissues including endometrium, gall bladder, small intestine, Peyer's patches, skin, spleen/brain, dendritic cells, and mammary gland. Interestingly, LGALS15 ESTs were highly represented in an endometrial cDNA library from Day 14 pregnant ewes (1.8%), as well as in gall bladder (1.1%), and small intestine (1.1%). In cattle, only 5 ESTs for the *Bos taurus* mRNA similar to ovine LGALS15 were found out of 1.3 million bovine ESTs. Of these five sequences, two were full length, forward and reverse from one clone from a male Holstein (BARC 9 library). This full-length sequence aligned to a region of bovine chromosome 18 and spanned four predicted exons. The other three sequences were from the placenta, but two of those sequences appeared to be chimeric ribonucleoprotein/LGALS15, suggesting that they were most likely cloning artifacts. The remaining placental sequence spanned three of the four predicted exons for LGALS15.

Primers were developed to amplify the entire coding sequence of *LGALS15* and used for RT-PCR analyses of total RNA isolated from endometria of cyclic and pregnant

sheep, goats, cattle, and pigs. PCR products were generated from endometria from sheep and goats, but not cattle or pigs. Sheep and goat LGALS15 were highly homologous at the mRNA (95%) and protein (91%) levels (Figure 3.1). Similarly, the bovine LGALS15-like sequence shared 86% and 77% identity to the ovine *LGALS15* mRNA and protein, respectively.

A search of the amino acid sequence of the LGALS15 proteins revealed the presence of a CRD characteristic of galectins [309]. The CRD is a consensus motif consisting of 13 amino acids [310] of which eight (H.N.R.V.N.W.E.R) play a critical role in binding sugars [311, 312]. As illustrated in Figure 3.1, comparison of the putative CRDs of ovine and caprine LGALS15 with the conserved CRD of other galectins indicated that four residues are identical (V62, N64, W71, E74) and three are conservatively substituted (R54, W56, K76). Similarly, comparison of the predicted CRD of bovine LGALS15 with the conserved CRD of other galectins found that five residues were identical (V63, N65, W72, E75, R77) and two were conservatively substituted (H54, R56). However, ovine and caprine LGALS15 substituted a P52 and the bovine LGALS15 an A52 for the first H residue of the consensus CRD. The C57 in ovine and caprine LGALS15 is different from prototypical galectins, but appears to allow for binding of mannose in LGALS10 [313]. However, a C residue at position 58 was not found in bovine LGALS15.

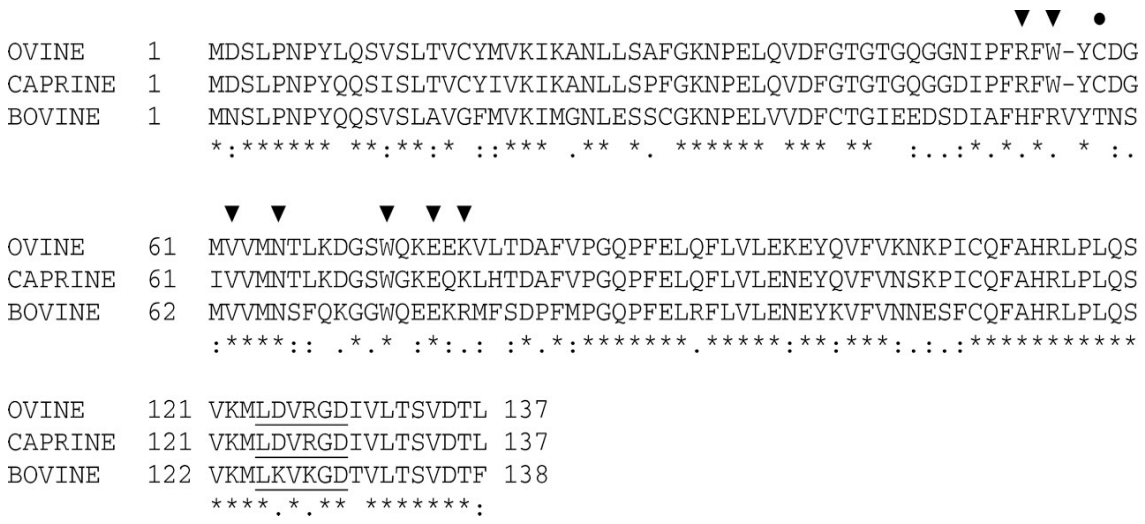


Fig. 3.1. MUSCLE alignments of the amino acid sequences of LGALS15 from ovine and caprine endometria compared to predicted bovine protein. The arrows (▼) denote the conserved residues forming the carbohydrate recognition domain (CRD) in prototypical galectin family members. The circle (●) denotes a conserved C residue critical for mannose binding in LGALS10. The underlined residues denote the conserved LDV and RGD recognition sequences for integrin binding near the C-terminus in ovine and caprine LGALS15 and closely related LKV and KGD sequences in the predicted bovine LGALS15.

Consistent with other galectins, none of the LGALS15 had apparent or predicted signal peptide, transmembrane domain, or glycosylation sites. A PROSITE search revealed two putative cell attachment sequences at positions 123 (LDV) and 126 (RGD) in ovine and caprine LGALS15 that are recognition sequences for integrin binding [296]. The putative CRD and LDVRGD recognition sequences were conserved in all cDNAs amplified from sheep and goat endometria (GenBank Accession Numbers EU009324 and EU009325). However, approximately 50% of the sheep and goat LGALS15 from each individual contained an LVV polymorphism next to the RGD sequence in the C-terminus (GenBank Accession Numbers EU009323 and EU009326). As shown in Figure 3.1, the bovine LGALS15-like protein contained LKVKGD sequences at position 124 instead of the LDVRGD or LVVRGD sequence found in ovine and caprine LGALS15. The KGD recognition sequence binds integrins similar to the RGD sequence [296].

All galectin protein sequences present in Uniprot [314] were downloaded and aligned with the translated ovine, caprine and bovine LGALS15 sequences (data not shown). It was clear from this alignment and the resulting phylogeny that LGALS15, CLC/LGALS10, and CLC2/LGALS14 were most closely related and most similar to LGALS4. This relationship is illustrated in Figure 3.2, where only the LGALS4 node from the comprehensive tree is displayed. Based on this result, the *LGALS15* genes are likely specific to the subfamily Caprinae of the family Bovinae. Further, the *LGALS15* genes and the primate *CLC/LGALS10*, *CLC2/LGALS14* and *LGALS13* genes arose from an ancestral duplication of *LGALS4*. Specifically, the predicted protein sequence from the *Bos taurus LGALS15*-like mRNA shares significant similarity to LGALS10, LGALS13, and LGALS14 from several species with no gaps and to ovine LGALS15 with one gap.

***LGALS15 mRNA and Protein Are Present in Endometria of Sheep and Goats, but Not Cattle or Pigs***

Steady-state levels of *LGALS15* mRNAs in endometria from cyclic (C) and pregnant (P)

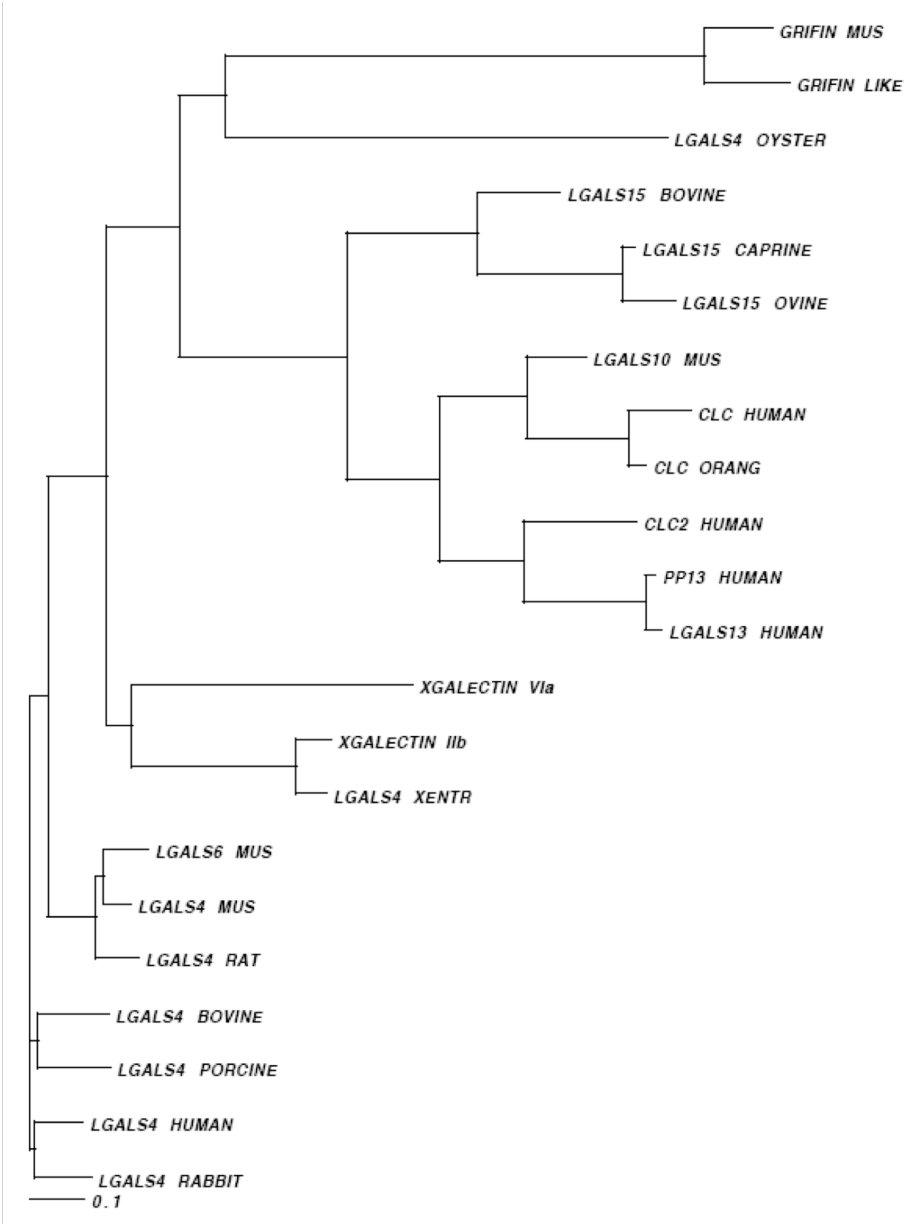


Fig. 3.2. Phylogenetic tree of relationships of LGALS15. The tree indicates relationships of LGALS15 to the other most closely related galectin superfamily members based on the Neighbor Joining method using the tree generated from the MUSCLE alignment. The branch lengths are proportional to an estimate of evolutionary change. The scale bar at the bottom denotes relative estimate of evolutionary distance.

goats were determined by slot blot hybridization analyses (Figure 3.3) and found to be affected ($P<0.01$) by day, status, and their interaction. In cyclic goats, endometrial *LGALS15* mRNA was low to undetectable before Day 13, increased (cubic effect of day, $P<0.01$) about 88-fold from Days 13 to 17, and then declined to Day 19. In pregnant goats, *LGALS15* mRNA levels were also low to undetectable before Day 13, increased between Days 13 and 17, and declined between Days 19 and 25 (cubic effect of day, $P<0.01$). Between Days 13 and 17, endometrial *LGALS15* mRNA levels increased about 88-fold in cyclic goats, compared to a 292-fold increase in pregnant goats (day x status, $P<0.0001$). Thus, endometrial *LGALS15* mRNA levels were not different between cyclic and pregnant goats between Days 11 to 15, but increased in pregnant compared to cyclic goats between Days 15 and 19, which correlates with the onset of definitive attachment of the trophectoderm to the endometrial LE in goats [315] and maximal production of IFNT by the caprine trophectoderm [316] and maximal production of IFNT by the caprine trophectoderm [316, 317].

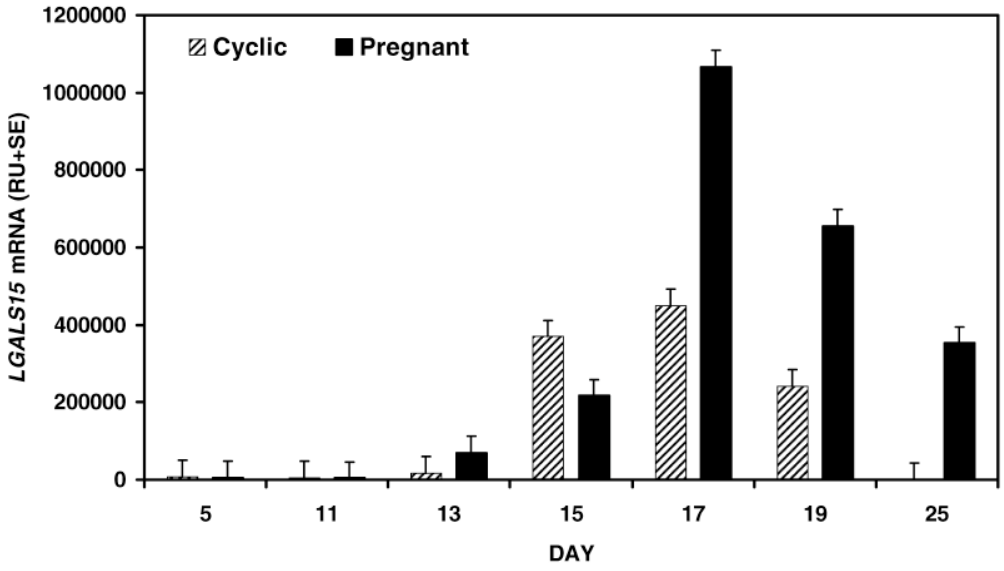


Fig. 3.3. Steady-state levels of *LGALS15* mRNA in endometria from cyclic and early pregnant goats. Steady-state levels of *LGALS15* mRNA were determined by slot blot hybridization analysis. In cyclic goats, *LGALS15* mRNA was low to undetectable from Days 5 to 11, increased about 88-fold from Days 11 to 17, and decreased to Day 19 (cubic effect of day, $P < 0.01$). In pregnant goats, *LGALS15* mRNA was low to undetectable from Days 5 and 11, increased about 292-fold between Days 13 and 17, and declined somewhat to Day 25 (cubic effect of day, $P < 0.01$). The abundance of endometrial *LGALS15* mRNA was higher in pregnant than cyclic goats on Days 17 and 19 (day x status, $P < 0.0001$). Data are expressed as LSM relative units with SEM.

*In situ* hybridization analyses found abundant *LGALS15* mRNA in endometrial LE and sGE of cyclic and pregnant uteri from goats and sheep (Figure 3.4). In contrast, no hybridization signal for *LGALS15* mRNA was detected in uteri of cyclic or pregnant cattle and pigs. In goats, *LGALS15* mRNA was first observed at low levels in LE, sGE and upper glands of endometria on Day 13 of both the estrous cycle and pregnancy. In cyclic goats, *LGALS15* mRNA was most abundant on Day 17 and then declined substantially to Day 19, whereas *LGALS15* mRNA in pregnant goats increased from Days 15 to 17 and remained abundant thereafter. *LGALS15* mRNA was not detected in conceptus trophectoderm. Thus, the presence of a conceptus increases *LGALS15* mRNA levels in caprine endometrium.

Overall changes in immunoreactive LGALS15 protein abundance in endometrial LE and sGE of goats paralleled changes in *LGALS15* mRNA in cyclic and pregnant goats (Figure 3.5). In both cyclic and pregnant goats, LGALS15 protein was localized primarily in the cytoplasm of endometrial LE and sGE. Consistent with an increase in *LGALS15* mRNA, the abundance of LGALS15 protein increased in LE and sGE after Day 13 and was readily apparent near and on the apical surface of endometrial LE by

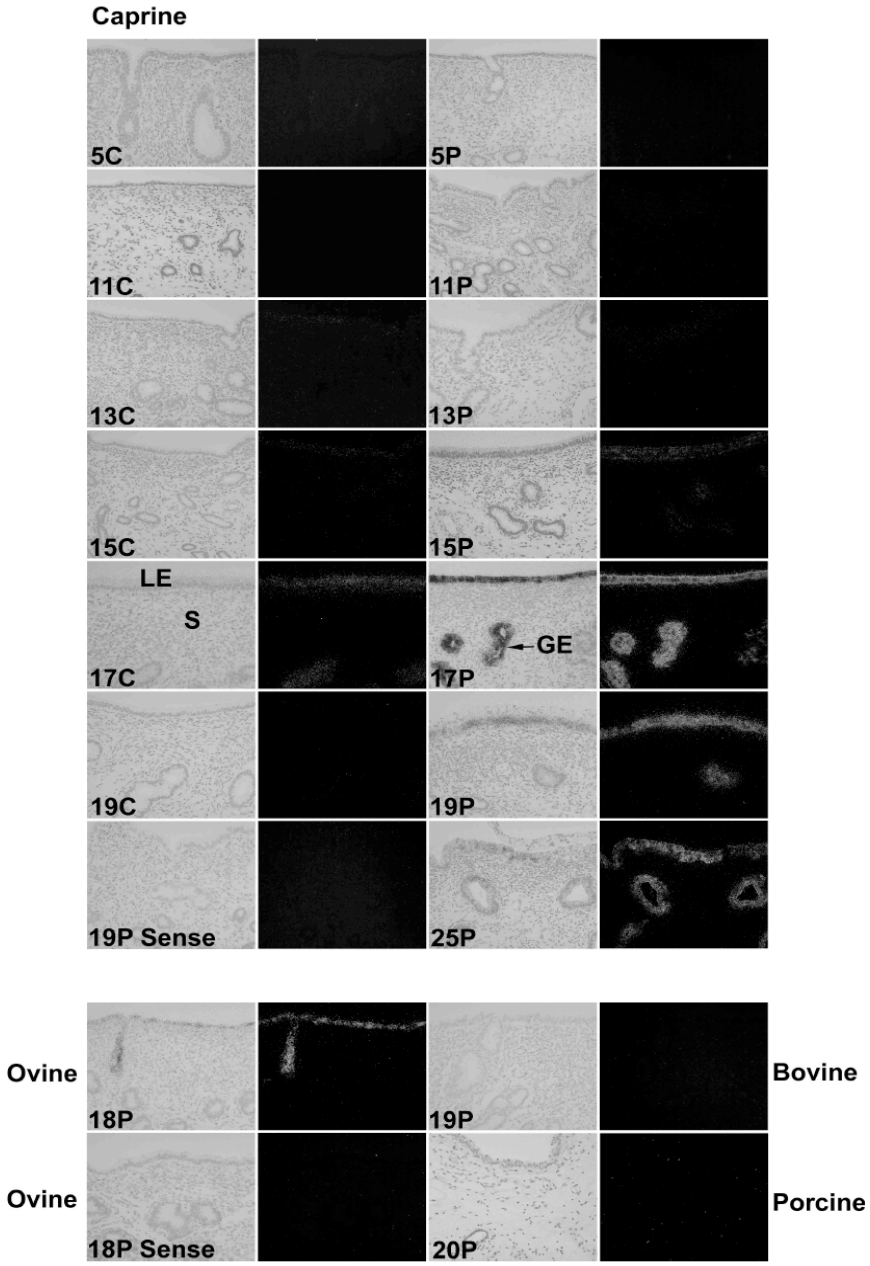


Fig. 3.4. *In situ* localization of *LGALS15* mRNA in the endometria of cyclic and pregnant goats. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) goats were hybridized with radiolabeled antisense or sense ovine *LGALS15* cRNA probes. *LGALS15* mRNA was detected only in endometrial LE and sGE of goats and sheep, but was not detected in either cattle or pigs. All representative photomicrographs are shown in bright field (left) and dark field (right) illumination at the same width of field (420 μm). LE, luminal epithelium; GE, glandular epithelium; S, stroma. Numbers in panels indicate days.

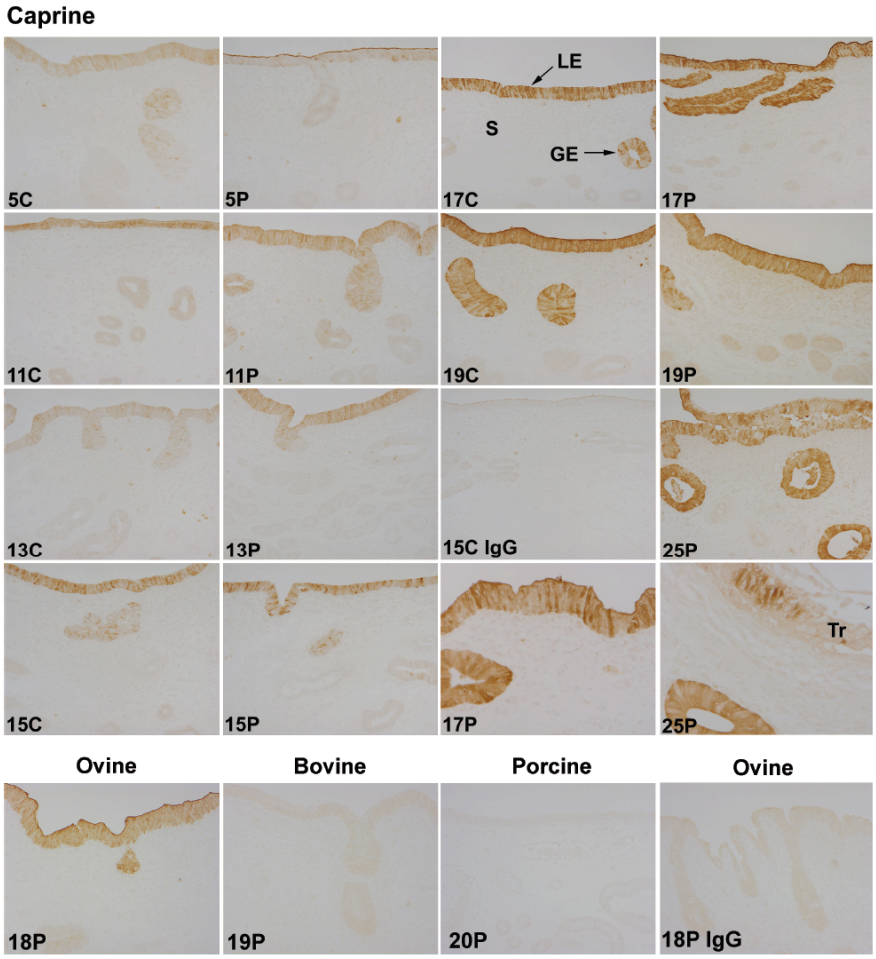


Fig. 3.5. Immunolocalization of LGALS15 protein in endometria of cyclic and pregnant goats. Note the presence of immunoreactive LGALS15 protein in endometrial epithelia and conceptus trophectoderm in pregnant goats and sheep, but not cattle or pigs. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Representative photomicrographs are shown at the same width of field (420 μm) with the exception of the higher magnifications of the caprine endometrium (right bottom) at width of field of 630 μm. Sections were not counterstained. S, stroma; Tr, trophectoderm. Numbers in panels indicate days.

Day 17 of the estrous cycle and pregnancy. Although *LGALS15* mRNA was not present in the conceptus, LGALS15 protein was detected in crystal structures within conceptus trophectoderm (Tr) as well as endometrial LE and sGE. Consistent with the lack of detectable *LGALS15* mRNA, no immunoreactive LGALS15 protein was detectable in bovine or porcine uteri.

***LGALS15 Promotes Attachment of Ovine Trophectoderm (oTr1) Cells***

Ovine trophectoderm (oTr1) cells isolated from Day 15 conceptuses were predominantly mononuclear and expressed *IFNT* according to results from RT-PCR (data not shown). A dose-dependent increase ($P<0.01$) in oTr1 cell attachment was induced in wells coated with increasing amounts of LGALS15 and bovine FN, but not BSA (Figure 3.6). An increase in oTr1 cell attachment also occurred in response to bovine FN as well as in response to all forms of ovine and caprine LGALS15, and LGALS15, and bovine FN induced similar increases in oTr1 cell attachment.

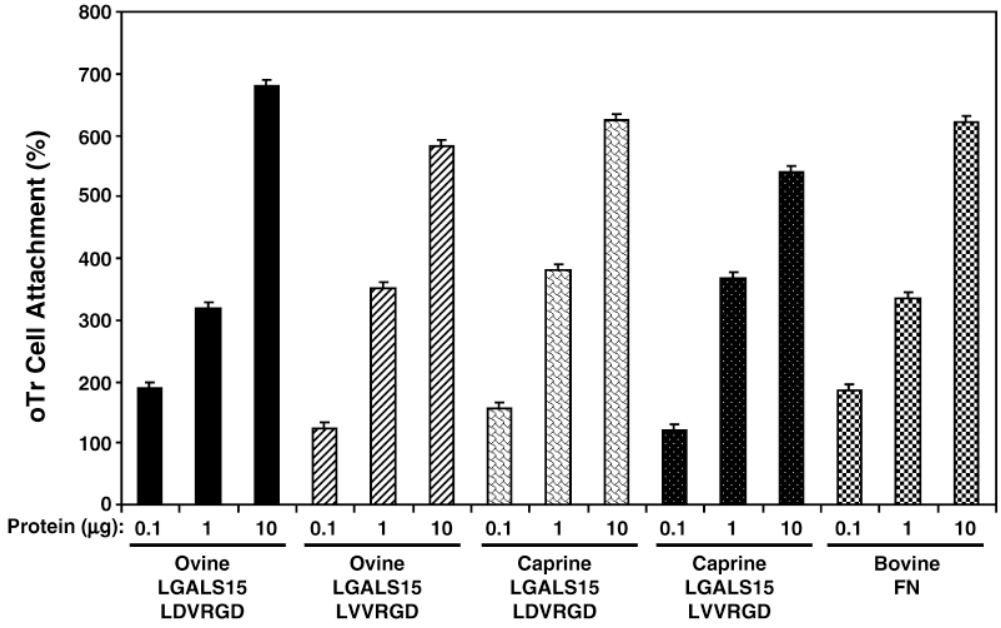


Fig. 3.6. Attachment function assays of ovine and caprine LGALS15. oTr1 cells were used in attachment function assays. Wells of suspension culture plates were coated with increasing amounts (0.1, 1, or 10 μg) of recombinant ovine and caprine LGALS15 or purified bovine fibronectin, but not BSA. Freshly prepared oTr1 (labeled oTr) cells were seeded into each well and allowed to attach for 1.5 h. Unattached cells were washed off, and cell number in each well determined. Data are expressed as percentage of attached oTr1 cells relative to BSA. The entire experiment was independently repeated at least three times with similar results.

## Discussion

Results of bioinformatic and RT-PCR analyses indicate that *LGALS15* is a unique member of the galectin family present in the genome of sheep and goats (Subfamily Caprinae) and cattle (Subfamily Bovinae), but not in pigs (Suborder Suina), which are Artiodactyls. Outside of the Artiodactyls, *LGALS15* was not detected in human, nonhuman primate, mouse, chicken, dog or any other species with a sequenced genome. These results suggest that *LGALS15* is a unique gene in sheep, goats and cattle. Given the lack of expression of *LGALS15* in the bovine uterus and very rare abundance in other tissues based on EST analysis, the bovine *LGALS15* gene may be a pseudogene, which can be defined as a defunct relative of known genes that are no longer expressed in cells [318]. Phylogenetic analyses of available galectins from a number of species (human, mouse, rat, dog, and cow) suggest that *LGALS15* is likely a paralog derived from another closely related galectin family member such as *LGALS10/CLC*, *LGALS11*, *LGALS13* or *LGALS14/CLC2*. Paralogs are genes related by duplication within a genome that evolve new functions, even if they are related to the original gene. Indeed, the LDV and RGD recognition sequences in LGALS15 of sheep and goats are conservatively substituted in cattle, but not present in the C-terminus of any other galectin. Interestingly, *LGALS13,* originally known as placental tissue protein 13 (PP13), was originally cloned from human placenta [319] and is a homologue of human eosinophil Charcot-Leyden Crystal (CLC) protein that is known as LGALS10. Moreover, ovine, caprine and bovine LGALS15 display highest similarity to human CLC [288, 289] and LGALS13 [290]. Thus, LGALS15 most likely evolved as a paralog

of LGALS13 or LGALS10/CLC in sheep, goats, and cattle. Functional studies of other galectins have implicated these proteins in cell adhesion, chemoattraction and migration, as well as cell growth, differentiation and apoptosis [291, 294, 320]. All of these biological activities are proposed to be important for ruminant blastocyst growth and elongation during the peri-implantation period of pregnancy [273, 274, 321].

The temporal changes in expression of endometrial *LGALS15* mRNA support the hypothesis that ovarian progesterone and conceptus IFNT regulate transcription of the *LGALS15* gene in endometrial epithelia of goat uteri as found in sheep [285, 286]. IFNT is the pregnancy recognition hormone in ruminants that acts on the endometrium to prevent development of the luteolytic mechanism, thereby maintaining the corpus luteum for production of progesterone [322]. The enhanced levels of *LGALS15* mRNA in endometria of pregnant goats on Days 15 to 19 of pregnancy as compared to cyclic goats parallels the increase in production of IFNT by the conceptus, which is produced from Days 16 to 21 and is maximal between Days 16 and 18 in goats (Gnatek 1989; Guillomot 1998). Intrauterine administration of IFNT increases *LGALS15* mRNA, but only in progesterone-treated ewes [286]. Indeed, several genes have been identified to be progesterone-induced and IFNT-stimulated specifically in ovine endometrial LE/sGE, including cathepsin L (CTSL) and cystatin C (CST3) [176, 177]. Thus, progesterone and IFNT act in concert to stimulate expression of a number of genes apparently important for conceptus survival, growth and implantation [36, 274, 285, 323].

Prototypical members of the galectin superfamily (LGALS1, LGALS2, LGALS5, LGALS7, LGALS10, LGALS11, LGALS13, LGALS14) have one conserved

CRD. Interestingly, results of phylogenetic analyses suggest that LGALS10, LGALS13, and LGALS15 were derived from LGALS4, which is a tandem repeat galectin with two CRDs [324]. Although the CRD of LGALS15 differs slightly from that in the prototypical galectins, it does possess the "jellyroll" structural fold similar to that found in LGALS10 and LGALS13 [325]. Galectins bind beta-galactosides via the CRD, but the carbohydrate binding specificity for each galectin appears to be different [326]. In addition to the CRD, sheep and goat LGALS15 also contains predicted cell attachment sequences (LDV and RGD) that could mediate binding to integrins in extracellular matrix proteins [296]. Galectins can also bind and activate integrins via their CRD [294]. In the present study, all forms of recombinant ovine and caprine LGALS15 increased attachment of mononuclear ovine trophectoderm cells to a similar extent as bovine FN. Although the LDV sequence next to the RGD sequence is an integrin binding site [296], there were no detectable differences in cell attachment function among the different polymorphic variants of ovine or caprine LGALS15 that contained the LVVRGD sequence instead of the LDVRGD sequence in the C-terminus. The temporal and spatial alterations in *LGALS15* mRNA and protein in the ovine uterus [286] and caprine uterus during pregnancy, combined with the *in vitro* attachment of ovine trophectoderm cells to recombinant ovine and caprine LGALS15, support the hypothesis that LGALS15 functions as a heterotypic cell adhesion molecule bridging endometrial LE and conceptus trophectoderm, which is required for blastocyst growth, elongation and attachment phase of implantation.

Both the RGD recognition sequence and perhaps the CRD of LGALS15 may be involved in cell attachment and adhesion via integrin binding and activation. Indeed, integrins are proposed to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation [327, 328]. During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on conceptus trophectoderm and apical surface of endometrial LE [329]. In goats, integrin subunits αv, α4, α5, β1, and β3 are expressed on conceptus trophectoderm and endometrial LE on Days 21 and 23 of pregnancy [330]. Thus, conceptus implantation in sheep and goats does not appear to involve changes in temporal or spatial patterns of integrin expression [329, 330], but appears to depend primarily on changes in expression of integrin ligands, such as LGALS15 and secreted phosphoprotein one (SPP1/osteopontin) [45, 274, 331]. Other galectins bind integrins, fibronectin, and laminin, because these extracellular matrix proteins are modified with beta-galactoside sugars [291, 292]. Indeed, FN and vitronectin (VN) are also expressed on conceptus trophectoderm and endometrial LE on Days 21 and 23 of pregnancy in goats [330]. In the goat, close contact between the conceptus trophectoderm and endometrial LE occurs between Days 17 and 18, with firm adhesion developing between Days 19 and 23 [315, 332]. This time period coincides with rapid elongation of the goat blastocyst to form a filamentous conceptus [332]. Blastocyst elongation has not been achieved *in vitro*, suggesting that a factor(s) present in the uterine lumen, perhaps a secreted protein like LGALS15 or SPP1, is required for blastocyst development into a filamentous conceptus. The idea that factors in uterine histotroph are required to

promote blastocyst growth and elongation in ruminants is supported by studies of asynchronous uterine transfer of embryos and trophoblast vesicles [278, 279], progesterone regulation of blastocyst elongation [280-282], and failure of conceptus development in UGKO ewes [283, 284]. In fact, blastocyst elongation has been hypothesized to require transient attachment, detachment and reattachment as the trophectoderm elongates from each side of the centrally located embryonic disc [273]. Thus, available evidence suggests that LGALS15 secreted by endometrial LE functions to promote blastocyst growth and elongation in sheep and goats by moderating adhesion of trophectoderm to endometrial LE via integrin binding.

As observed in sheep [286, 297], LGALS15 is detectable on the surface of the trophectoderm and within intracellular crystal structures of trophectoderm and the endometrial LE of the goat. LGALS10 was initially known as Charcot-Leyden crystal (CLC) protein because it formed distinctive hexagonal bipyramidal crystals in eosinophils that accounted for nearly 10% of the total cellular protein [288, 333]. Further, LGALS15 was immunologically identical to the novel 14K progesterone-modulated protein from the sheep uterus associated with crystalline inclusion bodies in endometrial LE and conceptus trophectoderm [185]. Subsequent immunogold electron microscopy analysis revealed the 14K protein was localized to large, membrane-bound rhomboidal or needle-shaped crystal structure, but not in the endoplasmic reticulum and Golgi body. Thus, Kazemi and coworkers [185] suggested that the protein was secreted by the endometrial epithelia and taken up by the conceptus from uterine histotroph. Indeed, needle-shaped crystalline structures have also been described in caprine

endometrial epithelial and trophectoderm cells [332], and their development correlates with the synthesis and secretion of an unidentified 15 kDa protein with a pI of ~6.0 from explant cultures in response to the conceptus [334]. Interestingly, development of *in vitro* produced bovine blastocysts transferred into sheep uteri resulted in the presence of crystals in trophectoderm cells [335, 336]. However, crystal-like structures were not observed in the trophectoderm of Day 15 or Day 19 bovine blastocysts produced by *in vivo* development in cattle [336]. Thus, it is not surprising that the *LGALS15* gene is expressed in endometria of ovine and caprine uteri, but not bovine uteri. The present results strongly indicate that LGALS15 is expressed by endometrial LE and sGE of ovine and caprine uteri, secreted into the uterine lumen, and then adsorbed to the surface of or internalized by conceptus trophectoderm where it forms crystals. Although the biological role(s) of LGALS15 crystals in the conceptus is not known, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interacting with specific intracellular ligands such as the oncogene product of Harvey sarcoma virus (H-RAS) and B-cell lymphoma 2 (BCL2) [337, 338]. Similar to LGALS15, a number of galectins are present in the cytoplasm and nuclei of cells, including LGALS1, LGALS3, LGALS7, and LGALS12 [295].

Collectively, available results support the hypothesis that *LGALS15* is uniquely expressed in uterine endometria of ruminants in the subfamily Caprinae (*Ovis aries* and *Capra hircus*) and secreted into the uterine lumen where it functions as an attachment factor important for pre-implantation blastocyst growth, elongation and attachment

phase of implantation. In mammals, implantation is fundamental to successful reproduction. Therefore, it is likely that other members of the galectin super-family are expressed in the uteri of cattle and pigs and exhibit similar functional roles as LGALS15 in the uteri of sheep and goats. Indeed, similar tissue distribution patterns and functional characteristics have been described for galectins expressed in bovine and porcine tissues [21, 142, 339, 340]. Future work will focus on the extracellular and intracellular roles of LGALS15 in endometria and conceptuses of sheep and goats and determine if other galectin family members are expressed in uteri of other mammals.

# CHAPTER IV

# COMPARATIVE ASPECTS OF THE RUMINANT *LGALS15* GENE: DETERMINANTS OF RESTRICTED EXPRESSION IN SHEEP AND GOATS

## Introduction

In domestic ruminants like sheep goats and cattle, the conceptus undergoes extensive growth and development in the uterus prior to implantation. The endometrial epithelia synthesize and secrete an extensive array of proteins and related substances, collectively termed histotroph [40, 41]. Histotroph is an ambiguous mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, adhesion proteins, and other substances [41]. The epithelial cells produce large amounts of secretory products during the luteal phase of the cycle and at the beginning of implantation [42]. The trophoblast cells are sites of concerted pinocytotic activity which increases as the blastocyst develops [186]. Thus it has long been hypothesized that regulatory molecules necessary for growth of the elongating conceptus were obtained from uterine histotroph.

Available results clearly support a role for the uterus and its secretions in conceptus survival and development in addition to the developmentally regulated secretion of the pregnancy recognition signal IFNT [25, 341]. There are many proteins hypothesized to have significant roles in conceptus implantaion, not the least of which belong to the multifunctional, evolutionarily conserved galectin superfamily. Importantly, a common function of galectins appears to be cell recognition as mediated by lectin-carbohydrate interactions, which are essential to many biological processes.

Galectins comprise a family of evolutionarily conserved animal lectins, which are defined by their affinity for β-galactoside enriched glycoconjugates and sequence homology in the carbohydrate recognition domain (CRD). The expression of galectins is modulated in both normal and abnormal tissues where protein-carbohydrate and/or protein-protein interactions allow them to mediate malignant transformation, tumor progression and metastasis [182, 183, 269, 342, 343]. Galectins may contribute to successful reproduction in mammalian species. They are expressed by the pre-implantation conceptus trophectoderm and in various embryonic organs during embryogenesis [16, 17, 23]. Additionally, galectins are expressed in the endometrium during peri-implantation period of conceptus development in mice, humans, cattle, sheep and goats [18-21]. Based on galectin fingerprinting studies in human endometrial tissue, *LGALS1* and *LGALS3* exhibit cell-specific and cycle-dependent expression patterns suggestive of roles during implantation [19]. Similarly, expression of *LGALS3BP* and *LGALS9* are restricted to the uterine LE during implantation [21] and *LGALS1* during estrus [142] in cattle.

Galectin 15 (LGALS15), the newest member of the galectin super family, was initially discovered in sheep abomasal tissue infected with the nematode parasite, *Haemonchus contortus* [24]. Transcripts for *LGALS15* are only expressed in the endometrial LE/sGE and are induced by progesterone and further stimulated by conceptus-derived interferon tau (IFNT) [3]. Temporally, *LGALS15* expression is coordinate with early conceptus elongation, growth and development during the peri-implantation period in the ovine uterus. Spatially, *LGALS15* mRNA is limited to

endometrial luminal (LE) and superficial glandular (sGE) epithelia and represents one of a growing list of interferon-stimulated genes (ISG) expressed by LE and sGE that lack both progesterone receptor (PGR) and STAT1. Therefore, LGALS15 gene expression is regulated by a novel non-classical IFN signaling pathway(s) [175-177]. Similar to other galectins, LGALS15 lacks a signal peptide; nonetheless it is an abundant component of the uterine histotroph [3].

The cellular and molecular mechanisms of how progesterone and IFNT regulate *LGALS15* gene transcription in the endometrial LE/sGE is not known. Transcription of other galectin family member genes is primarily regulated by epigenetic modification of their promoter DNA [266]. Transcriptional repression of the *LGALS3* gene *in vitro* in malignant prostate epithelial cells is regulated by DNA methylation status of the 5′ proximal promoter [28]. Epigenetic modifications such as DNA methylation of cytosine residues at CpG dinucleotides are common in the promoter/enhancer region of genes. Methylation at cytosine of CpG dinucleotides are considered stable modifications even if other repressive modifications are reversed [29]. These DNA modifications can thus be retained from one generation to the next in similar patterns. In addition to transcriptional repression and/or silencing, DNA hypermethylation of gene promoters is also responsible for tissue – specific patterns of gene expression [30, 31]. To date, most galectins studied exhibit tight regulation and expression is restricted to specific organs and cell types. There are few results showing a direct relationship between galectin gene expression and promoter methylation in normal tissue [344]; however, many galectin gene promoters have a CpG dinucleotide density that compel further studies on the role

methylation has on transcriptional regulation of galectins [32]. We hypothesize that liganded PGR inhibits transcriptional activation of the *LGALS15* 5′ promoter and epigenetic modification of the DNA are responsible for transcriptional silencing of the bovine *LGALS15* gene. Objectives were to: (1) characterize the *LGALS15* gene; (2) uncover how progesterone and IFNT regulate ruminant *LGALS15* gene transcription at the level of the promoter; and (3) determine if methylation of the bovine *LGALS15* gene promoter is responsible for transcriptional silencing in this species but not in sheep or goats.

## Materials and Methods

### *Cells and Reagents*

Human 2fTGH (parental), U3A (STAT1-deficient 2fTGH) fibrosarcoma cells (Pelligrini), and Madin-Darby bovine kidney cells (MDBK) [345, 346] were maintained in DMEM-F12 medium (Sigma–Aldrich Corp., St. Louis, MO) supplemented with penicillin/streptomycin sulfate/amphotericin B (PSA) solution (Invitrogen, Carlsbad, CA) and fetal bovine serum (Hyclone, Logan, UT) (5% FBS for 2fTGH and U3A and 10% FBS for MDBK cells). Recombinant ovine IFNT (IFNT) ($10^4$ antiviral units (AVU) per mg) was prepared as described previously [347]. Restriction endonucleases, T4 DNA ligase, cell culture lysis reagent and luciferase substrate were purchased from Promega (Madison, WI). Ex Taq$^{TM}$ polymerase (Takara, Kyoto, Japan) was used. Plasmid DNAs were purified by the alkaline lysis method with kits from Qiagen (Qiagen, Valencia, CA). Vent *Taq* polymerase (New England Biolabs, Beverly, MA) was used. R5020 was purchased from Perkin Elmer Life Sciences (Boston, MA).

Demethylating agent; 5-aza-2′-deoxycytidine (AZA) and histone deacetylase (HDAC) inhibitor; Trichostatin A (TSA) were from Sigma-Aldrich.

***Isolation and Cloning of Ovine LGALS15 5′-Flanking Promoter/Enhancer***

The ovine *LGALS15* gene was previously derived from an ovine genomic DNA library which was screened with the ovine *LGALS15* cDNA for identification and isolation of a clone containing ~15 kb of LGALS15 genomic DNA. Briefly, 100 ng of pBlueSTAR$^{TM}$ – 1 vector containing ~15 kb of genomic DNA insert was digested for at least 2 h with restriction endonucleases in the appropriate buffer. Physical mapping by restriction endonuclease digestion and Southern blotting were performed using standard methods [348]. A 2.3 kb fragment (flanked by SacI restriction endonuclease sites) was subcloned into the SacI multiple cloning site of the pCRII plasmid vector using T4 DNA ligase and sequenced on both strands using Sp6 (5′- ATT TAG GTG ACA CTA TAG -3′) and T7 (5′- TAA TAC GAC TCA CTA TAG GG -3′) primers. To determine the complete sequence, contigs were aligned and contiguous sequences determined.

***Cloning of Ruminant LGALS15 Gene Coding and Non-coding DNA***

Caprine and bovine LGALS15 gene 5′promoter/enhancer regions were cloned from genomic DNA (gDNA) collected from white blood cells of Boer goats and Red

Angus cows. Primers (forward: 5′- GTC GTT TAT CAG TAG ACA CAA GGA ATT GC -3′ and reverse: 5′- CGT CTC CAG CTG GGC CTT TCT TCC -3′) used to clone the caprine promoter were designed based on the ovine promoter sequence and primers (forward: 5′- TCA ACA AGG GCC ACC TTA AC -3′ and reverse: 5′- AGT CTT CAA CTG GGC CTT TCT TCC -3′) used to clone the bovine promoter were designed based on the published sequence, reference number: NW_001493613.1 on chromosome 18 and contig regions 28,039 to 30,028. All PCR reactions using gDNA as template were performed using Vent *Taq* polymerase (New England Biolab) polymerase (2.5 U) and 10X Vent *Taq* buffer. Sticky ends were generated by repeating PCR reactions using the product from the Vent *Taq* PCR reation as template with ExTaq polymerase (Takara Bio, Carlsbad, CA) and nested primers according to the manufacturer's recommendations. PCR amplifications were conducted as follows: 34 cycles of 95˚C for 30 sec, 54˚C (caprine) or 58˚C (bovine) for 1 min, and 72˚C for 1 min, resulting in an ~1.9 kb PCR product. Following T/A cloning (Invitrogen) of the DNA into pCRII plasmid vector, primer-walking experiments were conducted to determine the entire sequence of the insert DNA (Table 4.1).

Table 4.1. Sequencing of the LGALS15 promoter/enhancer region by primer walking. Primers were designed for both the sense and anti-sense strands. Overlapping sequences were visualized and contigs were assembled to form consensus sequences based on the quality of the peaks of the electropherogram. The odd number of the primer set indicates forward primers and the even number of the primer set indicates reverse primers. Primer walking experiments were always initiated using Sp6 and T7 primers thus primer sets represent primers needed to sequence the remaining insert DNA on either strand.

| *Primer Set* | *Forward (5′-3′)* | *Reverse (5′-3′)* |
|---|---|---|
| oP1/P2 | GCAATCACCTCAGATATGC | TGCCCAATCAGGAATCAGG |
| oP3/P4 | CATCTAGTCAAGGCTATGG | CATGAATCGCAGCACTCC |
| oP5/P6 | GAGTTGGTGAGGGACAGG | TCATGGCTGCAGTCTCC |
| oP7/P8 | CTTCAAAGTGTCACCTCTGC | CTCAAGAGGCAGGTCAGG |
| cP1/P2 | TCACTGTGGATGGTGATTGC | GACTGGTTGGATCTCCTTGC |
| cP3/P4 | AGGGTCAGGAGTGACTCAGC | GATGTTAGCAATTTGATCTCTGG |
| bP1/P2 | TCAACAAGGGCCACCTTAAC | GAGCCCTGCCCTCTATTCTT |
| bP3/P4 | AGATCAAATTGCCAACATCG | AAAGTGATGGGACCAGATTCC |
| bP5/P6 | GACTGCAAGGAGATCCAACC | ACCTGGATGAGAGTCCAAGC |

***5′-LGALS15 Luciferase Constructs***

Truncated LGALS15 luciferase constructs were created by PCR amplification. The *LGALS15* gene was analyzed for the presence of conserved transcription factor binding sites using the Transcriptional Element Search System (TESS). Weight matrix searches for cis-elements were conducted using recommended parameters [349]. Briefly, a maximum allowable mismatch of 10%, a minimum factor length of 8 bp, a minimum log-likelihood score (La) of 12 and a factor quality score (Qa) of 0.8 were used in determining valid transcription factor binding sites. Based on the TESS and RepeatMasker analysis, primers were designed to generate LGALS15 5′ deletions to be directionally subcloned upstream of a luciferase gene in the pGL3 Basic (Promega Corp., Madison, WI) reporter vector system. All constructs excluded the translation start site (ATG site; +41). This was accomplished by designing a common reverse primer starting at position +40 relative to the transcription start site at +1. For the ovine and caprine LGALS15 luciferase constructs, *SacI and XhoI* sites in the multiple cloning site (MCS) of either the pCRII or pGL3 Basic (Promega Corp., Madison, WI) vectors were digested and inserts directionally ligated upstream of the luciferase gene in the pGL3 Basic reporter. For bovine LGALS15 luciferase constructs, restriction endonuclease sites were engineered at the 5′ terminuses of forward and reverse primers. Forward primers contained *HindIII* sites (-2035, -1398, -145) and the common reverse primer contained *XbaI* sites (bov_5′UTR_+40). PCR products were gel purified and digested with *HindIII* or *XbaI* restriction endonucleases prior to ligation into pGL3 Basic vectors.

Plasmid DNA used for transfections was isolated by the alkaline lysis method and anion exchange chromatography (QIAGEN, Valencia, CA).

***Transient Transfection, Luciferase, and Bradford Protein Assays***

Immortalized 2fTGH or U3A cells were subcultured into 12-well plates (70–80% confluent) and transiently cotransfected (n = 4 wells/construct and treatment) with the indicated LUC reporter construct (0.5 mg/well) and pEF1-Myc/His-lacZ (0.05 mg/well) or PRB (0.05 mg/well) using the GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's recommendations. The transfected cells were placed in DMEM/F-12 medium with 10% FBS (phenol-free medium and charcoal stripped FBS if steroid treated) and then treated with roIFNt or left untreated (control) or treated with the synthetic progestin R5020 or vehicle (ethanol). Cell lysates were prepared in Cell Culture Lysis buffer (Promega). Luciferase and Bradford protein assays were performed according to the manufacturer's instructions using a Luciferase Assay System (Promega Corp.) and a Bradford Reagent Kit (BioRad), respectively, and measured with a luminometer. Each transfection experiment contained four replicates and was repeated in at least four independent experiments. All luciferase data were normalized against concentrations of total cellular proteins. The values for concentrations of total cellular proteins were corrected for differences in transfection efficiency among wells and plates within an individual transfection experiment. Normalized luciferase data were then used to calculate the effect of roIFNt or R5020 treatment on LGALS15 promoter activity.

***Pharmacological Demethylation of the LGALS15 Gene in Normal Cells***

Immortalized Madin-Darby bovine kidney cells (MDBK) [345, 346] were subcultured into 100 mm plates (10 x $10^4$ cells/100 mm dish, reaching 10% – 20% confluency in 24 h) and given daily (7 days) doses of either AZA (DMSO, 0.1 μM, or 10μM), TSA (DMSO, 50 nM, 250 nM, or 500 nM), or both. The medium was changed daily with fresh AZA, TSA, both, or DMSO treatments. Following treatment program, total cellular RNA was isolated from MDBK cells using Trizol (Gibco-BRL, Bethesda, MD) according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and the integrity of RNA was examined by gel electrophoresis in a denaturing 1% agarose gel. Briefly, cDNA was synthesized from total endometrial RNA (5 μg) using random and oligo-dT primers and SuperScript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl sterile water, and stored at -20˚C. The cDNAs were diluted (1:10) with sterile water prior to use in PCR reactions. The PCR reactions were performed using Ex Taq DNA polymerase (2.5 U) and 10X Ex Taq buffer (Takara Bio, Carlsbad, CA) according to the manufacturer's recommendations. The primers should have amplified ~234 bp or ~181 bp PCR products for LGALS15 or bIFNT, respectively. PCR amplifications were conducted as follows: 34 cycles of 95˚C for 30 sec, 57˚C (bIFNT primers) or 52˚C (Bt-like-LGALS15 primers) for 1 min, and 72˚C for 1 min. As a positive control, ACTB (beta actin) primers (forward: 5′-ATG AAG ATC CTC ACG GAA CG-3′; reverse: 5′-GAA GGT GGT CTC GTG AAT GC-3′) were used to amplify a cDNA of 270 bp.

### *Statistical Analysis*

The effect of the treatments on reporter construct activity in transient transfection assays was analyzed by least squares ANOVA using the General Linear Models procedure of the Statistical Analysis System (Cary, NC). A *P*-value of 0.05 or less was considered a statistically significant effect of treatment.

## Results

### *Characterizing the Ruminant LGALS15 Gene*

#### *Gene Structure*

Initial sequence analysis of the ovine *LGALS15,* 2.3 kb clone isolated from an ovine genomic DNA library, revealed the presence of the 5′ UTR, transcription start site, translation start site (ATG), exon I and part of exon II (GenBank DQ518347). The ovine, caprine and bovine LGALS15 genes have a very high sequence homology (Figure 4.1). Between sheep and goat sequence homology over the entire 5′promoter/enhancer and coding regions is ~95 %, while between either sheep or goat and cattle the homology is ~84%. The gene structure is similar to that of other galectins such as LGALS1 and

LGALS2 [140, 159]. In all cases, the entire protein is encoded by four exons (Figure 4.2) and the functional CRD is encoded entirely by exon III. The exons and introns are of equal length, spacing and sequence homology (Figure 4.2). The proximal promoter (-306/+1 in sheep; -312/+1 in goats; -146/+1 in cows) of the LGALS15 gene of all three species contains similar transcription factor binding sites (Figure 4.1). The proximal promoter is roughly defined by the presence of a repetitive DNA sequence which is closer to the transcription start site of the bovine LGALS15 gene (~145 bp) than the ovine or caprine LGALS15 gene (~300 bp). LGALS15 contains a TATA box at -28 in sheep and goat and -30 in cattle. Interestingly, exon one is short (-1/+52) and includes an ~41 bp 5′ UTR (-1/+40). Exons two (~80 bp), three (~208 bp) and four (~127 bp) of ovine, caprine and bovine *LGALS15* genes were similar in length and sequence homology. In the first to last exons of the LGALS15 gene, sequence homology is 94% between ovine and caprine and 88% between ovine or caprine and bovine LGALS15.

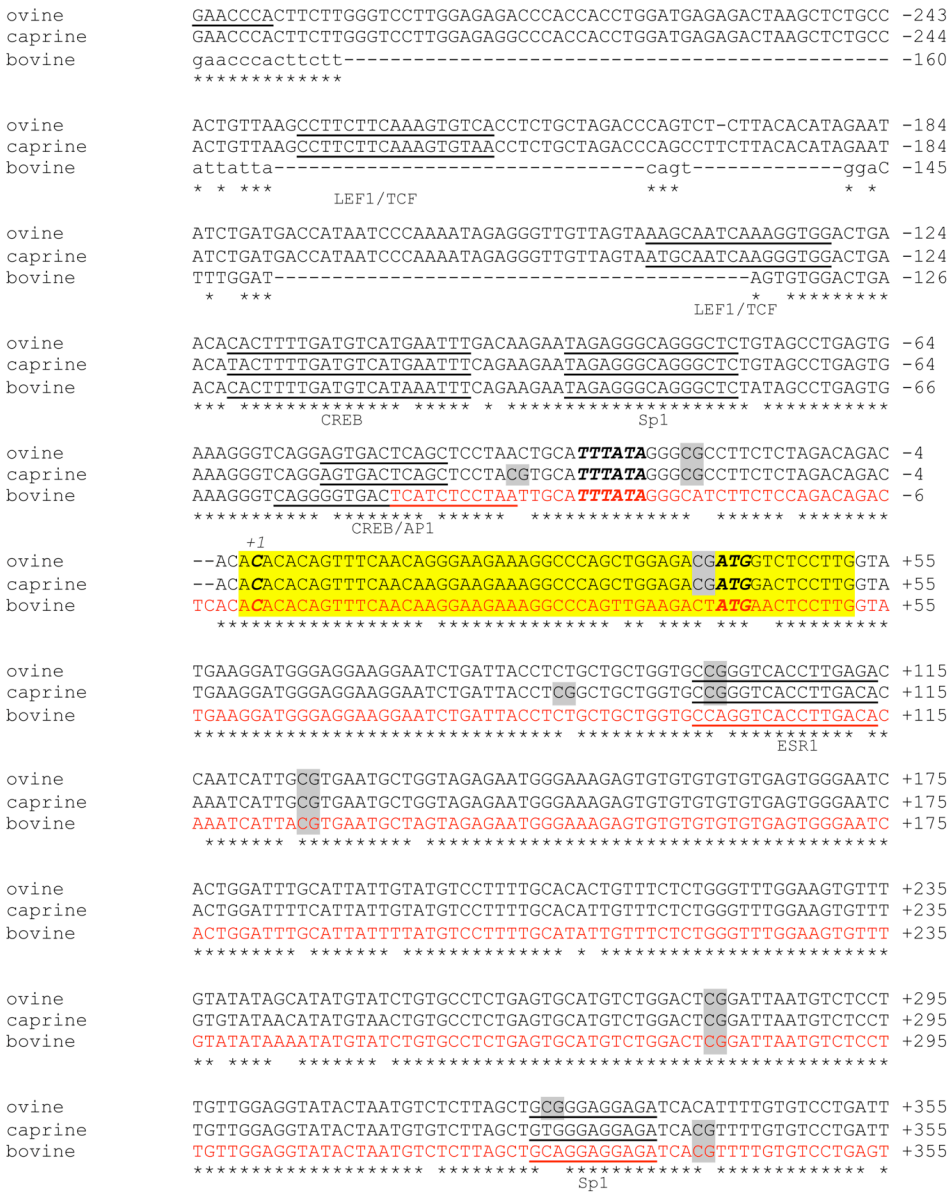


Figure 4.1. Partial alignment of ovine and bovine *LGALS15* gene 5′promoter/enhancer and coding regions. Sequence homology of the entire gene is ~ 85% between ovine or caprine and bovine species and ~ 94% between ovine and caprine species. The TATA box, transcription start site (+1), and translation start site are ***bold and italicized***. Putative transcription factor binding sites were determined using TESS (see Materials and Methods) and are underlined. The highlighted text indicates an exon (exon I is shown). Small case sequence represents the repetitive DNA sequence. CpG dinucleotides are highlighted grey and text in red indicates a CpG island.

*Transcription Factor Binding Sites*

The proximal promoter of all three species has a cAMP response element binding protein (CREB) (~ at -122) and an Sp1 (~ at -91) binding site immediately 5′ of an activator protein 1 (AP1) (~ at -54) binding site on both the sense and antisense strands (Figure 4.1). Weight matrix searches using the Transcription Element Search System (TESS) [349] revealed the presence of additional predicted transcription factor binding sites for the lymphoid enhancer binding factor 1 (LEF1), T-cell factor 1 (TCF1), Ets-1, NFκB, CCAAT, CEBP, ISRE, IRF2 and IRF7 within the repetitive DNA sequences. A glucocorticoid receptor-binding site (GRE) was observed spanning the 3′ terminal boundaries of the repetitive DNA sequence and the proximal promoter region in sheep and goats (-300), but not cattle. The ruminant LGALS15 promoter has putative AP1 binding sites (caprine promoter positions -443, -405; ovine promoter positions -437, -399; and bovine promoter positions -311, -273, -223) within a region of repetitive DNA in the 5′promoter region (Figures 4.1 and 4.2). In the coding region, similar transcription factors were observed. In the first intron of the LGALS15 gene, binding sites for estrogen receptor alpha (ESRI) at +102 on the antisense strand and Sp1 at +325 on the sense strand and in intron II at +700 on the sense strand were observed.

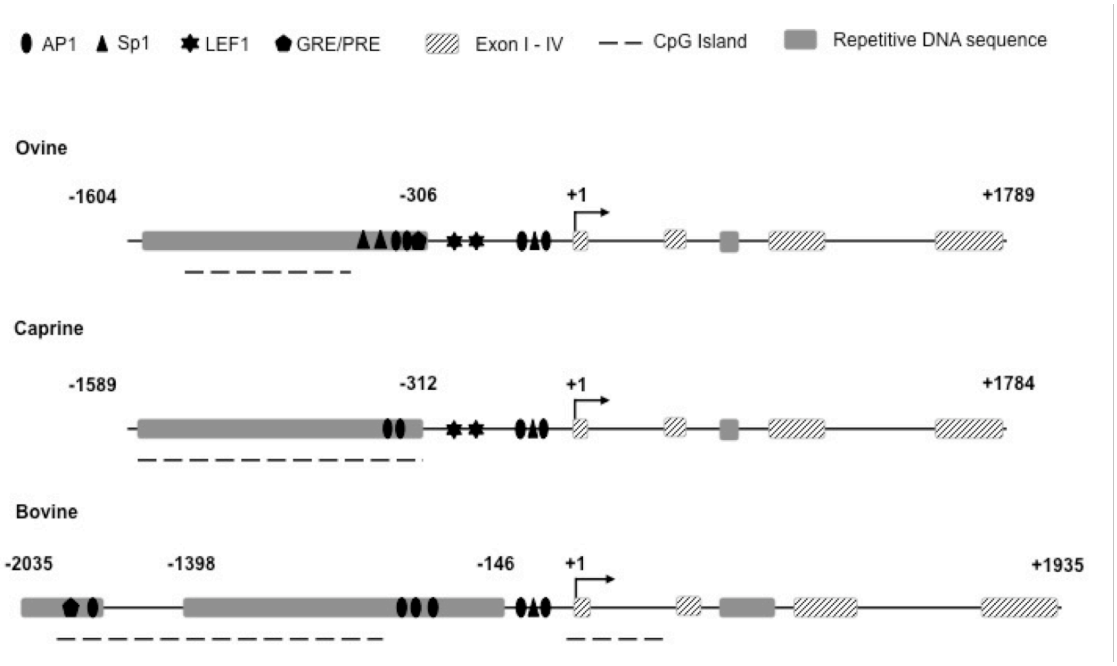


Figure 4.2. Illustration of ruminant *LGALS15* gene architecture, putative transcription factor binding sites, and cytosine paired guanine (CpG) islands and regions containing repetitive DNA sequences. Similar to other galectins, LGALS15 protein is encoded by four exons and the entire CRD is encoded by exon III. The exons are of equal length in all three species. Repetitive interspersed DNA sequences flank the 5'-promoter region of the ruminant LGALS15 gene, and between exons II and III. CpG islands (high ratio of CpG dinucleotides) of various lengths are also associated with the 5′ flanking region including one encompassing the transcription start site (+1) and exon I. Putative transcription factor binding sites are located within the repetitive DNA and within the proximal promoter region (-306/+1 in sheep; -312/+1 in goats; -146/+1 in cows). The proximal promoter region (approximately between the transcription start site and the repetitive DNA element) of bovine LGALS15 is shorter than that for sheep and goats. It contains similar putative transcription factor binding sites.

*Retrotransposable DNA Sequences*

Repetitive DNA sequences were found in the 5'-promoter region of the ruminant LGALS15 gene, and between exons II and III (Figure 4.2). Repeatmasker software identified an approximately 1.2-kb interspersed repetitive DNA element at -1528/-306, -1533/-313 and -1398/-146 in ovine, caprine and bovine promoter/enhancer regions, respectively and a 690-bp repetitive DNA element at -2454/-1765 in the bovine *LGALS15* promoter. In the promoter region, these repetitive DNA elements were of the non-long terminal repeat (RTE) variety. Interestingly, the repetitive DNA element within the bovine *LGALS15* promoter is closer to the transcriptional start site (~145 bp) than repetitive DNA elements within the ovine or caprine *LGALS15* promoters (~300 bp). The repetitive DNA within the *LGALS15* 5' promoter/enhancer region exhibited a high degree of homology with 95% conservation between ovine and caprine and ~88% between either ovine or caprine and bovine. Repetitive DNA between exons II and III was also similar in sequence homology and location. In sheep and goat there is a 50 bp simple repeat of $(CA)_n$ at position +677 and in cattle a 150 bp simple repeat of $(CA)_n$ at +676, $(GA)_n$ at +722, and a GA box at +796. Additionally there is a STAT1 binding site spanning the intron II to exon III boundary at +902 in sheep and goats and +890 in cattle.

*Correlative Indicators of Epigenetic Control*

CpG islands (high ratio of CpG dinucleotides) of various lengths are also associated with the 5' promoter/enhancer region including one encompassing the transcription start site (+1) and exon I in the bovine LGALS15 gene (Figure 4.1). A

search for putative CpG dinucleotide islands revealed CpG islands within the interspersed repetitive DNA of the *LGALS15* promoter of sheep (-1052/-503) and goat (-1533/-313) while there were two putative CpG islands within the bovine *LGALS15* promoter. The larger CpG island (-1826/-416) spans most of the two repetitive elements and a smaller CpG island encompasses the transcription start site and exon I (-48/+357). The CpG dinucleotide density of the ovine, caprine and bovine LGALS15 genes is 1.3%, 1.4% and 1.2%, respectively within about 3 kb of genomic DNA encompassing the transcription start site. For each species there are about 40 CpG dinucleotide pairs in both the 5′promoter/enhancer and gene coding and non-coding regions (about 3 kb). Not only are CpG dinucleotides located within the introns, but also within exons of the LGALS15 gene.

***Transient Transfection Analysis of the LGALS15 Promoter***

Transient transfection analyses determined that IFNT alone cannot activate the ovine LGALS15 promoter. A dose-response experiment to determine the effect of INFT alone on the ovine LGALS15 promoter was conducted in U3A cells (Figure 4.3). The basal promoter activity of the -1604 construct (includes the repetitive DNA) was consistently lower than that of the -323 or -184 constructs (Figure 4.2). Compared to control, there was no effect of IFNT treatment at any concentration on activation of the ovine LGALS15 promoter in U3A cells. The same results were obtained in a study using 2fTGH cells (data not shown). All subseqent studies were conducted in U3A cells.

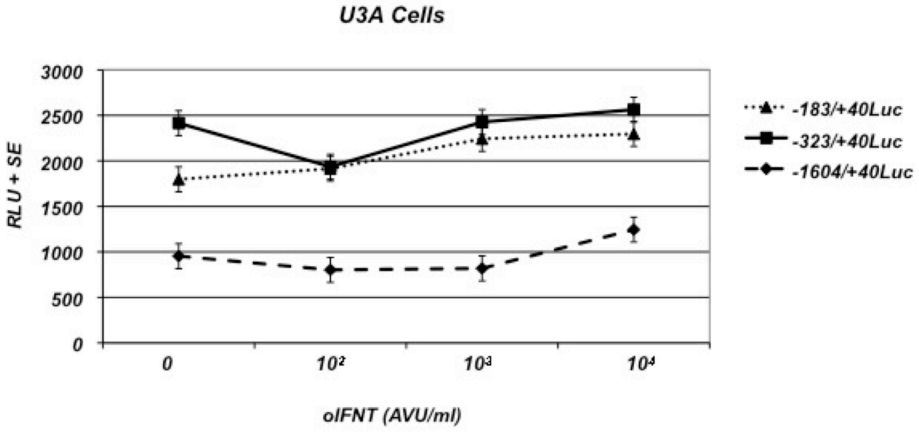


Figure 4.3. Effects of IFNT on ovine *LGALS15* promoter in U3A cells. Cells were cotransfected with LGALS15 constructs and pEF1-Myc/His-lacZ and treated with nothing, or recombinant ovine IFNT ($10^2$, $10^3$ or $10^4$ antiviral units), for 24 h, and luciferase activity was determined as described in Materials and Methods. Results are expressed as mean relative light units (RLU) with SE. Four replicate determinations for each treatment group were conducted in each experiment. Results of a representative experiment of three independent experiments with similar results are presented. Similar results were obtained using 2fTGH cells.

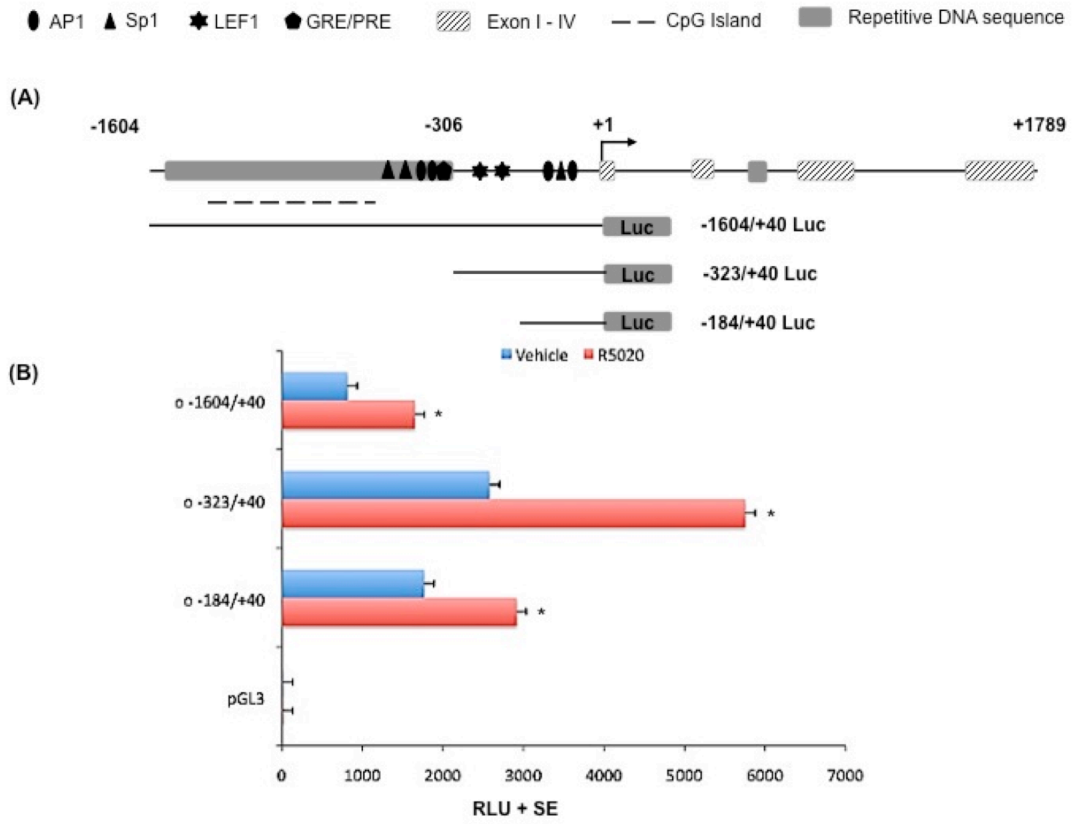


Figure 4.4. Ovine *LGALS15* promoter 5′ truncations and transient transfection analysis in U3A cells. (A) 5′ deletions of the ovine LGALS15 promoter/enhancer region and insertion upstream of the luciferase (Luc) gene in a reporter vector as described in Materials and Methods. The -1604 construct contains putative Sp1 (-497, -485) and AP1 (-437, -399) transcription factor binding sites within a repetitive DNA sequence. (B) Effects of progesterone on the ovine LGALS15 promoter in U3A cells. Cells were co-transfected with LGALS15 constructs and PRB and treated with vehicle or progesterone ($10^{-8}$M), for 24 h, and luciferase activity was determined as described in Materials and Methods. Significant induction ($P < 0.001$) is indicated with an asterisk, and results are expressed as mean relative light units (RLU) with standard error (SE). Four replicate determinations for each treatment group were conducted in each experiment. Results from a representative experiment of three independent experiments with similar results are presented.

Treatments including the synthetic progestin R5020 consistently increased promoter activity. Progesterone alone induced significant activity of the ovine LGALS15 promoter when compared to the vehicle control group ($P < 0.001$) (Figure 4.4). Similar results were observed for the caprine LGALS15 promoter (Figure 4.5). For both ovine and caprine LGALS15 promoter constructs a significant difference was observed between constructs in their response to treatments ($P < 0.001$). A small region of about 350 bp (-300/+40) appears to be critical for activation of the LGALS15 promoter. This region is defined as the proximal promoter because transient transfection analysis consistently showed higher basal promoter activity of this construct in the ovine and caprine LGALS15 gene. The bovine LGALS15 promoter had no activity in the luciferase reporter assay (Figure 4.5). If analyzed separately, there are differences in the activity of the -145/+40 Luc construct when compared to the -2035/+40 and -1398/+40 constructs.

***Methylation of the Bovine LGALS15 Promoter***

Pharmacological studies using MDBK cell treated with AZA alone or in combination with TSA indicated that transcriptional silencing of the bovine LGALS15 allele in these cells is not due to hypermethylation, histone deacetylation and subsequent modification of the chromatin resulting in heterochromatin formation. Results from RT-PCR studies were unable to amplify any bovine LGALS15-like or IFNT transcripts under any experimental conditions.

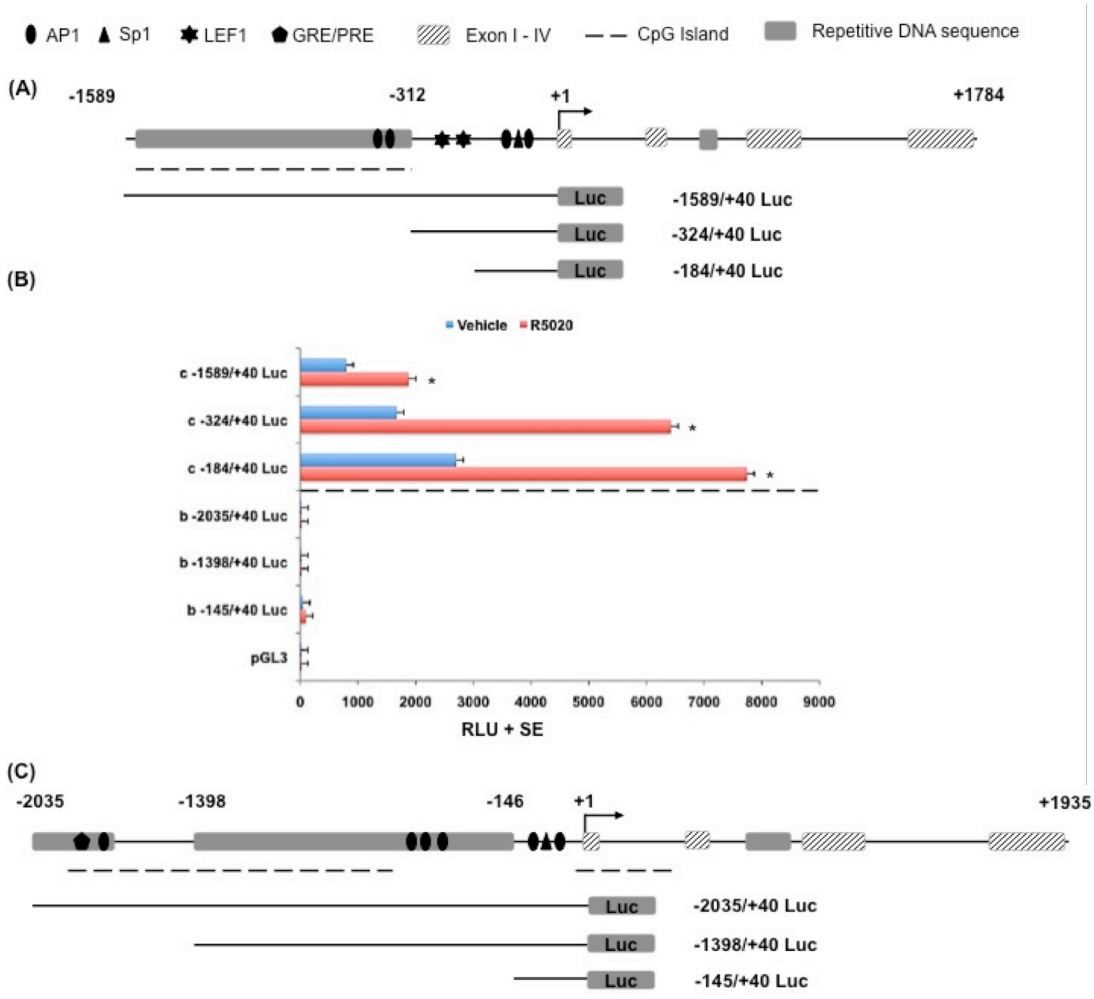


Figure 4.5. Caprine and bovine *LGALS15* promoter 5′ truncation and transient transfection analysis in U3A cells. (A)(C) 5′ deletions of the caprine and bovine LGALS15 promoter/enhancer region and insertion upstream of the luciferase (Luc) gene in a reporter vector as described in Materials and Methods. The caprine -1589 and bovine -2035 and -1398 constructs contain putative Sp1 and AP1 transcription factor binding sites within a repetitive DNA sequence. (B) Effects of progesterone on the caprine and bovine LGALS15 promoter in U3A cells. Cells were cotransfected with LGALS15 constructs and PGRB and treated with vehicle or progesterone ($10^{-8}$M), for 24 h, and luciferase activity was determined as described in Materials and Methods. Significant induction ($P < 0.001$) is indicated with an asterisk, and results are expressed as mean relative light units (RLU) with standard error (SE). Four replicate determinations for each treatment group were conducted in each experiment. Results from a representative experiment of three independent experiments with similar results are presented.

## Discussion

The LGALS15 gene is only expressed in uteri of sheep and goats, but is silenced in cattle [3, 350]. To understand the transcriptional regulation of the ruminant LGALS15, the ovine, caprine and bovine genes were cloned and sequenced. Subsequently, transient transfection analyses of 5′promoter/enhancer deletions were conducted to gain insight into transcriptional regulation of LGALS15 by progesterone and IFNT. Sequence analysis of the ruminant LGALS15 gene indicated a high degree of nucleotide homology and fundamental promoter characteristics. A supposed transcription start site (+1) was identified for all three promoters. Relative to the transcription start site, LGALS15 has a TATA box at ~ -30 (Figure 4.1). This finding parallels those of similar studies on other galectin family members such as LGALS1 and LGALS2 [159, 265], which are also prototype galectins. In fact, LGALS1 and LGALS15 exhibit similar spacing of their TATA box, which indeed directs promoter activation. Unlike the LGALS1 promoter, which also contains an initiator sequence (Inr) overlapping the TATA box [234], no Inr sequence was observed in the LGALS15 promoter. Additional similarities include the number of exons and the functional CRD being encoded entirely by exon III. The exon/intron structure of LGALS15 in all three species suggest they are derived from similar ancestral genes during speciation [351]. The ruminant LGALS15 5′promoter/enhancer region contains putative transcription factor binding sites proven vital for expression and secretion other galectins [352, 353].

The ruminant LGALS15 5′ promoter/enhancer region contains putative transcription factor binding sites for CREB, AP1, Sp1, and various ETS transcription factors (Figures 4.1 and 4.2). Activator protein 1 or Sp1 and ETS are important for the expression of other galectin family members and IFNT, respectively [352, 354, 355]. Activator protein 1 transcription factors are involved rapid responses of mammalian cells to stimuli that impact proliferation, differentiation, and transformation. The LGALS15 proximal promoter exhibits CREB and Sp1 binding sites immediately 5′ of an AP1 binding site, which are conserved in all three species. Interestingly, c-Jun can interact with Sp1 transcription factors, resulting in synergistic activation of some gene promoters [356]. The LGALS15 promoter may be activated by ERK- and/or p38-mediated mitogen-activated protein kinase (MAPK) or NF-κB pathways because transcription factor binding sites for C/EBP-β, Ets-1 and CREB were observed in the 5′ promoter/enhancer region. Indeed, the actions of type I IFNs can be mediated by activation of the MAP kinase and NF-κB pathways [73-75]. In the current *in vitro* studies, IFNT alone did not activate any of the LGALS15 promoter constructs transfected into human fibroblast U3A (STAT1 negative) or 2fTGH (STAT1 positive) cell lines lacking PGR. However, it is plausible that IFNT uses a MAP kinase or NF-κB signaling pathway to stimulate LGALS15 expression in endometrial LE and sGE cells during the peri-implantation period in sheep.

Transient transfection of the LGALS15 promoter into mammalian cells to analyze the effects of progesterone alone or in combination with IFNT showed no significant increase in promoter activity with combined treatments. *In vivo*, the combined actions of progesterone and IFNT resulted in an ~4 fold increase in LGALS15 mRNA abundance when compared to progesterone alone [3]. In these studies progesterone alone or in combination with IFNT could increase LGALS15 transcript abundance, but only in the context of down-regulated PGR. A fundamental difference between the current *in vitro* studies and the *in vivo* animal is the presence of liganded PGR directly interacting with the LGALS15 promoter.

The fact that IFNT alone cannot activate the LGALS15 promoter is not surprising. *In vivo* studies dissecting the individual and combined effects of progesterone and IFNT show that INFT stimulation requires a functional progesterone-PGR interaction [3]. In fact progesterone, acting through its endometrial PGR, is first required for the LE/sGE specific expression of non-classical ISGs including *LGALS15* [3, 176, 177]. Functional interactions with its receptor result in progesterone down-regulating PGR expression in the LE and GE. Paradoxically, progesterone induction of *LGALS15* expression in the endometrial LE and sGE on Day 12 of pregnancy coincides with the loss of PGR in these cells. Importantly, expression of PGR by stromal cells remains positive in the endometrium during this time. This suggests stromal/epithelial interactions in the endometrium in which progesterone acts through its stromal PGR to influence epithelial gene expression. Factors derived from the stroma and acting in a paracrine manner through their epithelial receptors are believed to mediate such

stromal/epithelial interactions [357]. Thus, progesterone likely induces *LGALS15* expression in the LE and sGE indirectly by interacting with stromal PGR.

In the ovine uterus, fibroblast growth factor 10 (FGF10) and hepatocyte growth factor (HGF) are specifically expressed in the stroma while the expression of their receptors, FGFRIIIb and c-met is confined to the uterine epithelia [358, 359]. Indeed, expression of FGF10 and c-met are increased by progesterone in the ovine uterus [360]. Available results indicate the effects of FGF10 are mediated by an ERK dependent cell signaling pathway [361]. It is feasible then, that LGALS15 expression in the LE and sGE is mediated by activation of a MAP kinase signaling pathway coordinated by the sequential actions of progesterone induced, stromal derived FGF10, and conceptus derived IFNT acting through their epithelial receptors.

Repetitive interspersed DNA sequences are widely distributed throughout the genome. When found in the 5′promoter region of a gene, they can influence the quality and quantity of gene transcription [362, 363]. This could explain the lack *LGALS15* expression in cattle. Repetitive DNA sequences are about 145 bp from the transcription start site in the bovine LGALS15 promoter. This is considerably closer to the transcription start site and all promoter constructs containing repetitive DNA sequences showed reduced basal activity (Figures 4.4 and 4.5). Additionally, simple repeats such as $(CA)_n$ and $(GA)_n$, observed in intron two of all ruminant species investigated, could influence transcriptional efficiency [364]. Importantly, the simple repeat observed in the bovine *LGALS15* gene is longer than those in either the caprine or ovine *LGALS15* genes. Potentially contributing to the lack of *LGALS15* expression in the bovine uterus.

Even though cell specific transcriptional regulation of other galectins is due to promoter methylation [28, 264], this does not hold true for LGALS15. Pharmacological studies on cells not expressing LGALS15 were designed to induce aberrant expression of the gene using demethylating (AZA) and/or acetylating (TSA) agents. No variation of concentrations of treatments or time of exposure resulted in LGALS15 gene activation. These results were not surprising when put in the context of the activity of these agents on normal cells. Studies show that the demethylating agent, AZA, affects normal cells differently than cancer cells resulting in a significantly lower number of genes being activated in normal cells [344]. Indeed this characteristic makes AZA (Decitabine) a useful anticancer drug for chemotherapeutic reactivation of genes suppressed in tumors.

Collectively, these results indicate that the LGALS15 5′promoter/enhancer contains putative transcription factor binding sites that suggest regulation by MAP kinase and/or NF-κB signaling pathways. The presence of a conserved retrotransposable DNA element in the 5′promoter/enhancer region negatively modulates *LGALS15* expression and physically defines a region essential for promoter activation. Comparatively, this region is substantially smaller for bovine LGALS15 than for ovine or caprine LGALS15. Transcriptional silencing of LGALS15 expression in bovine uteri is likely a result of the proximity of a retroelement to the transcription start site and not promoter hypermethylation. IFNT alone cannot stimulate LGALS15 promoter activity, even in cells lines inherently lacking PGR. A more detailed definition of the relationship

between IFNT and LGALS15 expression will require an *in vitro* model that better recapitulates the uterine milieu.

Future studies will focus on the role of retroelements in modulating LGALS15 expression in the uterus, defining the chromosomal location of the LGALS15 gene in ruminant species, and galectin fingerprinting to determine which members of the galectin-superfamily are expressed in the bovine uterus.

# CHAPTER V

## SUMMARY

Galectins are a family of secreted animal lectins with biological roles in cell adhesion and migration. In sheep, *LGALS15* is expressed specifically in the endometrial luminal (LE) and superficial glandular (sGE) epithelia of the uterus in concert with blastocyst elongation during the peri-implantation period. The studies herein examined *LGALS15* expression in uteri of cattle, goats, and pigs. Additionally, initial studies aimed at determining *LGALS15* gene structure and promoter activity were conducted.

Although the bovine genome contains a *LGALS15*-like gene, expressed sequence tags (ESTs) encoding *LGALS15* mRNA were found only for sheep, and full-length *LGALS15* cDNAs were cloned only from endometrial total RNA isolated from pregnant sheep and goats, but not pregnant cattle or pigs. Ovine and caprine *LGALS15* were highly homologous at the mRNA (95%) and protein (91%) levels, and all contained a conserved carbohydrate recognition domain and RGD recognition sequence for integrin binding. Endometrial *LGALS15* mRNA levels increased after Day 11 of both the estrous cycle and pregnancy, and were considerably increased after Day 15 of pregnancy in goats. *In situ* hybridization detected abundant *LGALS15* mRNA in endometrial LE and sGE of early pregnant goats, but not in cattle or pigs. Immunoreactive LGALS15 protein was present in endometrial epithelia and conceptus trophectoderm of goat uteri and detected within intracellular crystal structures in trophectoderm and LE. Recombinant ovine and caprine LGALS15 proteins elicited a dose-dependent increase in ovine trophectoderm cell attachment *in vitro* that was comparable to bovine fibronectin.

These results support the hypothesis that *LGALS15* is uniquely expressed in Caprinae endometria and functions as an attachment factor important for peri-implantation blastocyst elongation.

Galectins are a family of animal lectins with a high affinity to beta-galactosides commonly involved in cellular functions such as apoptosis, adhesion and migration. Galectin 15 (LGALS15), the newest member of the galectin superfamily, has a unique C-terminal RGD sequence and participates in integrin-mediated ovine trophectoderm cell attachment and migration. In the ovine uterus, *LGALS15* is expressed only by the endometrial LE and sGE, induced by progesterone between Days 10 and 12 of the cycle and pregnancy, and then stimulated by interferon tau (IFNT) from the conceptus after Day 14 of pregnancy.

Progesterone induction of *LGALS15* apparently involves progesterone down-regulation of progesterone receptor (PGR) in the endometrial LE/sGE. During early pregnancy, the classical janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is not active in the endometrial LE/sGE. Therefore, IFNT utilizes a non-classical signaling pathway, likely the MAP kinase pathway [73, 74], to increase transcription of genes, including *CST3, CTSL, HIF2A, LGALS15*, and *WNT7A*, specifically in the endometrial LE/sGE. The LGALS15 promoter may be activated by ERK- and/or p38-mediated mitogen-activated protein kinase (MAPK) or NF-κB pathways because transcription factor binding sites for C/EBP-β, Ets-1 and CREB were observed in the 5′ promoter/enhancer region (Figure 5.1). Indeed, the actions of type I IFNs can be mediated by activation of the MAP kinase and NF-κB pathways [73-75].

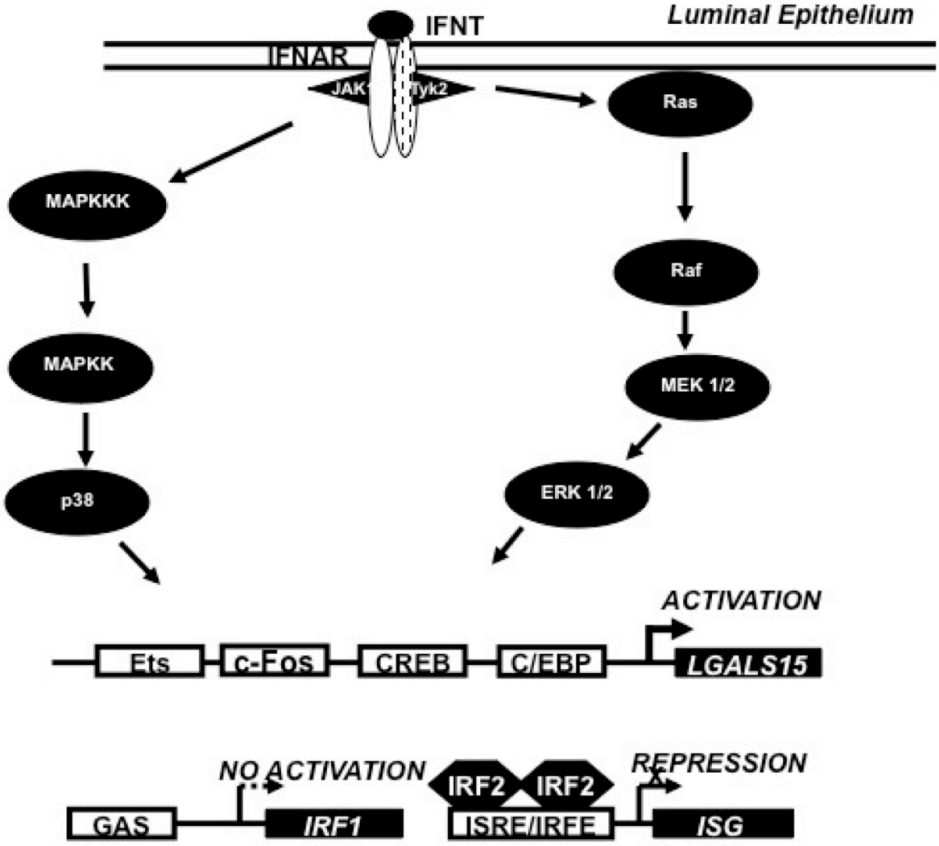


Figure 5.1. Schematic illustrating the current theory on IFNT signaling in the ovine or caprine endometrial luminal (LE) or superficial glandular epithelia (sGE). The non-classical actions of type I IFNs in the endometrial LE and sGE are likely mediated by the initiation of the p38 and/or ERK mitogen-activated protein kinase (MAPK) pathways. The ruminant LGALS15 5′ promoter/enhancer region contains putative transcription factor binding sites for CREB, AP1, C/EBP, and various ETS transcription factors suggesting activation by MAPK signaling pathways. Activation of type I IFNAR by IFNT thus may initiate ERK- and/or p38-mediated MAPK pathways resulting in the transactivation of the LGALS15 5′ promoter/enhancer region in the endometrial LE and sGE. LE and sGE expression of *LGALS15* is concomitant with expression of IRF2, a potent repressor of classical ISGs.

Although the *LGALS15* gene is present in ovine, caprine and bovine species, it is only expressed in uteri of sheep and goats. Our working hypothesis is that *LGALS15* expression is regulated by epigenetic mechanisms and activated PGR inhibits transcription of the *LGALS15* gene in sheep and goat uteri.

An ovine genomic DNA library was screened with the ovine *LGALS15* cDNA, and a clone containing ~15 kb of genomic DNA was isolated. A 1.63 kb portion of the 5′ promoter/enhancer region of the ovine *LGALS15* gene was sequenced. The 5′ promoter/enhancer region of the caprine *LGALS15* gene was cloned using primers designed from the ovine sequence and caprine genomic DNA used as a template. The 5′ promoter/enhancer region of the bovine *LGALS15* gene was cloned using primers designed from *Bos taurus* genomic contig NW_001493613.1 and bovine genomic DNA as a template. Relative to the predicted transcriptional start site (+1), the proximal promoters of sheep (-305/+1), goat (-312/+1) and cattle (-145/+1) *LGALS15* contain a TATA box at -28 in sheep and goat and -30 in cattle. Repeatmasker software identified an approximately 1.2-kb interspersed repetitive DNA element at -1528/-306, -1533/-313 and -1398/-146 in ovine, caprine and bovine promoter/enhancer regions, respectively and a 690-bp repetitive DNA element at -2454/-1765 in the bovine *LGALS15* promoter. Interestingly, the repetitive DNA element within the bovine *LGALS15* promoter is closer to the transcriptional start site (~145 bp) than repetitive DNA elements within the ovine or caprine *LGALS15* promoters (~300 bp). The repetitive DNA within the *LGALS15* 5′ promoter/enhancer region exhibited a high degree of homology with 95% conservation between ovine and caprine and ~88% between either ovine or caprine and bovine. In all

three species, exon structure and length are similar. Interestingly, exon one is short (-1/+52) and includes a ~41 bp 5′ UTR (-1/+40). Exons two (~80 bp), three (~208 bp) and four (~127 bp) of ovine, caprine and bovine *LGALS15* genes were similar in length. Additionally, 94% (ovine:caprine) or 88% (ovine or caprine:bovine) sequence homology was in the first to last exons of the *LGALS15* gene.

A search for putative CpG islands revealed their presence within the interspersed repetitive DNA sequences in all three species but in the bovine LGALS15 gene, a CpG island encompassed the proximal promoter, transcription start site, and exon I. Using the Transcription Element Search System (TESS), the *LGALS15* promoter of sheep, goat and cattle all contained predicted transcription factor binding sites for activator protein-1 (AP1), lymphoid enhancer binding factor 1 (LEF1), T-cell factor 1 (TCF1), Sp1, C/EBP-β, Ets-1, CREB glucocorticoid receptor (GR) and estrogen receptor alpha (ESR1), Future studies will utilize the *LGALS15* promoter to discover how progesterone and IFNT regulate expression of selected genes in the endometrial LE/sGE during pregnancy.

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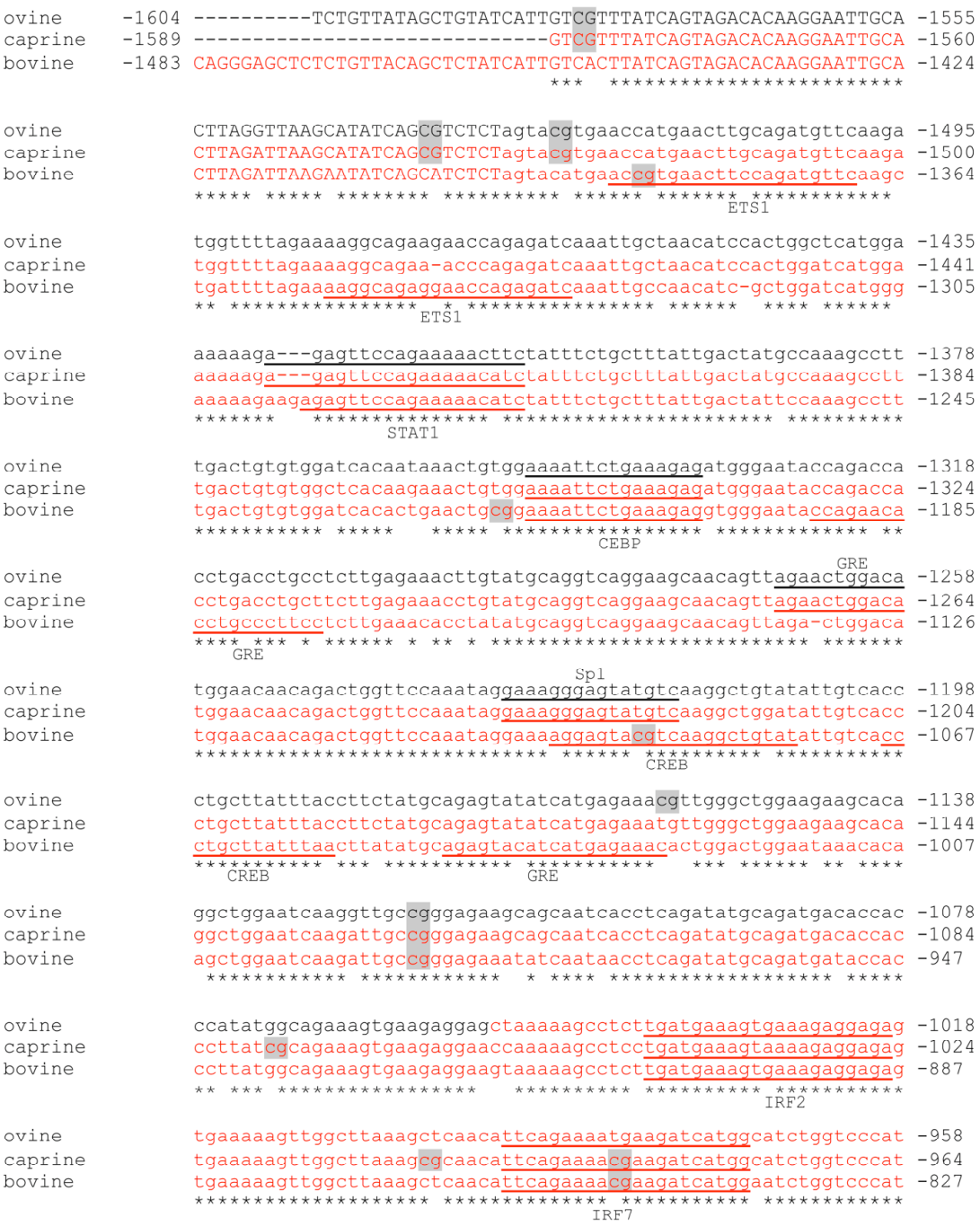
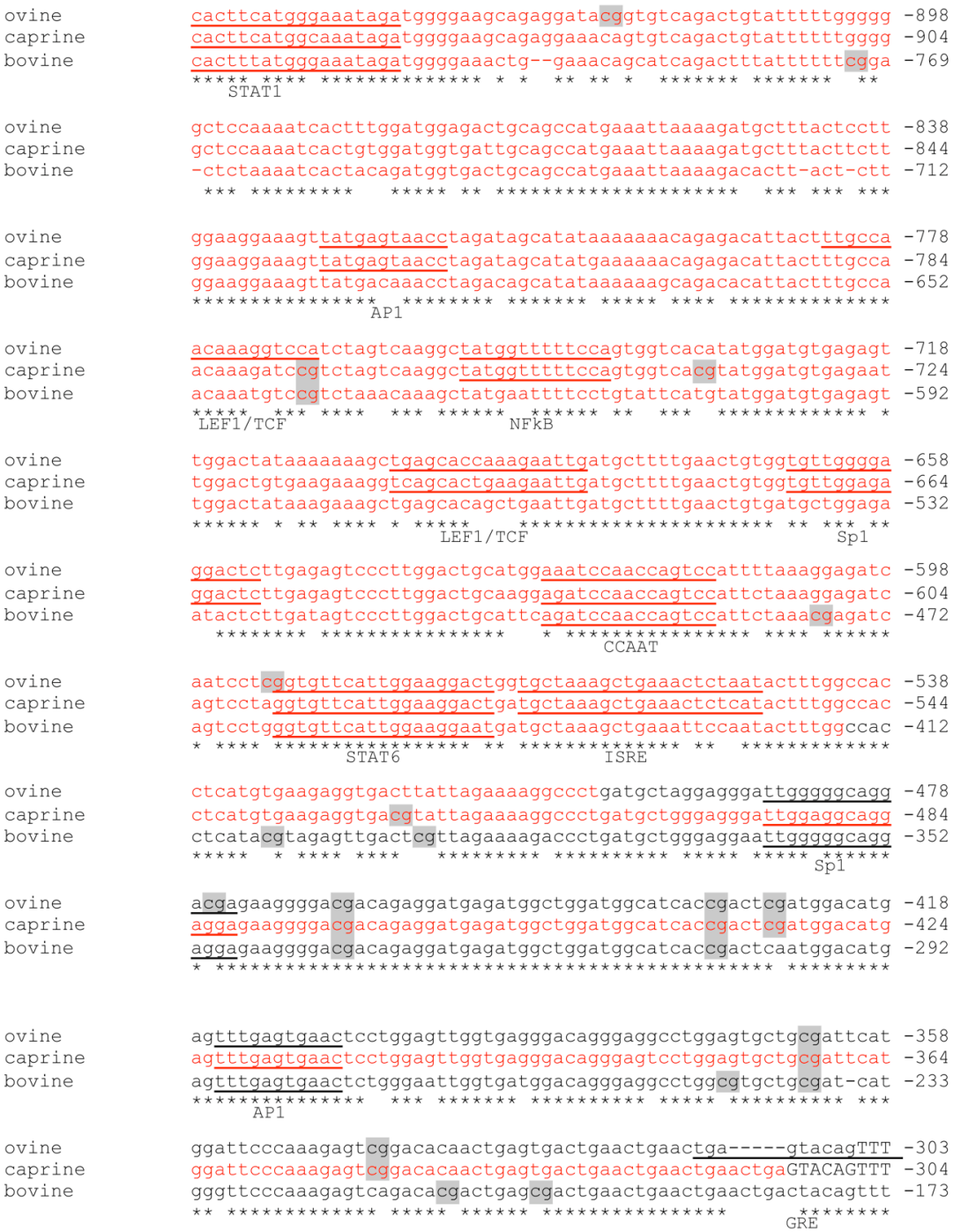
# APPENDIX I

## ALIGNED AND ANNOTATED RUMINANT *LGALS15* GENE SEQUENCES

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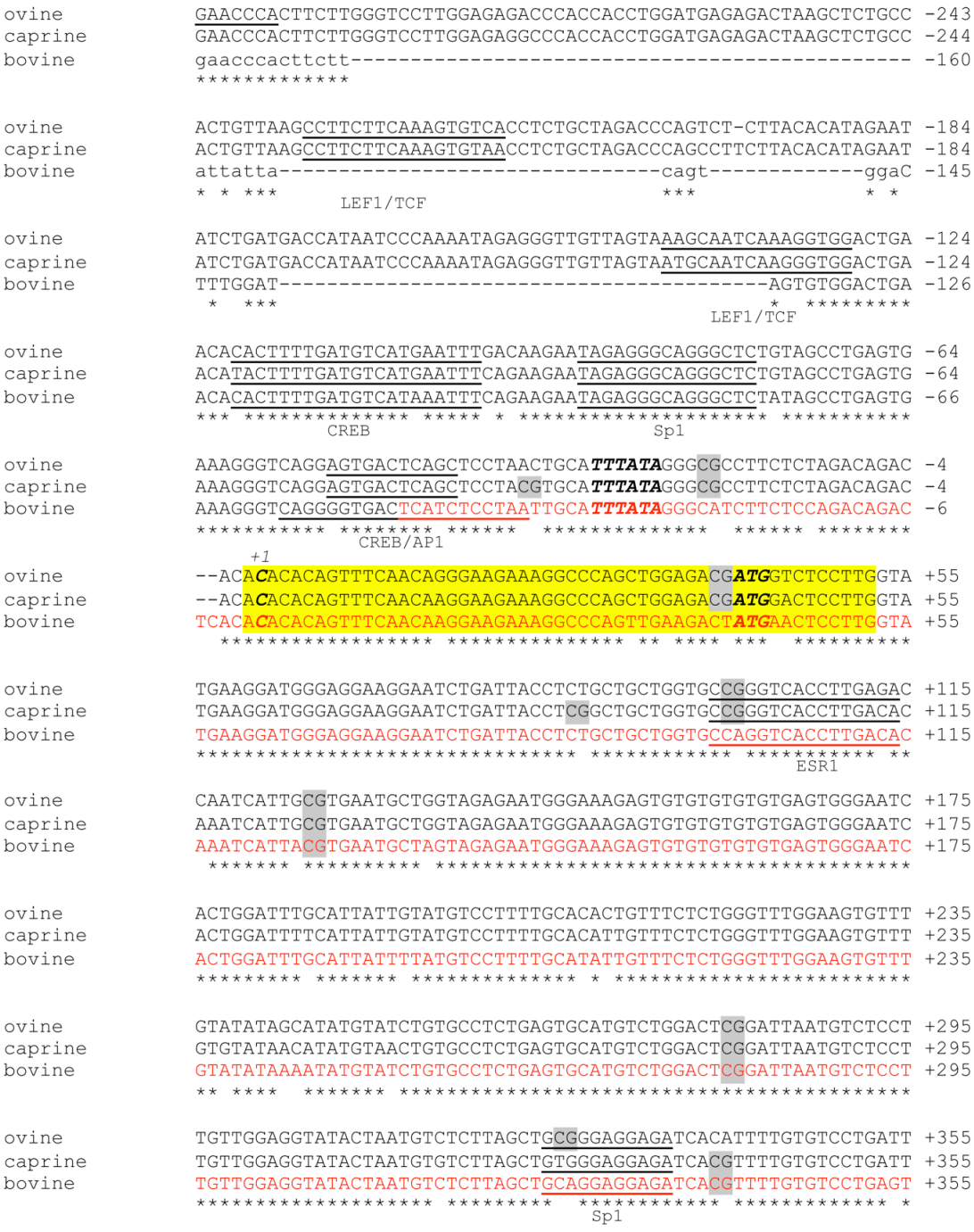
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bovine gaacccacttctt---------- -160
LEF1/TCF
ovine ACTGTTAAGCCTTCTTCAAAGTGTCACCTCTGCTAGACCCAGTCT-CTTACACATAGAAT -184
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LEF1/TCF
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LEF1/TCF
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CREB
Sp1
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CREB/AP1
+1
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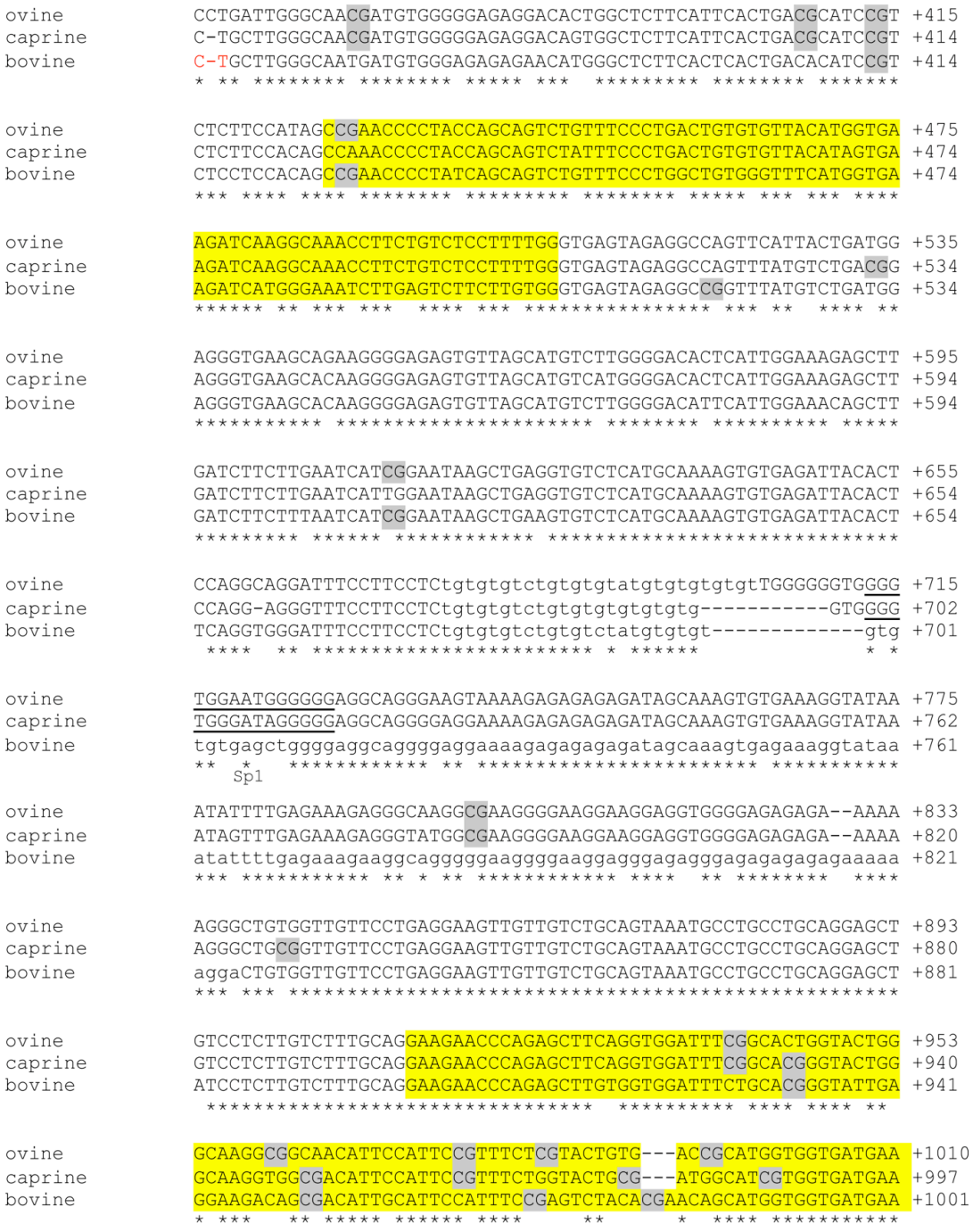
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bovine         CTCCAGGGTGTGGAGCGCCATGGCCATGTTGGGGCTGCTGTGGAGAAACACACGGAGAGT +1181
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bovine         CCAACTCTGATGTGATCACAACAGACCAATTTTTTGTTTGTT---TTGTATTTTTAACAC +1238
               ****************  *** *********** * ******   ***** *** *****

ovine          ATGTGCTTTTTAAAATTGAAGTGTCATTGATTTCCCTTACTGTGTGAATCTCAGGTGCAC +1305
caprine        ATGTGCTTTTAAAAATTGAAATGTCATTGATTTCCCTTACTGTGTGAGTCTCAGGTGCGC +1294
bovine         ATGTGCTTTTTGAAATTGAAGTATCATTGATTTACCTTACCATGTGAGTTTCAGGCGTAC +1298
               ********** ******** * ********** ******  ***** * ***** *  *

ovine          AGCAGAGTGACTGAGCACCTTGTCCCCGTATAGCATAGGCCTCTATACTCAGGATTTTGT +1365
caprine        AGCAGAGTGATTGAGCACCTTGTCCTCGCATAGCATAGGCCTCTATACTCAGGATCTTGT +1354
bovine         AACAGAGTGGTGGAGCCCCTTGTCCCTGTATAGCACAGGCATCTATACTCAGGATCTTGT +1358
               * *******   **** ********  * ****** **** ************** ****

ovine          GTGCTGTAC------------------AATTAGAGATTCTGTGGTGAACTCCACACAGCT +1407
caprine        GTGCTGTGC------------------AATTAGAGATTCTGCGGTGAACTCCACACAGCT +1396
bovine         GTGCTGTACTCAGTGTGTGTGCTGTACAAGTAGAGATGCTGAGGTGAACTCCACACAGCT +1418
               ******* *                  ** ******* *** ******************
                        C/EBP alpha

ovine          CTCCCTGTATCGGACAGTCAGCAGTGTGGAGGGAGACATGCAGGCCCCCTGCCCTCAGGG +1467
caprine        CTTCCTGTATCAGACAGTCAGCAGTGTGGAGGGAGACATGCAGGCCCCCTGCCCTCAGGG +1456
bovine         CTTACTGTATCGGACAGTCAGCAGTGTGGAGGGAGACGTGCAGGCCCCCTGCCCTCAGGG +1478
               **  ******* ************************* **********************

ovine          CAAAGCAGGGGTGGCAGCTCTGGAGGCAAGGAAGTGGACATGGAGGATGATTCCTACAGC +1527
caprine        TAAAGCAGGGTTGGCAGCTCTGGAGGCGAGGAAGTGGACATGGAGGATGATTCCTATAGC +1516
bovine         TAAAGCAGGGATGACAGCTCTGGAGGCGAGGAAGTGGACATGGAGGATGATTTCTACAGC +1538
                ********* ** ************* ************************ *** ***

ovine          AGTGTCTTGGCAGGATGTCCAGGAG---AATTTCTGCTTTGCTTTTACAGAGTAAAGAAA +1584
caprine        AGTGTCTTGGCGGGATGTCCAGGAGGAGAATTTCTGCTTTGCTTTTACAGAGTAAAGAAA +1576
bovine         ACTGTATTGGCAGGATGTCCTGGAGGAGAATTTCTGCTTTGCTTTTACAGAGTAAAGAAA +1598
               * *** ***** ******** ****   ********************************

ovine          TTTTAGAGAAGGCAATGAAAATAGGAAAGGGAAAGGGGAGG---CCTAATTGTTCTGAGC +1641
caprine        TTTTACAGGAGGCAATGAAAATAGGAAAGGGAAAGAGGAGGAGGCCTAATTGTTCTGAGC +1636
bovine         TTTTACAGAAGGCAATGAAAGTAGGAAAGGGAAAGAGGAGG---CCTAATCGTTCTGAGC +1655
               ***** ** *********** ************** *****   ****** *********
```

```
ovine           TCACAAGAAATATATCTCCTTCCTTAGGTGTTTGTGAAAAACAAGCCCATCTGCCAGTTT +1701
caprine         TCACAAGAAATATATCTCCTTCCTTAGGTGTTTGTGAATAGCAAGCCCATCTGCCAGTTT +1696
bovine          TCACAAGAAATACATCTCCTTCCTTAGGTGTTTGTGAATAACGAGTCCTTCTGCCAGTTT +1715
                *********** ************************ * * ** ** ***********

ovine           GCGCACCGCCTGCCCCTGCAGTCTGTGAAAATGCTGGATGTGAGGGGAGATATCGTGCTG +1761
caprine         GCCCACCGCCTGCCCCTACAGTCTGTGAAAATGCTGGATGTGAGGGGAGATATCGTGCTG +1756
bovine          GCCCACCGCCTGCCCCTACAGTCTGTGAAAATGCTGAAGGTGAAGGGAGATACTGTGCTG +1775
                ** ************** ****************** * **** ********  ******

ovine           ACTTCAGTGGATACGTTATAAGGGGCGG +1789
caprine         ACTTCAGTGGATACGTTATAAGGGGCGG +1784
bovine          ACTTCAGTGGATACATTTTAAGGGGCAG +1803
                ************** ** ******** *
```

# APPENDIX II

## COMPLETE CAPRINE, OVINE, AND BOVINE *LGALS15* GENE SEQUENCES

*Caprine LGALS15*

GTCGTTTATCAGTAGACACAAGGAATTGCACTTAGATTAAGCATATCAGCGTCTCTagtacgtg
aaccatgaacttgcagatgttcaagatggttttagaaaaggcagaaacccagagatcaaattgc
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*Ovine LGALS15*
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*Bovine LGALS15*

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TG

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Education/Training:

| Institution | Degree | Year | Field |
|---|---|---|---|
| Texas A&M University | Ph.D. | 2009 | Physiology of Reproduction |
| Prairie View A&M University | M.S. | 2004 | Animal Science |
| Prairie View A&M University | B.S. | 2002 | Biology |

Honors and Awards:

2008 Invited Speaker: "The Mighty Have Fallen. The Fallen Have Become Mighty; Significant Contributions by the Negligible Ninetieth" Prairie View A&M University Department of Biology Annual Research Symposium, Prairie View, Texas.

2007 Mauro Procknor Memorial Award, Interdisciplinary Faculty of Reproductive Biology, Texas A&M University, College Station, Texas.

2007 Burroughs Wellcome Minority Affairs Award, Society for the Study of Reproduction, San Antonio, Texas.

2006 Frontiers in Reproduction, Molecular and Cellular Concepts Course, Marine Biological Laboratory, Woods Hole, Massachusetts.

2006 Carl Storm Underrepresented Minority Fellow, Gordon Research Conference on Reproductive Tract Biology, Connecticut College, New London, Connecticut.

2005 FASEB/MARC Fellow, Society for the Study of Reproduction, Laval University, Quebec, Canada.