BIOLOGICAL FUNCTIONS OF GALECTIN 15 (LGALS15)

IN THE OVINE UTERUS

A Dissertation

by

JENNIFER LYNN FARMER

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

December 2008

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee:	Fuller W. Bazer
	Thomas E. Spencer
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ABSTRACT

Biological Functions of Galectin 15 (LGALS15) in the Ovine Uterus.

(December 2008)

Jennifer Lynn Farmer, B.S., Clemson University

Co-Chairs of Advisory Committee: Dr. Fuller W. Bazer
Dr. Thomas E. Spencer

Galectins are proteins with 15 known members found in nearly all living organisms. They share a conserved CRD that binds beta-galactoside sugars, and functions to cross-link glycoproteins as well as glycolipid receptors on the surface of cells to initiate biological responses. Functional studies on the extracellular and intracellular roles of galectins implicate them in cell adhesion, chemoattraction and migration as well as growth, differentiation and apoptosis. Therefore, studies were conducted to identify functional roles of galectin 15 (LGALS15) during the peri-implantation period of pregnancy in the sheep.

The first study was designed to develop and characterize primary ovine trophectoderm cell lines for the study of the biological functions of LGALS15. Once characterized, these cell lines were used to investigate the role of LGALS15 in trophectoderm gene expression, development, growth, and survival. Two primary trophectoderm cell lines (oTr1 and oTrF) were developed, and they had characteristics similar to *in vivo* conceptus trophectoderm relative to gene expression, morphology, and

migration and proved suitable as an *in vitro* model to investigate functional roles of LGALS15.

The second study investigated LGALS15 function in trophectoderm cell adhesion. A dose-dependent increase in oTr cell attachment to LGALS15 was found that could be inhibited by cyclic GRGDS, but not GRADS, peptides. Mutation of the LDVRGD integrin binding sequence of LGALS15 to LADRAD decreased its ability to promote oTr cell attachment, whereas mutation of the CRD had little effect. LGALS15 induced formation of robust focal adhesions in oTr cells that were abolished by mutation of the LDVRGD sequence.

The third study tested the hypothesis that LGALS15 is a secreted regulator of trophectoderm development and gene expression, as well as growth, migration, and apoptosis of trophoblast. LGALS15 moderately increased cellular proliferation, partially inhibited staurosporine elicited apoptosis, stimulated migration that was dependent on Jun N-terminal kinase (JNK), and initiated differential gene expression of oTr cells.

Collectively, these results support the hypothesis that LGALS15 has a biological role in the peri-implantation stage of early pregnancy in the ovine uterus and stimulates trophectoderm cell gene expression, migration and attachment via integrin binding and activation which are critical to blastocyst elongation and implantation.

DEDICATION

To my loving and supportive parents

James and Catherine Farmer

And to my big sister

Katie

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I would like to thank my mentors, Drs. Fuller Bazer and Thomas Spencer, for their guidance and support throughout my time at Texas A&M University, and for their example of hard work and excellence. I consider them my friends as well as mentors. I would also like to thank my committee members, Drs. Robert Burghardt and Gregory Johnson, for unselfishly giving their time and expertise over the years. The research presented here could not have been done without the help and support of both past and present members of the lab and our lab manager Dr. Jo-Anne Fleming. Thank you so much for your patience, assistance, and friendship. I would also like to thank my part time roommate Ken Widmer who kept me sane while studying for my preliminary exams. Special thanks to Nahum Puebla for his support and advice throughout my time at Texas A&M, I do not think I could have done it without him.

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CHAPTER I

INTRODUCTION

The most important evolutionary advances in reproduction came with the development of viviparity and placental formation which permits vital stages of embryonic development to either begin or begin and be completed within a part of the parent's body [1]. Yolk is the principal—and sometimes only—source of nutrition available to support embryogenesis in all non-mammalian vertebrates and is vital during the early stages of embryogenesis in monotremes and marsupials [2]. In Eutherian species, however, embryogenesis, from the time it hatches from the zona pellucida (ZP), is directly dependent on maternal nutrients. In Eutherian mammals, including sheep, viviparity requires progesterone to maintain pregnancy, and the development of a trophoblast that expresses properties independent of the developing embryo. The trophectoderm forms the outermost cell layer of blastocysts, which attaches to the uterine luminal epithelium (LE) and serves to transport nutrients to the conceptus (embryo/fetus and associated membranes) [3].

Prior to placental formation, the conceptus is dependent on endometrial secretions as its major source of nutrients. Endometrial secretions, along with molecules selectively transferred from maternal circulation into the uterine lumen, collectively termed histotroph, regulate conceptus survival and growth during pregnancy [4-7].

This dissertation follows the style of Biology of Reproduction.

Components of histotroph play major roles in initiating conceptus development from a spherical blastocyst, to a tubular, and then filamentous conceptus. These components also aid in conceptus attachment to the uterine LE. The uterine gland knock out (UGKO) ewe, where endometrial glands fail to develop and there is a reduction in uterine LE to produce uterine secretions, cannot support conceptus development beyond Day 14 of pregnancy [6, 8].

Superficial implantation is mediated through binding of bridging ligand secretory proteins from glandular epithelium (GE) and/or LE to receptors on the apical surface of the conceptus trophectoderm. Guillomot and colleagues [9] proposed that the adhesion cascade followed by most mammalian blastocysts includes: 1) shedding of the zona pelucida; 2) precontact and blastocyst orientation; 3) apposition; 4) adhesion; and 5) endometrial invasion. True endometrial invasion by blastocysts occurs in humans and mice, but not pigs or ruminants [9, 10]. For all species, however, adhesive proteins secreted from LE and/or GE, as components of histotroph, facilitate the initiation of conceptus adhesion and implantation.

In sheep, secreted phosphoprotein one (SPP1) and glycosylated cell adhesion molecule one (GLYCAM-1) are secreted by uterine epithelia and bind to integrin subunits and L-selectin, respectively, to promote adhesion of conceptuses to uterine LE [11, 12]. Galectins are another family of proteins implicated in implantation. Galectins are secreted animal lectins which contain a conserved carbohydrate recognition domain (CRD) responsible for binding beta-galactosides, thereby cross-linking glycoconjugate receptors on the surface of cells and initiating biological responses [13-15]. This ability

to bind various carbohydrates has implicated several members of the galectin family in cell-cell adhesion, and in some cases, adhesion between parasite/bacteria and host [16]. Many galectins are expressed both intracellularly (cytoplasm and nucleus) and extracellularly (cell surface and secretions), and are often secreted and/or integrated into the extracellular matrix (ECM) [17]. Galectins lack known secretory signal peptide sequences and are secreted in a non-classical manner. Functional studies on extracellular and intracellular roles of galectins have implicated them in cell growth, differentiation and apoptosis, in addition to adhesion, chemoattraction and migration [18]. All of these biological effects are critical for apposition, attachment and adhesion stages of conceptus implantation.

The 15 known members of the galectin family share a high level of evolutionary conservation [19] and can be divided into three groups: 1) prototype galectins (galectins-1, 2, 5, 7, 10, 11, 13, 14, and 15) existing as monomers or noncovalent homodimers with one CRD; 2) chimera-type galectins (galectin-3) containing a nonlectin domain linked to a CRD; and 3) tandem-repeat-type galectins (galectins-4, 6, 8, 9, and 12) with two distinct CRDs [20]. Members of this gene family are present in species ranging from sponges to mammals.

The most recently discovered member of the galectin family was first identified as being induced in the sheep intestine as part of the immune response to parasitic infection by *Haemonchus contortus* and initially given the name *ovgal11* [21]. It was confirmed to be a novel member of the galectin family of proteins and was renamed LGALS15 [22]. Interestingly, LGALS15 is a component of the crystalline inclusions in

conceptus trophectoderm and uterine LE of ruminants [22, 23]. In addition to a conserved CRD, LGALS15 contains specific integrin recognition sequences (LDV and RGD) proven to mediate integrin binding in ECM proteins [24-26]. *LGALS15* was found to be a major gene expressed by uterine LE and superficial ductal glandular epithelium (sGE) during the peri-implantation period and regulated by progesterone and interferon tau (IFNT) [22]. LGALS15 is abundant in histotroph and increases between Days 12 and 16 of pregnancy in association with conceptus adhesion, implantation and pregnancy recognition [22].

A primary cause of embryonic loss during early pregnancy is the inability of the conceptus to bind to LE and begin the delicate processes of implantation and development in species with invasive implantation [27, 28], whereas in sheep and other species with noninvasive implantation most embryonic loss occurs during the period when the spherical blastocysts must transition into filamentous conceptuses [29]. In sheep, this morphological transition corresponds to the time of onset of LGALS15 expression.

Knowledge of the complex and synchronized relationships between secretions of endometrial epithelia and trophectoderm that influence conceptus development could provide new targets to improve embryonic development in culture and diagnosis of endometrial dysfunction that leads to infertility. Identification of useful molecular and cellular markers of uterine endometrial function and receptivity to implantation by the conceptus are of particular importance.

CHAPTER II

LITERATURE REVIEW

ESTABLISHMENT AND MAINTENANCE OF PREGNANCY

Successful establishment of pregnancy requires a pregnancy recognition signal for the maintenance of a functional corpus luteum, endometrial differentiation that transforms the uterine environment into a receptive state, and proper attachment and adhesion of the conceptus trophectoderm to uterine LE to initiate implantation. This review focuses on mechanisms for the establishment and maintenance of pregnancy in sheep including survival and implantation of the conceptus (embryo/fetus and associated extraembryonic membranes). Particular focus will be placed on functions of adhesive proteins and *galectin 15* (LGALS15).

Luteolytic mechanism

Ruminants are spontaneous ovulators with an estrous cycle that includes proestrus, estrus, metestrus and diestrus with ovulation occurring late during estrus or early metestrus [30-32]. The ovulated follicle on the ovary goes through transformations during metestrus, diestrus and proestrus – first from corpus hemorrhagicum to a corpus luteum (CL) capable of secreting progesterone and oxytocin, then to corpus albicans following luteolysis. Luteolysis is the functional and physical breakdown of the CL and is dependent on oxytocin (OXT) binding to its receptor to induce pulsatile release of the uterine luteolysin prostaglandin F2α (PGF) [33]. Regression of the CL allows the ewe to

return to estrus and begin a new estrous cycle. The estrous cycle in ruminants is dependent on uterine secretion of luteolytic pulses of PGF by endometrial LE and sGE which express both oxytocin receptors (OXTR) and prostaglandin-endoperoxide synthase 2 (PTGS2), which is the rate limiting enzyme for production of PGF [34-36].

The process of luteolysis requires the sequential effects of estrogen (E2), OXT and progesterone (P4) acting through their respective receptors [30, 37]. At estrus, E2 from the developing Graafian follicle stimulates an increase in expression of estrogen receptor alpha (ESR1), progesterone receptor (PGR) and OXTR in the uterus [31, 32]. During diestrus, secretion of P4 increases as the CL develops. P4 binds to PGR in endometrial LE/sGE and this inhibits *ESR1* and *OXTR* expression for a period referred to as the "progesterone block" where P4 also acts on the uterine epithelium to increase phospholipid stores and PTGS2 expression for conversion of arachidonic acid to PGF [38], [30, 39]. The mechanism whereby P4 inhibits expression of ESR1 directly and OXTR either directly or indirectly is not well understood [30]. Continuous exposure of the uterus to P4 for 8 to 10 Days down regulates PGR expression in the LE/sGE after Days 11 to 12 of the cycle [39]. Thus, P4 loses its ability to block ESR1 and OXTR expression and endometrial epithelia become sensitive to the effects of E2 from developing follicles and to increases in abundance of ESR1 that induces OXTR formation. This allows pulses of OXT from the posterior pituitary and/or CL to induce pulsatile release of PGF that culminates in luteolysis [39-41].

Loss of PGR from uterine epithelia during mid-diestrus and prior to implantation has been documented in sheep, cattle, pigs, monkeys, skunks, humans and mice [31, 42-

47]. PGR down regulation in LE/sGE occurs prior to implantation in all species studied and correlates with a reduction in anti-adhesive mucin glycoprotein one (MUC1) and induction of LGALS15 and SPP1. Thus, loss of epithelial PGR stimulates events necessary for an environment conducive to implantation [48].

IFNT as the maternal recognition of pregnancy signal

In 1969, Roger Short introduced the idea that the conceptus secretes a molecular signal to sustain the CL and ensure its own survival and termed this phenomenon "maternal recognition of pregnancy" [33, 49]. In sheep, the maternal recognition of pregnancy signal was discovered to be a protein known as interferon tau (IFNT) [50, 51]. Before the current nomenclature of IFNT was adopted, IFNT was originally known as Protein X, trophoblastin, and later as ovine trophoblast protein 1 (oTP-1) [52]. IFNT is expressed by the mononuclear cells of the trophectoderm of the elongating ruminant conceptus on Days 10 to 21-25 of pregnancy with maximal expression occurring on Days 14 to 16 [53-57]. It primarily functions as a secreted molecule from the conceptus that acts directly on the endometrium, but has also been shown to be present in the uterine vein, which induces interferon stimulated genes (ISGs) in extrauterine tissues such as the CL, and in circulating immune cells during the time of maternal recognition of pregnancy [58, 59]. In bovine *in vitro* derived embryos, IFNT was reported to be produced without maternal influence, but not to the extent that is observed in embryos *in utero* where it is about 1000-fold more abundant [60].

IFNT has been found in cattle, sheep, goats, musk oxen, gazelles, giraffes and deer [61]. The development of IFNT is thought to have originated when interferon omega (IFNW) underwent gene duplication that resulted in IFNT having a reorganized promoter and a novel 3' end [61]. The expression of IFNT is unique in at least three aspects: lack of viral inducibility, restricted localization to embryonic trophectoderm, and sustained high-level synthesis over several days [61]. It is hypothesized to be upregulated by cytokines, granulocyte macrophage-colony stimulating factor (CSF2) and interleukin 3 (IL3), which are all produced by the endometrium and are readily available to the conceptus [62].

The antiluteolytic function of IFNT was originally thought to involve the stabilization of PGR to maintain the progesterone block on ESR1 and OXTR expression or to inhibit expression of ESR1 and OXTR directly. Available evidence now clearly indicates that IFNT acts in a paracrine fashion to suppress transcription of *ESR1* directly and *OXTR* indirectly in the endometrial LE/sGE, thus abrogating the luteolytic mechanism to maintain secretion of P4 from the CL [63]. In addition IFNT affects cytokines important for inhibiting fetal rejection such as interferon gama (IFNG) and interleukin 4 (IL4) and also reduces the proliferative response of lymphocytes to IL2 [58]. It also increases PTGS2 production of prostaglandin E2 (PGE2) in the endometrium, which is a putative luteotrophic agent [64].

IFNT is a member of the Type I family of interferons that bind to Type 1 receptors (IFNAR) present in all cells of the endometrium, with highest expression in the LE [65]. IFNAR subunits 1 and 2 (IFNAR1/IFNAR2) form a receptor complex on the

surface of cells and IFNT-mediated association of IFNAR subunits facilitates cross-phosphorylation and activation of Janus kinase (JAK), which, in turn, phosphorylates the receptor and creates a docking site for signal transducers and activators of transcription (STAT) [66]. Using the JAK/STAT signaling pathway IFNT acts on uterine GE and stroma to regulate expression of ISGs [67]. ISGs are hypothesized to play roles in endometrial remodeling to the receptive state for implantation and conceptus development [37, 68-70]. IFNT induces expression of a wide variety of genes with various functions important for implantation including: interferon stimulated gene 15 ubiquitin-like modifier (ISG15); oligoadenylate synthetase (OAS); ubiquitin-like modifier activating enzyme 7 *(UBA7);* interferon induced transmembrane proteins 1-3 *(IFITM1-3*); myxovirus resistance protein 1 and 2 *(MX1* and *MX2);* colony stimulating factor 1 *(CSF1);* interferon regulatory factor 1 and 2 *(IRF1* and *IRF2); STAT1* and *STAT2*, which are involved in signal transduction, cell metabolism, regulation of endometrial secretory activity, uterine remodeling, adhesion and other mechanisms important to successful pregnancy [71].

Most classical ISGs are expressed in endometrial stroma and middle to deep glands with few IFNT-stimulated genes being expressed by LE and sGE [70, 72-75]. Restrictions on expression of ISGs in the uterine LE and sGE by IFNT is due to expression of IRF2, a potent repressor of gene transcription, which is constitutively expressed in the LE and sGE and increases during early pregnancy [72]. There is also the lack of critical factors in the JAK–STAT–IRF signaling pathway important for IFNT signaling in LE and sGE (STAT1, STAT2 and interferon regulatory factor nine (IRF9)),

but these components of the JAK-STAT cell signaling pathway are present in GE and stromal cells. However, *WNT7A*, cathepsin L (*CTSL*), cystatin C (*CST3*), hypoxia-inducible factor 2A (*HIF2A*) and *LGALS15* are genes identified in endometrial LE and sGE that are induced by IFNT or induced by P4 and stimulated by IFNT through an unknown non-classical cell signaling pathway [22, 70, 76].

Uterine glands

Postnatal uterine morphogenesis includes development of glands, endometrial folds, organization of the intercaruncular stroma and growth of the caruncular areas and myometrium [77, 78]. Ovine uterine development is not completed until postnatal Day (PND) 56 with the completion of adenogenesis [79]. However, the glands may not be fully developed until they undergo puberty, and perhaps pregnancy induced glandular hypertrophy and hyperplasia [80, 81]. Events that occur during the developmental process of uterine adenogenesis in the neonatal period affect uterine capacity and embryotrophic potential of the adult uterus [82]. Possible regulators of adenogenesis include members of the WNT signaling pathway, fibroblast growth factors-7 and -10 (FGF7 and FGF10), hepatocyte growth factor (HGF), insulin-like growth factors one and two (IGF1 and IGF2) and the activin-follistatin system [35, 83-85]. Adenogenesis is independent of ovarian influences, but does involve coordinated changes in epithelial phenotype and expression of ESR1 [77]. The ECM can also affect patterns of branching morphogenesis through control of the cell cycle, apoptosis and related changes in stromal and epithelial gene expression that define such developmental programs [82].

Maternal uterine gland hyperplasia occurs between Days 15 to 50 of pregnancy, after which glands undergo hypertrophy leading to increased surface area and greater secretory capacity after Day 60 of pregnancy [80]. This functions to provide increased histotroph to support the developing conceptus. A severvomechanism has been described in sheep for development of endometrial glands where sequential actions of ovarian steroid hormones, pregnancy recognition signals and lactogenic hormones from the pituitary and/or placenta act on receptors present on the endometrial GE to stimulate adenogenesis [82, 86]. During pregnancy, sequential exposure of the uterine GE to E2, P4, IFNT, chorionic somatomammotropin hormone one (CSH1) and placental growth hormone one (GH1) stimulates uterine gland morphogenesis and differential secretory functions [87, 88].

Ewes treated with exogenous non-metabolizable progestin from birth to PND 56 fail to undergo normal adenogenesis and have a uterine gland knock out (UGKO) phenotype, [8, 82, 89]. UGKO ewes have an absence of uterine glands, smaller uteri, less LE and fewer endometrial folds [90]. Adults can become pregnant, but cannot maintain pregnancy past Day 14 [6, 8, 89], presumably due to the absence of histotroph to support conceptus development beyond the pre-implantation stage [8]. Conceptus recovered from UGKO ewes are retarded in development and produce little IFNT [8].

Histotroph

Histotroph is a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, nutrients, transport proteins and other substances secreted by

the uterus and used to nurture the preimplanted conceptus [4, 81]. Histotroph was first described by Aristotle in the 3rd century BC, later investigated by William Harvey in the 17th century and, in 1882, by Bonnett who applied the concept to ruminants [82]. Uterine factors involved in conceptus nutrition fall into two categories: 1) factors secreted from the uterine glands or LE as histotrophic nutrition; and 2) factors responsible for increasing number and size of uterine blood vessels for increased blood flow and increased hematotrophic nutrition [3]. In humans, histotroph appears to be a primary source of nutrition for conceptus development during the first trimester before mechanisms for hematotrophic nutrition are established [90, 91]. Evidence collected over the last century from studies of primate and sub-primate species verified the unequivocal role of uterine gland secretions in survival and overall health of the conceptus [6, 92]. In marsupials, carnivores and roe deer, the state of endometrial secretory activity is proposed to regulate the status of blastocysts in delayed implantation or become activated after termination of delayed implantation [93, 94].

During ruminant pregnancy, the combined effects of histotrophic and hematotrophic nutrition support development of the conceptus, the onset of pregnancy recognition signaling and fetal-placental growth [3]. Uterine secretions are particularly important in ruminants, pigs and horses, which have prolonged preimplantation periods [95]. During later stages of pregnancy, uterine glands undergo extensive hyperplasia and hypertrophy that increases histotrophic support for the developing conceptus [3].

Histotroph is absorbed by the placenta, transported into fetal circulation and cleared by the kidney into the allantois via the urachus [4, 7]. The allantois was

historically thought to be a reservoir for waste; however, it is now apparent that it plays an important role in fetal nutrition [96]. Histotroph is taken up by specialized areas of the placenta called areolae, developed from the chorioallantois, which form over the mouths of uterine glands and transport secretions by fluid-phase pinocytosis across the areolae and into the fetal circulation [3, 4]. In pigs, the number of areolae is related to the birthweight of the fetus [97]. In laboratory rodents, several components of uterine histotroph, including leukemia inhibitory factor (LIF) and calcitonin produced exclusively by uterine glands, are necessary for conceptus survival, growth and establishment of uterine receptivity to implantation [82, 92].

Histotroph is produced by both the GE and the LE, with GE production predominating due to its abundance [98]. The cell-type of origin determines the nature and role of the proteins being secreted. For instance, secretions from the LE appear to have a larger role in implantation while secretions from the GE are involved in nutrition and early conceptus development [7]. Uterine milk proteins (SERPIN), SPP1, GLYCAM1, stanniocalcin one (STC1) and LGALS15 are major components of histotroph and are hypothesized to play major roles in stimulating conceptus elongation, adhesion and attachment [74, 88, 99-101]

IMPLANTATION

Overview of early events in ruminant conceptus implantation

Pregnancy in domestic ruminants (sheep, cattle, goats) begins at the blastocyst stage and involves coordinate pregnancy recognition signaling and conceptus

implantation (**Figure 2.1**). Ruminants, unlike rodents and primates, have an extended pre-attachment phase lasting up to two weeks. A variety of molecules important for adhesion, signaling, transcription, cell cycle progression and DNA replication coordinate conceptus and uterine development, differentiation and structural formation during this critical period [62]. Pre-implantation in ruminants consists of a highly coordinated and sequential number of events including shedding of the ZP, pre-contact and blastocyst orientation, apposition, adhesion and central or noninvasive superficial implantation [9].

After fertilization, the ruminant zygote develops to the blastocyst stage while surrounded by the ZP. The ZP is not critical to development progression as early removal of the ZP does not alter blastocyst survival, but the ZP does function to keep the embryo in a non-adhesive state, facilitating transport through the oviduct and into the uterus [92]. The preimplantation embryo migrates through the oviduct while undergoing several cell divisions to the morula stage before passing through the utero-tubal junction into the uterus. Shortly thereafter, the blasocyst undergoes it first perceivable differentiation with formation of the trophectoderm cell layer and extra-embryonic endoderm. The blastocyst consists of a fluid filled blastocoele, the outer trophectoderm cell layer with adhered extraembryonic endoderm, and the inner cell mass (ICM). After differentiation of the ICM and trophoblast, the blastocyst hatches from the ZP and acquires the ability to implant. The ICM will then become the embryo which gives rise to the yolk sac, allantois and amnion while the trophoblast becomes the chorion of the placenta. The trophectoderm, along with the somatic mesoderm (the portion of the embryonic mesoderm associated with the body wall derived from splanchnic (visceral

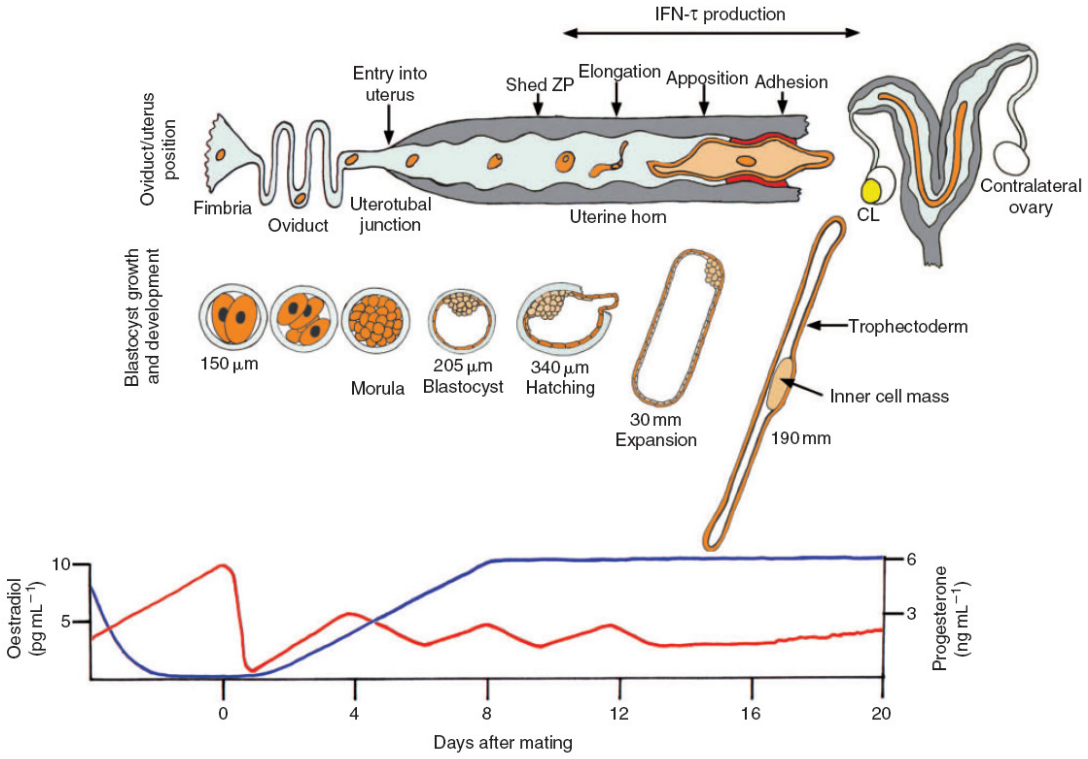

FIGURE 2.1

Schematic of events occurring during early pregnancy in sheep (Drawn by Dr. Greg A. Johnson, Texas A&M University). Location in the reproductive tract and stages of development of embryos, blastocysts and conceptuses in ewes along with changes in concentrations of estradiol and progesterone in maternal blood. After ovulation, oöcytes enter the oviduct, are fertilized at the ampullary-isthmic junction and enter the uterus around day 4 post-mating. By day 9, blastocysts shed the zona pellucida and expand and elongate into tubular and filamentous conceptuses between days 12 and 16. During elongation and implantation, IFNT is produced by mononuclear trophectoderm cells [102].

mesoderm), forms the chorion. The allantois originates as an evagination of the fetal hind gut starting on Day 16 of pregnancy and ultimately expands to fuse with the chorion to form the chorioallantois [103]. The vascularized chorioallantois is lined on its external surface by cells of the trophectoderm [58] that forms intimate contact with the maternal endometrial LE and sGE to create a functional placenta.

In sheep, the conceptus remains free floating in the uterine environment for up to two weeks with rapid elongation occurring between Days 12 and 16 [29]. Superficial implantation begins on Days 13 to 14, but the process of placentation is not completed until Days 60 to 70 [9, 81]. Ruminant placentae exhibit discrete areas of increased attachment in the placentomes, formed by interaction of cotyledons of the chorioallantois with caruncles of the endometrium, which function as sites of hematotrophic nutrient exchange, while areas of more superficial attachment in the interplacentomal areas have areolae that function as sites for histotrophic nutrition [58].

Pre-contact

The pre-contact stage begins soon after the blastocyst hatches from the ZP and lasts until about Day 15. Starting on day 12, the spherical or slightly tubular blastocyst begins to elongate. By Day 13, it has elongated markedly, reaching a length of 10–22 mm [104]. By Day 14, the filamentous conceptus is approximately 10 cm long and is loosely immobilized to the LE, but can still easily be flushed from the uterus. The primitive streak appears at this stage and the somites soon thereafter [104]. By Day 15,

the conceptus is in contact with several developing caruncles in both uterine horns [10]. The process of elongation of the ovine conceptus coordinates with production of IFNT.

Apposition

During apposition, different types of cell contacts can be observed. The most frequent involves the apical plasma membrane of trophectoderm cells, which line up against the apical surface of the microvilli of uterine LE [10]. The apical surface of the mononuclear trophectoderm cells is modified to form microvillar processes that interdigitate with similar structures on the maternal side creating an area of close contact between the maternal uterine LE and conceptus trophectoderm [105]. The second type of attachment involves areas of the LE and trophectoderm that lack microvilli, and in these areas the membranes are discontinuously apposed. In sheep, apposition occurs first in the vicinity of the inner cell mass and spreads toward the extremity of the elongated conceptus [104]. Uterine glands are also sites of apposition as the trophoblast develops papillae that, between Days 15 and 20, extend into the mouths of uterine glands and function as anchors for attachment and elongation as well as sites for uptake of histotroph [106].

Adhesion

Adhesion begins on Day 16 and continues to Day 22 of pregnancy in ewes [10]. As the trophectoderm becomes more firmly attached to the uterine LE, expression of IFNT decreases in a coordinated manner with mononuclear cell attachment and removal

of the LE [107]. The trophoblast differentiates into giant binucleate cells (BNC) and mononuclear trophectoderm cells by Day 16, but only mononuclear trophectoderm cells are thought to adhere to the endometrial LE [104]. Binucleated cells make up 15 to 20% of the total trophectoderm cellular population [108]. Wimsatt (1951) and Greenstein (1958) were the first to observe that the trophectoderm is made up of mononuclear (involved in nutrient exchange) and BNC (involved in hormone production) [109]. Both cell types express features typical of classical epithelial cells. They are located on a basal lamina and are connected to each other by junctional complexes including tight junctions [110]. The BNC are hypothesized to originate from cells that underwent mitosis without cytokinesis [105] and are not uniformly distributed, but tend to form in small clusters [111]. BNCs produce hormones that may play important roles in stimulating endometrial gland morphogenesis and differentiation during pregnancy to facilitate growth and development of the conceptus [58]. They produce prolactin in humans and rodents and prolactin-related protein one (PRP1), CSH1, pregnancy-associated glycoproteins (PAGs), estradiol and progesterone in ruminants [58]. BNCs migrate across the trophectoderm-maternal interface to fuse with mononuclear LE cells to form trinucleate hybrid cells [111]. Large syncytial plaques occur when binucleate, and perhaps mononucleate, cells continue to merge with the trinucleate cells to form large syncytial plaques with as many as 20 to 25 nuclei [106, 112]. The syncytial plaques eventually cover the caruncular surface and aid in formation of the placentome [104]. As adhesion progresses and becomes more and more stable, and cells of the trophectoderm begin to fuse to each other and with the LE, they form multinucleated

syncytia that immobilize the conceptus to the uterine wall. Wooding hypothesized in 1984 that the purpose of syncytial plaques was to decrease the distance between fetal and maternal capillaries for increased efficiency for nutrient exchange [111]. A schematic illustration of the pre-attachment, apposition and adhesion stages of superficial implantation in sheep can be seen in **Figure 2.2**.

Uterine receptivity and conceptus elongation

Most embryonic loss in sheep occurs due to failed fertilization (approximately 10-15%) or failure of spherical blastocysts to make the transition to filamentous conceptuses during the preimplantation period (approximately 25%) [113]. Conceptus elongation corresponds to IFNT production and successful implantation. Implantation deficiencies are also thought to be the main reason why artificial reproductive techniques (ART) only result in pregnancy 20-30% of the time despite high rates of success in creating fertilized embryos *in vitro* [114]. Despite improvement in techniques to achieve *in vitro* fertilization, pregnancy rates associated with ART have only improved marginally, thus there must be unrecognized factors specific to the uterine environment that are not well understood and are limiting [92]. The window of implantation is a period of time when molecular and hormonal conditions of the uterus are conducive to implantation of blastocysts [114]. The most fundamental feature of this process is synchrony between the developmental stage of the embryo/blastocyst and the uterus [114, 115]. Uterine receptivity is dependent on fetal-maternal crosstalk; however, mechanisms for two-way communication between blastocysts and the uterus are not well

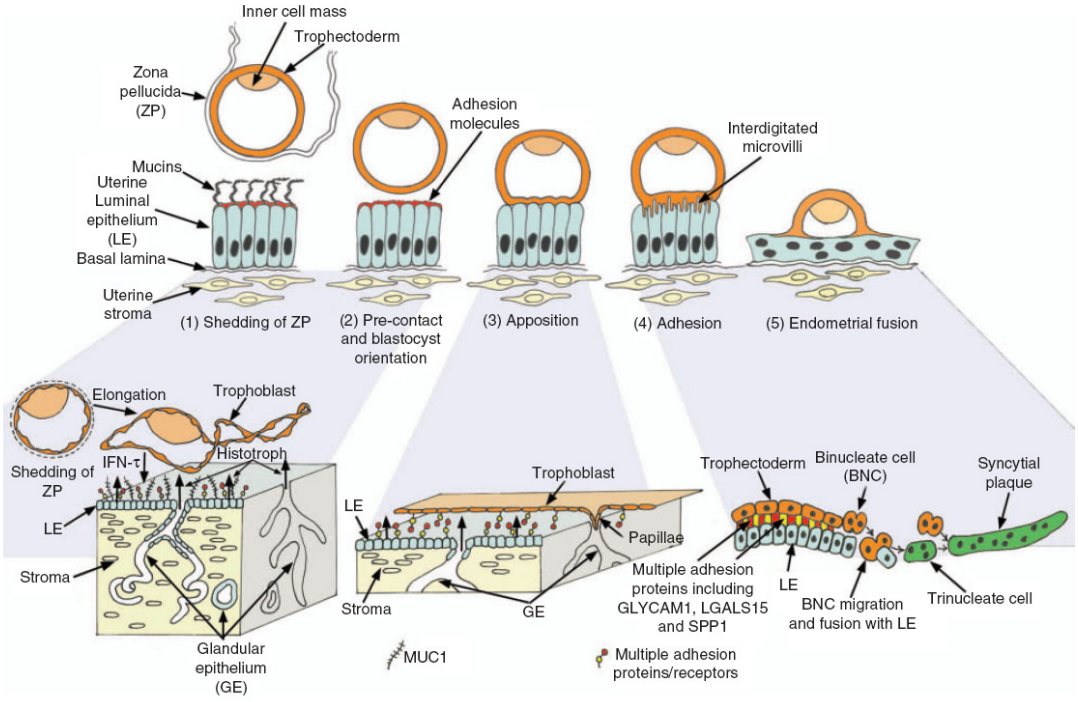

FIGURE 2.2

Schematic illustration of the pre-attachment, apposition, and adhesion stages of superficial implantation in sheep (Drawn by Dr. Greg A. Johnson, Texas A&M University). Pre-attachment events involve shedding of the zona pellucida by blastocysts, followed by expansion and precontact orientation and expansion of blastocysts. Prior to implantation, expression of MUC1 is down-regulated in LE to expose integrin subunits and/or carbohydrate receptors which facilitate adhesion. Apposition involves extension of conceptus trophoblast papillae into the necks of the uterine glands to act as anchors to aid in elongation of trophectoderm and to serve as sites for uptake of histotroph. Adhesion between the apical surfaces of conceptus trophectoderm and LE is mediated by uterine secretory proteins, such as secreted phosphoprotein 1 (SPP1) and glycosylation-dependent cell adhesion molecule-1 (GLYCAM1), binding to receptors and by LGALS15 which is proposed to cross-link beta galactosides on glycoproteins and glycolipids. Trophoblast BNC fuse with uterine LE to form syncytial plaques, but are not invasive beyond this single cell layer [102].

understood. Hormones, growth factors, cytokines and modulators of cell adhesion have been implicated in implantation [114].

The process of blastocyst elongation and implantation is thought to require cellular growth and proliferation along with adhesion of the trophectoderm to the uterine LE, which acts as a scaffold, to allow the conceptus to transition from the spherical to the elongated filamentous form [3]. Elongation begins on Day 12 and is completed by Day 16 at which time implantation begins and secretion of IFNT is at a maximum [9, 104]. Cross-communication between conceptus and uterus involves paracrine interactions between IFNT from conceptus trophectoderm on the endometrium, endothelial cells and immune cells [31]. Implantation is dependent on uterine receptivity and in sheep, several genes appear to play important roles in uterine receptivity such as *IFNT*, endogenous Jaagsiekte sheep retroviruses (*enJSRVs*), *CTSL*, *CST3*, *SPP1*, *IRF1 and 2*, and *LGALS15*. IFNT induces or stimulates expression of a number ISGs in the uterus that are hypothesized to play important biological roles in uterine receptivity and conceptus implantation. Most of the classical ISGs are induced or increased only in stroma cells and the epithelia of middle to deep glands in the ovine uterus [48, 68]. However, several nonclassical ISGs such as *WNT7A*, *CTSL*, *CST3*, *HIF2A* and *LGALS15*, are induced by P4 and increased by IFNT specifically in LE and sGE of the endometrium and implicated in regulation of uterine receptivity and conceptus development [66].

MODULATORS OF IMPLANTATION

Integrins

Integrins are glycoproteins that serve as receptors for extracellular matrix ligands that modulate cell functions. They are one of the best characterized immunohistochemical markers for uterine receptivity to implantation [116]. ECM and intergins are hypothesized to be responsible for conceptus attachment to the uterine wall. This effect is blocked during the non-receptive phase by the expression of mucins, which sterically hinder interaction of the trophectoderm with molecules of the ECM due to their large structure and extensive glycosylation [99, 117]. Integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed by conceptus trophectoderm and are localized to the apical surface of uterine LE in sheep [99]. The endometrium in humans and pigs has both constitutive and cycle-dependent expression of integrins and is the only tissue known with hormonal regulation of integrin expression [33].

Uterine receptivity in sheep does not appear to be dependent on the expression of integrins, but may be influenced more by expression of ECM proteins such as SPP1 and LGALS15, which are ligands for heterodimers of these integrin subunits [11]. Recently, human uterine Hoxa10 was demonstrated to induce the expression of uterine epithelial integrin subunit β3, and the lack of expression of this integrin subunit could explain sterility in Hoxa10 mutant mice [62]. The Arg-Gly-Asp (RGD) sequence present on many ECM proteins is a well known integrin recognition sequence and proteins expressing it have been implicated in trophoblast adhesion to the ECM. In fact, studies in mice have shown that RGD peptide inhibitors reduce implantation as do injections of

a monoclonal antibody for αvβ3, but it is not known if the blastocyst or uterus or both is the source of these effects [92]. The αvβ3 integrin heterodimer has many functions including activation of matrix metalloprotein protein 2 (MMP2) and serves as a matrix destabilizer [92]. Although many integrin heterodimers are expressed in the uterus, the apical localization of αvβ3 and αvβ5 integrins in human, baboon, rabbit, pig and sheep uterine LE make them candidates for mediating trophectoderm-epithelial interactions [117-119]. Another piece of evidence for the important role of integrins during implantation is from studies of human, baboon and pig, where expression of integrin β3 coincides with the window of uterine receptivity to implantation [118, 119].

Since the blastocyst and LE both contain integrins on their respective apical surfaces, a reciprocal and cooperative role in attachment is suggested. Relevant ligands for the integrins αvβ3 and αvβ5 may function as bridging ligands between the conceptus and maternal uterine LE [120]. Possible ligands for the integrins on the maternal surface include fibronectin, vitronectin, SPP1, laminin, thrombospondin, LGALS15 and perlecan. In addition, αvβ3 may also interact and activate specific matrix metalloproteinases in the extracellular matrix of the uterus, thereby implicating integrins in the transition between attachment and epithelial cell penetration by trophectoderm of the blastocyst in species with invasive implantation [121].

Most integrins activate focal adhesion kinase (FAK) and thereby src family kinases (SFKs), causing phosphorylation and signaling from p130-CAS and paxillin. A subset of integrins (α1β1, α5β1, and αvβ3) also activate the adaptor protein Shc [122]. Activated FAK and SFKs along with receptor tyrosine kinases (RTKs) activate several

signaling cascades. For example, actin polymerization occurs from downstream activation of Rac, Cdc42, p21-activated kinase (PAK), Wiskott–Aldrich syndrome protein (WASP)-family proteins, ARP2/3 complex and LIM kinase (LIMK). Other signaling molecules include myosin light chain kinase (MLCK), Rho effectors Rho kinase (ROCK), and mammalian diaphanous (mDIA) to regulate bundling and contraction of actomyosin fibers; PAR6 and protein kinase C (PKC) and Cdc42 to control cell polarity during migration; Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) to promote cell migration [123].

SPP1

Secreted phosphoprotein 1 (SPP1, also known as osteopontin) is an acidic single chain phosphorylated glycoprotein component of the ECM that ranges in length from 264 to 301 amino acids [124]. SPP1 contains the well-characterized integrin recognition sequence (RGD) and other cryptic sequences that interacts with cell surface receptors, including integrins, to mediate cell adhesion, migration, differentiation, survival and immune function [124]. Expression of SPP1 has been found in uteri of sheep, goats, pigs, cows, mice, baboons and humans [100, 101, 119, 125-127]. Recent studies have focused on the role of SPP1 in sheep as a progesterone-induced secretory product and pigs as an estrogen-induced secretory product of the uterine GE that binds receptors on the apical surface of the uterine LE and conceptus trophectoderm to stabilize adhesion between the uterus and conceptus for implantation and stimulate changes in conceptus

morphology [126, 128, 129]. The expression pattern and abundance of SPP1 correspond to periods of conceptus growth and development [124].

Focal adhesions

Focal adhesions are areas of close apposition and thus presumably anchorage points of the plasma membrane of a cell to the substratum over which it is moving. They are always associated with cytoplasmic microfilament bundles that are attached via several proteins to the plasma membrane. Cells use integrins to adhere to the ECM and link to components of the actin cytoskeleton to form focal adhesions. Integrins are transmembrane molecules that make up the extracellular component associated with the ECM and the intracellular component that associates with adhesion complex proteins (viniculin, talin, paxillin, tensin and more than 150 other proteins which all associate with actin filaments) [130, 131]. There are several variations of focal adhesions based on size: small (0.5 – 1.0 μm) "dot like" adhesions known as focal complexes; elongated (3 – 10 μm), streak like complexes that associate with actin and myosin containing filament bundles (stress fibers) known as focal contacts or focal adhesions; and tensin-enriched fibrillar adhesions involving fibronectin fibrillogenesis [130].

Focal adhesions not only anchor cells to a substrate, but are also involved in molecular cell signaling cascades [130] such as touch receptors that respond to mechanical stimuli to affect characteristics of the cellular microenvironment [130]. They are individual mechanosensors and their elongation reveals the local balance between the force on the cell and the rigidity of the ECM [130]. Matrix adhesion

formation is regulated by signals from G-proteins of the Rho family. However, formation of punctate focal complexes depends on Rac signaling [130] and activation of RhoA is required for formation of mature focal adhesion complexes and associated stress fibers. RhoA triggers downstream activation of targets such as Rho associated kinase (ROCK) and forming homology protein, Dia 1 (a mammalian homolog of *Drosophila* Diaphanous protein) shown to mediate effects of Rho on matrix adhesions and the actin cytoskeleton [130]. Self-assembly of protein complexes are favored when the cell is under stress and not favored when the pulling forces are relaxed [130]. The force used to stimulate focal adhesion formation can come from inside the cell or from an extracellular source [130].

In the ruminant synepitheliochorial placenta, restricted trophoblast invasion requires complex interactions of integrins with proteins of the ECM and integrin receptors of neighboring cells for the formation of focal adhesions [132]. Conceptus elongation involves focal adhesion formation as it migrates and stretches along uterine LE. LGALS15 stimulates focal adhesion formation via its RGD integrin recognition sequence, where it binds to integrins expressed on the LE and conceptus trophectoderm, and thus functions as an adhesive protein important to conceptus development.

GALECTINS

Features of the galectin superfamily of proteins

Galectins are proteins with 15 known members found in nearly all living organisms, including mammals, drosophila, zebrafish, fungi and arabadopsis, but

mysteriously absent from yeast. Candidate galectins have also been found in viruses such as lymphocystis disease virus and a few adenoviruses [18, 133]. They all share a conserved CRD that binds beta-galactoside sugars, and functions to cross-link glycoproteins as well as glycolipid receptors on the surface of cells to initiate biological responses [13, 15, 134]. Functional studies on the extracellular and intracellular roles of galectins implicate them in cell adhesion, chemoattraction and migration as well as growth, differentiation and apoptosis [135, 136]. The presence of galectins in so many evolutionarily divergent species suggests that they play a role in cell function, but, on the other hand, the presence of so many types in each species suggests that they have evolved to participate in a multitude of specific functions [133].

Lobsanov (1993) coined the term "galectin" and declared that "membership in the galectin family requires fulfillment of two criteria: affinity for beta galactosides and significant sequence similarity in the carbohydrate-binding site, the relevant amino acid residues of which have been identified by X-ray crystallography" [18]. In mammals, one can place proteins into families based on their sequence similarity to the canonical CRD with high accuracy. This is not the case in non-mammalian species. Thus it is best to keep both sequence motif and beta-galactoside binding in the definition and to use the term "galectin-like" for proteins that have a similar sequence motif but do not bind beta-galactosides [18]. Galectin was a term meant to replace the previous category of "S-type" lectins [18]. The S indicates the dependence on thiols (reducing conditions) for activity, which was a property of LGALS1, the first member discovered and the best studied, but this was not found to be the case for many of the subsequent members of the

family. Now S-type is sometimes used to designate a larger protein family to which galectins belong or a subgroup of galectins; however, both are incorrect as no such group has been defined [18]. Other common characteristics of galectins are their localization to the cytosol of cells and their ability to be secreted, despite the absence of a known signaling peptide, through a non-classical pathway. They can also be translocated to the nucleus or to other cellular compartments [137].

All galectins have a core sequence of about 130 amino acids and many of them are highly conserved [133]. The CRD is usually contained between residues 30 and 90 and encoded by one exon [133]. The CRD can act on its own or in conjunction with the CRD of another galectin, which is in contrast to other protein domains which often occur together with other domain types in the same peptide [138]. Hirabayashi and Kasai (1993) proposed the categorization of galectins, based on the CRD, into three groups: 1) prototype galectins (galectins-1, 2, 5, 7, 10, 11, 13, 14 and 15); 2) chimera-type galectins (galectin-3); and 3) tandem-repeat-type galectins (galectins-4, 6, 8, 9 and 12) [20]. Prototype galectins exist as monomers or noncovalent homodimers with one CRD while chimera type galectins contain a non-lectin domain linked to a CRD and tandem-repeat-type galectins have two distinct CRDs with distinct specificities [20]. Native galectins occur in monomer, dimer, or higher multimer forms depending on conditions such as concentration of ligand [18].

Galectins were first discovered through studies to test the hypothesis that cell-cell adhesion utilized carbohydrates. Beta-galactosides were used initially due to the ease of obtaining them for biochemical experiments and their presence on the surface of cells.

Beta-galactoside affinity columns were used to screen cell lysates. Bound proteins from tissue extracts eluted with lactose were analyzed and thus galectins were found [18]. The CRD is composed mostly of beta chains; six strands form the concave side and five form the convex side. Carbohydrates bind to the concave side to five distinct sites (A, B, C, D, E). Subsite C is the defined beta-galactoside binding site and the most conserved. Site D is the disaccharide binding site and is the second most conserved, but the structural requirements are much less and can be fulfilled by a wider range of molecules and preference in this site is one source of variation among members of the family [18]. A second source of variation is in their abilities to accommodate saccharides (GlcNAc, Gal, GalNAc, NeuAc), and this is handled by subsite B [18]. A few reports indicated that galectins can bind mannose [18]. This means galectins that show weak binding to beta-galactosides like LGALS10 and galectin-like proteins may have other specificities [18]. The amino acids found in the C and D subsites are the only residues highly conserved among members of the family. Galectin CRDs are, on average, 80% conserved. LGALS2 and LGALS9 are slightly more diverse and only about 70% conserved and LGALS1 and LGALS3 are slightly more conserved at 87% [18]. Other parts of the CRD may be conserved among species for particular galectins and have other interesting binding activities. Galectin homologies are most present in mammals, but can be present in plants suggesting an ancient origin [139]. LGALS1, 2, 3, 4, 7, 8, 9, 12, GRIFIN and HSPC159 have orthologues in humans, mice and rats, as well as other mammalian species. They are different enough to indicate that they diverged well before mammals and can be regarded as separate galectins and galectin-like proteins.

However the remaining galectins and galectin-like proteins appear in only a few species [18]. LGALS10, LGALS13, and placental protein 13-like protein (PPL13) have only been identified in humans with no apparent orthologues in the mouse or rat and are about 40% identical to LGALS15 [18].

Galectins have been adapted to interact with other proteins (not just beta-galactosides) in a lectin-independent manner, thus increasing their diversity and influence on cellular mechanisms [139]. Galectins have properties typical of other cytosolic proteins: 1) synthesized on ribosomes; 2) lack of a signal peptide for secretion; and 3) acetylated N-termini [18]. LGALS3 may be able to be phosphorylated, but other than that no other post-translational modifications have been shown with certainty for any members of the galectin family.

Members of the galectin family are ones of multiplicity and diversity; the former from occurrences of common localization and functionality and the latter from the variety of participating cellular processes of each member. They do not have a specific receptor, but recognize a group of proteins having oligosaccharides to allow them to interact with different cell types and cell surface proteins and to exert effects both extra- and intracellularly [139]. Two facts need to be considered regarding the extracellular function of galectins: 1) there is no evidence to suggest that any galectin is destined to be an extracellular molecule as none of them have a secretory signaling sequence; and 2) some appear to require reducing conditions to have a biological effect which raises the question of whether they can function in the oxidative extracellular environment [139]. Their extracellular functions have been investigated and they can bridge cells

(heterotypic and homotypic bridging ligand) via cross-linking to glycoconjuagtes that contain beta-galactosides [133, 137]. Their intracellular functions may be the predominant site of action where they function in pre-mRNA splicing, which is dependent on the N-terminal domain rather than the CRD [140]. They also appear to interact with multiple proteins in the cytosol and perhaps play important roles in a variety of cell signaling cascades [139, 141]. The next question is how important is the CRD in those protein interactions and signaling cascades. There is no evidence that galectins recognize intracellular glycoconjugates; however, saccharides (particularly O-linked varieties) are present in the cytoplasm and may function as ligands for galectins [139].

There is significant interest in using galectins or galectin inhibitors in therapeutic and biomedical research, particularly in the cancer field [137]. Because galectins are involved in modulation of cell adhesion, growth, apoptosis, immune response and angiogenesis, their expression might have a critical role in tumor progression and immune evasion [137]. They are effector molecules in humoral innate immune responses [142] and in sponges and mammals, galectins are part of an alternate complement activation system that leads to opsinization and engulfment of microbes or formation of pores in the microbial membrane causing cell lysis [142].

Many of the well-documented functions of galectins in the immune system have strong similarities to events in blastocyst implantation. Molecules involved in immune function stimulate a complex cell signaling network involving vascular dilation, increased permeability and blood flow, exudation of fluids and plasma proteins and

leukocyte migration into inflamed regions; all functions associated with members of the galectin family [143]. Similar functions (vascular dilation, increased permeability, chemotaxis, and increased secretory activity) are also required for implantation. During inflammation the initial binding of leukocytes to the endothelium is mediated by galectins cross-linking of sialylated Lewis antigens present on the endothelium to molecules present on leukocyte membranes. Lewis antigens are also present in uterine endometrium [143, 144]. Up-regulation of galectins during inflammation on both neutrophils and endothelium results in weak cell-cell interactions, slowing the speed of circulating neutrophils and allowing them to roll on the endothelium [142]. The rolling process is an initiation signal for firm, integrin-mediated cell attachment, which further slows the cell at the endothelial surface and allows it to flatten out. This firm adhesion must be preceded by galectin-mediated rolling [143]. This bears a striking resemblance to the rolling, apposition and attachment phases of implantation which are also dependent on weak followed by strong attachment and integrin interactions [27, 145].

Genetically modified mice have allowed documentation of interesting roles for LGALS1 and LGALS3 in neural development and modulation of the immune system [142]. However, it has been difficult to ascertain functions for other galectins in mice in which specific galectin genes have been deleted due to the redundancy of functions among this family of genes. *Lgals1* and *Lgals3* null mice do not exhibit defects in implantation, fertilization, or embryo survival under normal housing and husbandry conditions [18]. The two galectins do not substitute for one another since the double mutant mouse is viable; however, these mice have subtle defects in their immune and

olfactory systems [18]. There is no evidence for functional redundancy among members of the galectin family *in vivo*, perhaps due to their specificity in tissue localization and biochemical properties. The fact that double or triple null mice survive suggests that galectins function as optimizing molecules that are not essential to development [18].

LGALS1 and LGALS3

LGALS1 and LGALS3 are the most studied members of the galectin family and, while each family member is unique with specific functions, insights and similarities can be drawn from results of a few intensive studies that may apply to all members. Their intracellular functions include regulation of cell proliferation, differentiation, apoptosis and RNA processing while their extracellular functions include activation of autocrine or paracrine mechanisms and/or direct mediation of homotypic and heterotypic cell interactions and adhesion to the ECM (**Figure 2.3**) [139]. LGALS1 and LGALS3 play a role in T-cell homeostasis and survival in T-cell mediated immune disorders, acute inflammation and microbial infections [146]. They may also have minor functions in implantation where LGALS1 and LGALS3 are expressed in the trophectoderm and have been implicated in the process of implantation in mice. However, LGALS1 and LGALS3 null mice do not have an implantation defect although there are some developmental issues in the adult mice immune and olfactory systems [137].

LGALS1 can promote and/or inhibit cell growth in different cell types, which may or may not depend on its CRD [147]. It can function in a carbohydrate dependent or independent fashion and have positive or negative effects depending on cell type. The

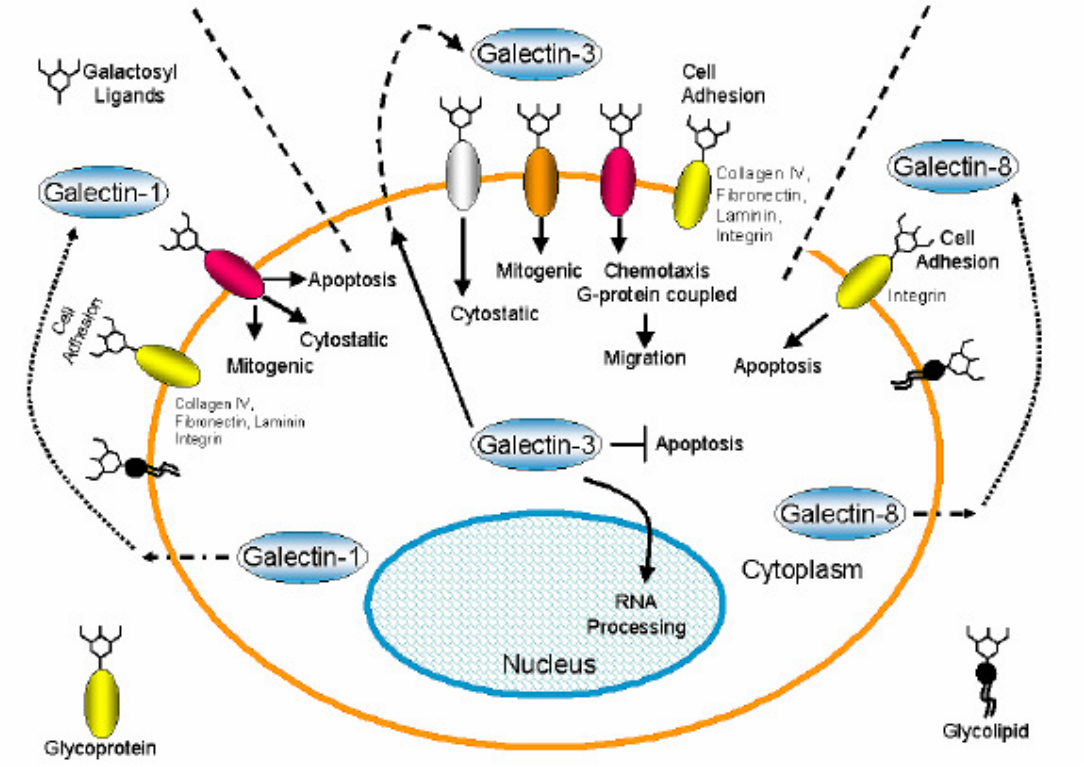

FIGURE 2.3
Biological functions mediated by three of the most well characterized members of the galectin family; galectins-1, -3, and -8. Secreted LGALS1 promotes cell adhesion by binding to glycolipid and glycoprotein receptors to induce mitogenesis, cytostasis and apoptosis. Secreted LGALS3 also binds to cell-surface glycoconjugates to promote cell adhesion, as well as induction of mitogenesis, chemotaxis and cell migration. Intracellular galectin-3 also promotes RNA processing and is anti-apoptotic. Secreted LGALS8 binds to glycoconjugates to promote cell adhesion and apoptosis [139]

CRD is usually utilized when LGALS1 is in high concentrations, while the CRD independent functions occur at low concentrations of LGALS1. LGALS1 is expressed in epithelia and connective tissue of the embryonic gonads [137]. It interacts with α7 and β1 integrin subunits to inhibit cell adhesion through an interference mechanism, although cell survival was reported to be normal initially, the inability of the cell to adhere may have led to increased cell death via apoptosis [148-150]. Transcriptional regulation of LGALS1 includes both an upstream and downstream position-dependent cis-element [151]. It has one SP1 site and a consensus initiator element, which partially overlaps a non-canonical TATA box. *LGALS1* has two known start sites and there is direct initiation of transcription of RNA from both start sites [152]. The upstream transcription start site contributes more than one-half of the *LGALS1* mRNA. The 5' end is very GC rich and formation of a hairpin structure which influences translation of the *LGALS1* mRNA is possible [152].

The abilities of LGALS3 to stimulate or inhibit cell growth and promote angiogenesis *in vivo* are dependent on its CRD region [139, 153]. A role in transcriptional activity has also been described for LGALS3 in thyroid tissue [139]. An intracellular role in regulating apoptosis, perhaps through the use of B-cell lymphoma two (Bcl-2) in mitochondria has been reported [154], as has its ability to inhibit cell adhesion by interacting with laminin and other extracellular matrix proteins [139]. Over expression of LGALS3 *in vitro* resulted in loss of cell adhesion and G1 arrest without detectable cell death [139]. However, LGALS3 can stimulate adhesion of neutrophils to laminin in an integrin-independent manner and, at high concentrations, it induces

neutrophil adhesion to fibronectin that appears to be dependent on β2 integrin [143]. LGALS3 is presumed to play a key role in the formation of tight junctions between the cells to maintain polarity and it has been directly implicated in terminal differentiation of epithelial cells where it binds to and polymerizes a high molecular weight glycoprotein known as hensin, which maintains polarity of cells in the differentiated state [155]. LGALS3 is involved in the modulation of both weak and strong adhesion between cells and ECM and cell-cell interactions [155]. It can be internalized as well as externalized via a mechanism that can be inhibited by the antibiotic filipin, but not chlorpromazine, suggesting internalization via the caveolae membrane microdomains and not clathrin coated pits [155]. LGALS3 can also mediate integrin clustering on the surface of cells to increase binding [155]. (**Figure 2.3**).

Other members of the galectin superfamily of proteins

LGALS2. *LGALS1* and *LGALS2* genes each contain 4 exons with similar intron placement; however, the genomic upstream region, which contains sequences characteristic of regulatory elements, is different [156]. *LGALS2* promoter region contains few known regulatory elements [137]. One known recognition site is AML, a master regulator of hematopoiesis [137]. LGALS2 binds to lymphotoxin-alpha (LTA) and a single-nucleotide polymorphism in LGALS2 is significantly associated with susceptibility to myocardial infarction [157]. This genetic substitution affects transcription of *LGLAS2 in vitro*, potentially leading to altered binding affinity to LTA,

which affects degree of inflammation. Smooth muscle cells and macrophages in human atherosclerotic lesions express both LGALS2 and LTA [157].

LGALS7. This member of the galectin family is an early transcriptional target of the tumor suppressor protein p53 [158] which suggests a pro-apoptotic function shared by many of the other galectins. It functions intracellularly upstream of JNK activation and cytochrome C release and might regulate expression of genes that modulate the redox status of cells to favor apoptosis [139]. LGALS7 affects proliferation and differentiation of epithelial cells [137] and has a role in reepithelialization of corneal wounds [159].

LGALS8. LGALS8 induces apoptosis in human lung carcinoma cell lines [139]. It has six known isoforms with three belonging to the prototype group and three to the tandem repeat group [137]. Recombinant LGALS8 remains active even when extracted and purified in the absence of reducing agents which suggests that it can function extracellularly for prolonged periods of time in a non-reducing environment without being inactivated [160]. Cell adhesion stimulated by LGALS8 triggers integrin-mediated cell signaling cascades such as tyrosine phosphorylation of FAK and paxillin [160]. In contrast, high concentrations of soluble LGALS8 interacts both with cell surface integrins and other soluble ECM proteins, to inhibit cell-matrix interactions [160]. Unlike fibronectin which will bind most integrins, LGALS8 is selective and interacts only with α3, α4, α6, β1 and β3 [161].

LGALS9. LGALS9 was recently shown to be a marker of human uterine endometrial function [162]. It is regulated during the menstrual cycle and the cells responsible for this regulation are the epithelial cells of the endometrial glands. In human early-pregnancy decidua, LGALS9 is also expressed abundantly in epithelial cells [162]. It induces apoptosis of thymocytes and melanoma cells, as in many other immune cells, such as T cells, B cells, and monocytes [163]. LGALS9 only acts as a proapoptotic agent on activated $CD4^+$ and $CD8^+$ T cells but not on inactivated ones [163].

LGALS10. LGALS10, also known as Charcot-Leiden crystal (CLC) protein [137], was originally excluded from the family due to its inability to bind beta-galactosides, but changes in criteria now allow lectins, like LGALS10, which bind mannose instead of lactose to be included [137]. LGALS10 is an abundant lysophospholipase of eosinophils and it occurs naturally as hexagonal bipyramidal crystals in human tissues and secretions. It is associated with increased numbers of peripheral blood or tissue eosinophils in parasitic and allergic responses [164]. LGALS15, which is closely related to LGALS10, also exists in crystals in ovine uterine LE and trophectoderm [22, 95, 165].

LGALS13. LGALS13, also known as placental protein 13 (PP13), was first cloned from human term placenta and is predominantly expressed by the syncytiotrophoblast [166, 167]. It has demonstrated endogenous lysophospholipase activity [168, 169] and can elicit depolarization of trophoblasts as well as liberation of linoleic and arachidonic acids from the trophoblast membrane [170]. Maternal serum levels of LGALS13 during the

first-trimester appear to be a reasonable marker for risk assessment for preterm preeclampsia [167]

Over the whole family, galectin expression varies by tissue type, developmental stage and pathological condition (particularly with cancer) [137] and most galectins interact with integrins. LGALS3 binds α1β1 [171]; LGALS1 and LGALS3 interact with α7β1 [149] and αmβ2 [160]. In addition to binding glycoconjugates, many members of the galectin family also share a key quality, they can be localized both intracellularly (cytoplasm and nucleus) and extracellularly (ECM and secreted) [172]. Nuclear localization of LGALS1, LGALS3, LGALS7, LGALS10, LGALS11, LGALS12, LGALS13 and LGALS14 has been reported [21, 172].

LGALS15

LGALS15 was discovered in uteri of pregnant ewes by Gray [173] while identifying molecular and cellular markers of endometrial function using gene profiling techniques and an endometrial cDNA library from the uterus of Day 14 pregnant ewes. Interestingly, approximately 1.4% of the 5,000 ESTs sequenced from the cDNA library were highly similar to *ovgal11*, a previously uncharacterized member of the galectin family of secreted animal lectins [22]. OVGAL11 was originally described as being induced in gastrointestinal tissue and secreted into the intestinal lumen in response to inflammation and eosinophil infiltration after infection of sheep with the helminth *Haemonchus contortus* [21]. Sequence analysis showed it to be a novel member of the galectin family and thus it was renamed LGALS15.

The amino acid sequence of endometrial LGALS15 contains a CRD similar to members of the galectin superfamily [174] (**Figure 2.4**). The CRD of other members of the galectin superfamily are composed of 13 amino acids, and 7 of these (H-N-R-------N-----W--E-R) play important roles in sugar binding [175, 176]. The putative CRD of LGALS15 has three of the seven amino acids important for sugar binding (N64, W71 and E74) and two are conservatively substituted (R53 and K76). Another amino acid of possible importance is C57, it is different from prototypical galectins but appears to allow for binding of mannose in LGALS10 [22, 177]. Similar to other galectins, LGALS15 does not have glycosylation sites, transmembrane domains, or predicted signal peptides for secretion; however, unlike other galectins, LGALS15 contains two potential integrin-binding domains at positions 123 (LDV) and 126 (RGD) [178]. (**Figure 2.4**)

LGALS15 is expressed by LE and sGE of intercaruncular endometrium of pregnant ovine uteri from Day 10 of pregnancy to term, including periods of implantation, placentation and uterine involution post-partum [95]. Temporal and spatial expression profiling revealed that *LGALS15* exhibits a similar expression pattern to other interferon stimulated genes in that it is detected only after Day 10 of the estrous cycle in LE and sGE coincident with the loss of PGR [22]. In cyclic ewes, expression decreased by Day 16 with re-emergence of PGR, but remained high in pregnant ewes in which PGR continues to be absent from LE and sGE throughout pregnancy [22].

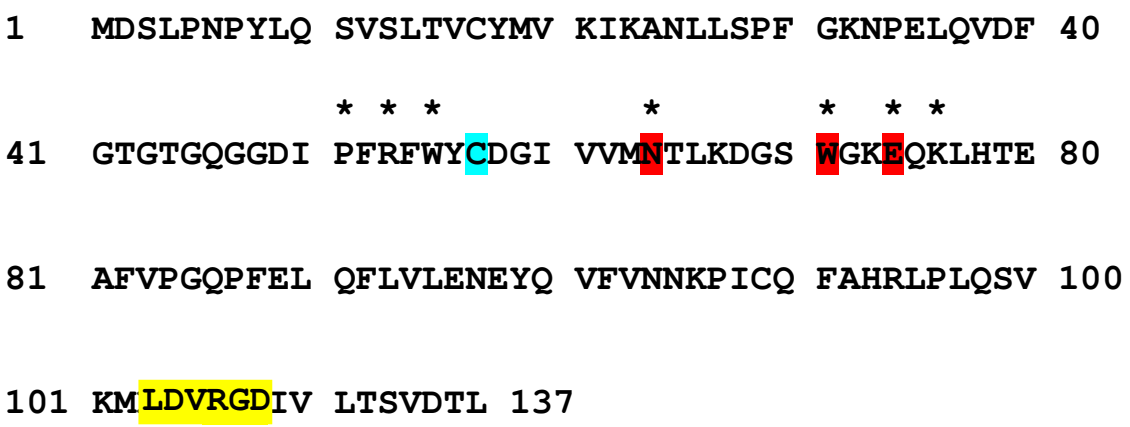

FIGURE 2.4
Amino acid sequence of LGALS15. The asterisks denote the conserved residues forming the carbohydrate recognition domain (CRD) found in prototypical galectin family members. Amino acids highlighted in red are identical to those conserved in CRD of other members of the galectin family. Residue C57 (highlighted in blue) is not well conserved, but is found in LGALS10, LGALS15's closest relative, and is predicted to function in mannose binding. Amino acids highlighted in yellow are predicted to be involved in integrin recognition and binding, a unique feature of LGALS15.

Studies of ovarectomized ewes treated with exogenous P4 and intrauterine IFNT or P4 and intrauterine control proteins and a PGR antagonist revealed that *LGALS15* is a P4-induced and IFNT-stimulated gene [22]. This corresponds to temporal and spatial aspects of expression in pregnant ewes in which LGALS15 increases to Day 16 when IFNT secretion by the conceptus is at a maximum. Further, pregnant ewes treated with P4 prior to onset of endogenous P4 production showed accelerated growth of the conceptus and IFNT secretion as well as LGALS15 expression [179].

Expression of LGALS15 is limited to LE and sGE during placentation in ewes when trophoblast giant BNCs begin to differentiate and fuse to LE to form multinucleated syncytia which replaces the LE. During that time *LGALS15* exhibits a variegated pattern of expression only in the remaining LE [81, 111]. The LE returns between Days 40 and 60 of pregnancy and *LGALS15* expression increases [95]. *LGALS15* mRNA cannot be detected in the placentomes which are devoid of LE [95]. Expression during uterine involution post-partum revealed that it takes at least four weeks to restore the LE and, during that time, *LGALS15* expression is variable [95]. LGALS15 protein has a nucleocytoplasmic distribution in LE and sGE, concentrated at the apical surface of these cells and is abundant in uterine secretions [22]. LGALS15 protein was also found in trophectoderm and allantoic fluid and, since it is not produced by trophectoderm cells, it must be taken up from the uterine lumen during the peri-implantation period and by both inter-placentomal chorion and chorionic areolae during later stages of gestation. Studies of unilaterally pregnant ewes revealed that LGALS15

protein was present in secretions of both the gravid and the non-gravid uterine horns [95].

LGALS15 is synthesized and secreted from the endometrial LE and sGE as a component of histotroph in ewes which accumulates between the fetal maternal interface and conceptus trophectoderm [23]. It was also found that LGALS15 was the previously unknown 14 kDa protein in intracytoplasmic crystalline inclusions in LE and trophectoderm cells [22, 23, 95]. These crystalline inclusions have been described for sheep [23, 180], mice [181], rabbits [182, 183] and humans [182]. Similar crystalline inclusions occur when Day 7 bovine blastocysts are transplanted into sheep uteri for 7 to 9 days, but not naturally in bovine trophectoderm or endometrial epithelium [184]. Crystalline inclusion bodies in trophectoderm increase in number and size between Days 10 and 18 of pregnancy [95]. Biological roles of components of the crystalline inclusion bodies are believed to include modulation of cell growth, differentiation and apoptosis through actions as pre-mRNA splicing factors and interactions with specific intracellular ligands such as Ras and BCL-2 [185]. Other studies showed that galectins can form crystals while bound to a ligand and that the ligand will leach out of the crystal over time, so the crystalline inclusion may function to store and slowly release nutrients [186].

Analysis of uterine flushings indicate that secreted LGALS15 binds to itself to form multimers similar to other members of the family and is most abundant in uterine flushings between Days 14 and 16 of pregnancy [95]. The role of multimerized LGALS15 is not known. LGALS15 is hypothesized to be: 1) packaged as crystalline

inclusion bodies in LE; 2) secreted into the uterine lumen as a component of histotroph; and 3) taken up by conceptus trophectoderm and packaged into crystalline inclusion bodies [95]. It is also hypothesized that LGALS15 secreted by the LE and sGE accumulates in the uterine lumen, is absorbed by the placenta and transported to the fetal circulation from which it may be cleared by the kidney and enter the allantois via the urachus to be utilized there or recycled into the fetal circulation [4, 7]. LGALS15 is not found in amniotic fluid [95].

Results of bioinformatic and RT-PCR analyses indicate that *LGALS15* is a unique member of the galectin family that is present in the genome of sheep and goats (Subfamily Caprinae) and cattle (Subfamily Bovinae), but not in pigs (Suborder Suina), which are Artiodactyls [187]. Outside of the Artiodactyls, *LGALS15* was not detected in human, nonhuman primate, mouse, chicken, dog, or any other species with a sequenced genome [187]. Despite the presence of *LGALS15* in the genome of cattle *LGALS15* mRNA was not found to be expressed in the uterus of cows, indicating the bovine *LGALS15* gene may be a pseudogene [188].

Several functions for LGALS15 may exist. Perhaps it has effects on the immune system during pregnancy to protect the conceptus, which may be similar to its role in *Haemonchus contortus* parasite infection by altering innate and/or adaptive immune functions [20]. LGALS15 may be a progesterone-induced and IFNT-stimulated factor that modulates and promotes placental growth [189]. Perhaps of greater interest, LGALS15 may mediate conceptus-endometrial interactions during implantation [22]. This could occur due to binding and cross-linking between LGALS15 and beta

galactosides on glycoproteins and glycolipids, which stimulates cellular mechanisms and signaling cascades leading to adhesion of the conceptus to maternal uterine epithelia and conceptus survival and growth [104]. (**Figure 2.2**)

CHAPTER III

EFFECTS OF GALECTIN 15 (LGALS15) ON AN OVINE PRIMARY TROPHECTODERM CELL LINE

INTRODUCTION

In mammals, the trophectoderm is the earliest differentiating tissue and contributes to the formation of extraembryonic membranes that are necessary for embryonic implantation and survival [190]. In addition to its role in attachment and implantation, the trophectoderm is important for fluid transport into the internal membranes which form the developing blastocyst [191, 192]. From Days 9 to 20 of gestation the ovine embryo and trophectoderm will undergo dramatic growth and differentiation from a spherical blastocyst to tubular then filamentous conceptus phenotype [191]. During this time of rapid growth the trophectoderm cells begin to secrete IFNT, which functions as the pregnancy recognition signal in ruminants [74]. As embryonic development progresses, the mesoderm and endoderm associate with the ectodermal derived trophectoderm to form the chorion. Ultimately, the chorion will become intimately associated with the allantois and the uterine epithelium to form the placenta [190, 193-195]. However, despite the important roles of the tropphectoderm in early embryonic survival and establishment of pregnancy there are many aspects of its physiology which remain unclear due to the difficulty of examining the trophectoderm in isolation from other extraembryonic tissues.

Recently, a new galectin family member and component of uterine histotroph, LGALS15, was discovered in the ovine endometrium [173]. LGALS15 was originally identified in ovine intestinal epithelium as being induced in response to infection by *Haemonchus contortus*, a nematode parasite [196]. *LGALS15* mRNA is expressed in the uterine LE and sGE after Day 10 of pregnancy and is induced by P4 and stimulated by IFNT [173]. Stimulation of blastocyst growth and development in response to early exogenous P4 treatment was strongly associated with increases in endometrial *LGALS15* mRNA and LGALS15 protein in uterine flushings [197]. Although *LGALS15* mRNA was found exclusively in the endometrial LE and sGE of the uterus, LGALS15 protein was detected predominantly in the chorion/trophectoderm of the placenta, as well as in the allantoic fluid. However, the exact role of LGALS15 in trophectoderm function is unclear.

Galectins are a unique family of proteins with both intra- and extra-cellular functions including cell proliferation, differentiation, motility, adhesion, apoptosis and pre-mRNA splicing [13, 15, 134]. Extracellularly, LGALS15 is a potent stimulator of trophectoderm cell migration and adhesion *in vitro* via integrin binding and focal adhesion formation which are critical for successful conceptus implantation [198]. Intracellularly, immunogold electron microscopy revealed that LGALS15 was localized to large, membrane-bound rhomboidal crystal structures of unknown function within the endometrial LE and conceptus trophectoderm [199]. LGALS15 has been proposed to stimulate the formation of crystals within the endometrial LE as well as be secreted into the uterine lumen as a part of histotroph. The conceptus trophectoderm then presumably

absorbs extracellular LGALS15 wherein it becomes a component of intracellular crystals [23]. In ovine conceptus trophectoderm, crystals were found to increase in number and size between Days 10 and 18 of pregnancy corresponding to times of highest LGALS15 production, rapid growth and elongation of the conceptus and implantation [180].

The presence of crystals in uterine histotroph between maternal and fetal intercotyledonary membranes, is well documented [200, 201]. The P4-induced crystal structures in the endometrium and/or conceptus trophectoderm have been described in sheep [23, 180, 200], mouse [181], rabbit [182, 183] and human [182]. The biological role(s) of crystals containing LGALS15 in uterine epithelia and conceptus trophectoderm is not known; however, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interactions with specific intracellular ligands such as Ras and Bcl-2 [14, 185].

LGALS15 amino acid sequence has high homology to galectin 13 (LGALS13 also known as placental protein 13, PP-13) [173]. LGALS13 is expressed in human placenta and is suggested to be a useful marker of pre-eclampsia since LGALS13's intracellular roles include establishing the ratio of vasoconstriction/vasodilatation agents, and may function similarly in the placenta [168]. LGALS13 also elicits calcium depolarization in human trophoblast cell lines to stimulate a cascade of changes such as the liberation of fatty acids and the elevation of prostaglandin production [202]. LGALS15 also shares high amino acid homology with galectin 10 (LGALS10 also known as Charcot Leyden Crystal protein) [173]. LGALS10 is a major autocrystallizing

constituent of human eosinophils and basophils, comprising approximately 10% of the total cellular protein in these granulocytes [168]. Interestingly, both LGALS10 and LGALS13 have lysophospholipase activity and promote strong cytolytic and membrane perturbing properties [203] which has been proposed to protect the trophectoderm during implantation [168].

The goal of this study was to characterize a primary ovine trophectoderm cell line and then use it to investigate the role of LGALS15 in trophectoderm gene expression, development, growth, and survival.

MATERIALS AND METHODS

Isolation, culture and characterization of trophectoderm cells

All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A&M University. Mature Suffolk-type ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16 to 18 days) as described previously [204, 205]. At estrus, ewes were mated to intact rams. The conceptuses were recovered by flushing the uterine lumen with 20 ml sterile PBS (pH 7.2) supplemented with 100 U/L penicillin and 100 µg/L streptomycin. The inner cell mass was removed from the conceptuses whenever possible, and the remaining tissues were carefully minced, pooled, and placed in trophoblast growth medium, containing DMEM/F-12 supplemented with 10% fetal bovine serum, glutamine (2 mM), insulin (700 nM), pyruvate (1.0 mM), non-essential amino acids (0.1 mM), and

antibiotics (50 U/L penicillin, 50 µg/L streptomycin). Cells were maintained in a 5% CO_2 environment at 37°C. Two different ovine trophectoderm cell lines were established and designated as oTr1 and oTrF. The oTr1 cell line was established from a Day 16 conceptus and maintained in tissue-culture in treated plastic dishes, whereas oTrF cell line was established from a Day 14 conceptus and maintained on collagen-coated plastic dishes (Cohesion, Palo Alto, Ca). Fluid-filled trophoblastic vesicles, which spontaneously developed in culture, were physically ruptured with sterile 28-gauge needles to enhance the generation of a cellular monolayer. This primary culture was propagated on the same support by serial trypsinizations. Cells were transferred to tissue culture flasks and propagated for six passages prior to use.

RNA extraction, reverse transcription, and polymerase chain reaction analysis

RNA extracted from oTr1 and oTrF primary ovine trophectoderm cell lines were converted to cDNA and analyzed by PCR for molecular markers found in trophectoderm cells *in vivo*. Total cellular RNA was isolated from tissues 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% formaldehyde-agarose gel. Expression of trophectoderm molecular markers was determined by reverse transcription-polymerase chain reaction (RT-PCR) using methods described previously [80]. Briefly, cDNA was synthesized from total oTr1 and oTrF 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 before use in PCR reactions. The PCR reactions were performed using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) according to manufacturers' recommendations. The PCR conditions and amount of template cDNA used in each reaction were optimized for each primer set to ensure linear amplification of the target. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining using an Alpha Innotech imaging system. See **Table 3.1** for primers and conditions. As a control for genomic contamination, cDNA was produced with and without reverse transcriptase. Beta-actin was used as a loading control and cDNA made from a Day 16 conceptus functioned as a positive control.

Production of rabbit antibodies to ovine LGALS15

Antibodies to the ovine LGALS15 protein in rabbits were commercially produced using the recombinant ovine LGALS15 protein as an immunogen. Rabbit anti-ovine LGALS15 IgG was purified from serum of immunized rabbits using an ImmunoPure (A/G) IgG Purification kit (PIERCE, Rockford, IL).

Wound healing assay

The oTr1 cells were plated in 6-well plates (Corning Costar Corning, NY) with trophectoderm growth medium and allowed to grow to confluency. A sterile pipette tip was used to remove cells by scratching a straight line down the center of each well.

TABLE 3.1
PCR primers and conditions.

Primer	Sequence of forward and reverse primers (5'-3')	GenBank accession no.	Annealing temperature (C)	Product size (bp)
DKK1	CACTCCAGATTTTCGGAAGG AAGTCAAGTGTGCACCAAGC	BF107232	54.0	301
FZD6	GGCTGAAGGTCATTTCCAAG TGAACAGGCAGAGATGTGGA	NM008056	54.5	230
GSK3B	TCCATTCCTTTGGAATCTGC AGCAGACGGTACAAAGTGC	CB465245	54.5	445
HYAL2	GAAGGGACACGTGGAACACT TTTCGGGAGACCTCAACATC	NM001009754	60.0	460
GAG	GTTTTCCTCGCCACTACTCTTATT AGTGTCTAATTCCTATGCCGATGTT	DQ838494	49.5	228
ENV	ATGCCGAAGCGCCGCGCTGG TCACGGGTCGTCCCCCGCAGC	XM001789148	49.5	473
CSH1	AGGGCATAAACTCCGAATCC CAGGGAGGACTGTTCTGACC	NM001009309	49.5	506
PAG1	TCACCAGTCTTCCACCTTCC CTACCCCACCAAACATCACC	M73961	49.5	285
CDH1	GGCTGAGTTGGACAGAGAGG TCATTGCGAGTCACTTCAGG	AY508164	55.0	720
IFNT	GGAAACTCATGCTGGATGC AAGGTGGTTGATGAAGTGAGG	X56343	49.5	450
B-Actin	ATGAAGATCCTCACGGAACG GAAGGTGGTCTCGTGAATGC	AF129289	55	270

Remaining cells were washed and incubated at 37 °C with fresh medium and monitored at 12 h intervals up to 72 h for cellular migration into the cleared area.

LGALS15 uptake assay

Four-well chamber slides (Nunc, Rochester, NY) were seeded in triplicate with oTr1 cells at approximately 60% confluency in serum and insulin-free oTr growth medium and incubated for 24 h. Fresh medium containing LGALS15 (1 μg/ml, 1 ml per well) was added and cultured for another 12 h. Cells were then washed three times and fixed. Using methods described previously [72, 75], cells were fixed in –20°C methanol, permeabilized with 0.3% Tween 20 in 0.02 M PBS, blocked in antibody dilution buffer (two parts 0.02 M PBS, 1.0% BSA, and 0.3% Tween 20, and one part glycerol) containing 10% normal goat serum, and incubated overnight at 4°C with either rabbit anti-recombinant ovine LGALS15 polyclonal IgG or rabbit IgG at a dilution of 1:100 . Immunoreactive protein was detected using a fluorescein-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Sections were then rinsed and overlaid with a coverslip and Prolong Antifade mounting reagent (Molecular Probes).

RESULTS

oTr1 and oTrF primary cell lines express genes characteristic of trophectodern cells in vivo.

Overall, expression of genes in both oTr1 and oTrF was similar to that of trophectoderm cells obtained from a Day 16 conceptus (**Figure 3.1**). In particular, oTr1

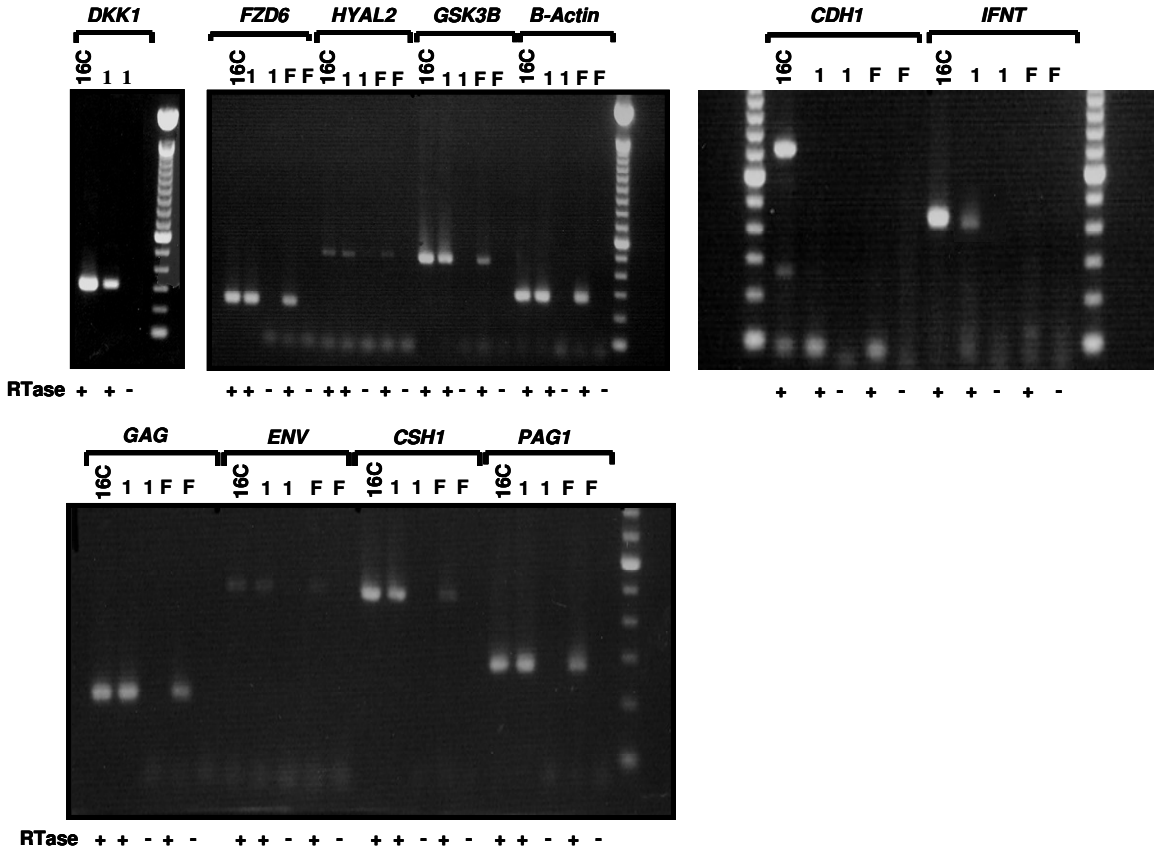

FIGURE 3.1
In vitro gene expression of *in vivo* trophectoderm cell markers. E-cadherin (*CDH1*), interferon tau (*IFNT*), endogenous Jaagsiekte Sheep Retroviruses (enJSRVs) *env* and *gag*, *HYAL2* receptor for enJSRVs Env, ovine placental lactogen (*oPL*), pregnancy associated glycoprotein 1 (*PAG1*) *and* other genes associated with the Wnt signaling pathway (*FZD6, GSK3B, DKK1*) mRNA in oTr cell lines. Day 16 conceptus (16C) along with beta-actin was used as positive controls.

and oTrF expressed endogenous Jaagsiekte Sheep Retrovirus envelope and gag (*enJSRV env* and *gag*) genes, as well as its receptor *Hyal2* which stimulate multinucleated syncituim formation in sheep [206]. Both cell lines also expressed ovine placental lactogen (*CSH1*) and pregnancy associated glycoprotein (*PAG1*) which are produced by giant binucleated cells of the trophectoderm *in vivo* [207-209]. Genes associated with the Wnt signaling pathway (frizzled homolog 6 (*FZD6*), glycogen synthase kinase 3 beta (*GSK3B*), and dickkopf 1 (*DKK1*)) which is an important regulator of uterine morphogenesis, uterine receptivity to the embryo, and blastocyst implantation were also expressed in oTr1 and oTrF [205]. However, only the oTr1 cell line expressed *IFNT* and neither cell line is expressing E-cadherin (*CDH1*). Since IFNT production is a hallmark of trophectoderm cell viability at the time of implantation only oTr1 cells were used for the remaining experiments.

oTr1 cells migrate as single cells

oTr1 cells were seeded onto 6-well tissue culture treated plates and allowed to grow to 100% confluency. Following physical removal of cells from the bottom of cell culture dishes, single oTr1 cells were observed in open spaces starting at 12 h (**Figure 3.2**).

Uptake of LGALS15 by oTr1 cells

Treatment of oTr1 cells with LGALS15 (10 μg/ml) resulted in uptake of this protein, as detected by indirect immunofluorescence, as punctate concentrations of

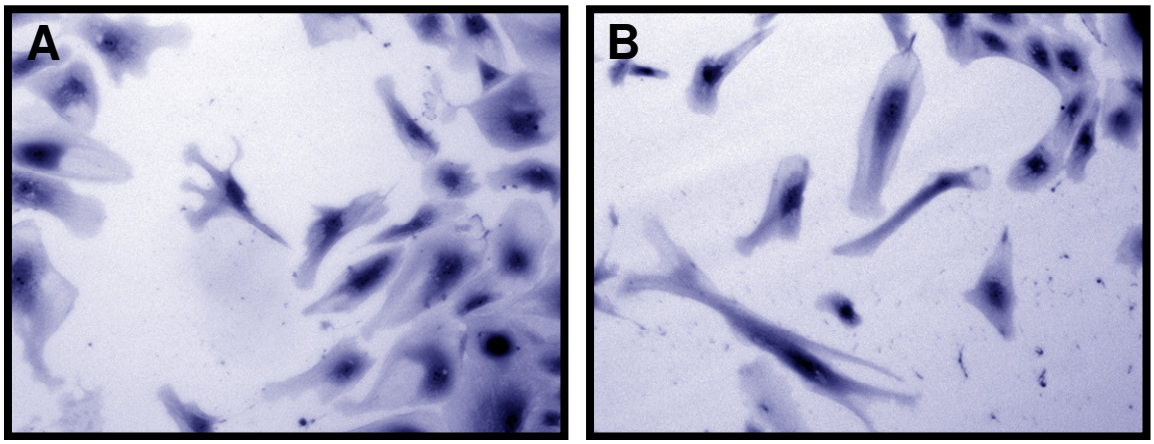

FIGURE 3.2
Trophectoderm cell migration into open areas created for a wound healing assay. A) oTr cell migration into space created when 100% confluent cells were removed by scratching down the plate. B) Image highlighting single cell migration.

protein throughout the cytoplasm. Control cells were not incubated with LGALS15 and, therefore, showed no immunostaining for this protein, confirming that oTr1 cells do not express endogenous LGALS15 (**Figure 3.3**). Colocalization experiments with anti-clathrin antibodies to determine the mechanism of LGALS15 uptake into oTr1 cells were unsuccessful (data not shown).

DISCUSSION

Results presented here describe a primary trophectoderm cell line derived from peri-implantation ovine conceptuses. Due to the complex nature of the trophectoderm and its critical roles in implantation, growth and development of the conceptus, a primary cell line is a valuable resource for deciphering gene and protein expression in isolated trophectoderm cells in response to specific treatments. Indeed, two trophectoderm cell lines (oTr1 and oTrF) had similar physiological properties and gene expression profiles to conceptus trophectoderm suggesting that they are suitable models for such investigations. In the sheep conceptus, trophoblast giant binucleated cells (BNCs) first appear on Day 14 [111] and are thought to arise from mononuclear trophectoderm cells by consecutive nuclear divisions without cytokinesis [106]. BNCs produce *CSH1* and *PAG1* and, by Day 16 of pregnancy, represent 15-20% of trophectoderm cells [210]. The oTr cell lines displayed typical trophectoderm cell morphology with both mononuclear cells and a small population of BNC which persisted for more than 20 passages. Furthermore, oTr cells expressed genetic markers of trophectoderm cells *in vivo*, such as *IFNT*, enJSRVs *env* and *gag*, *HYAL2* receptor for

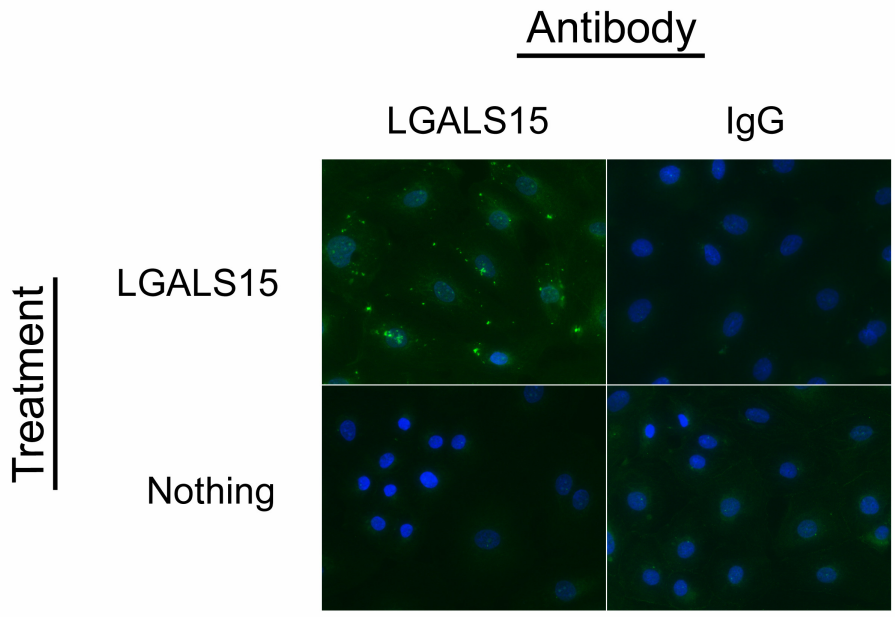

FIGURE 3.3
Uptake of exogenous LGALS15 by trophectoderm cells. Four-well chamber slides (Nunc, Rochester, NY) were seeded in triplicate with oTr1 cells at approximately 60% confluency in serum and insulin-free oTr growth medium and incubated for 24 h. Fresh medium with no treatment or fresh medium containing LGALS15 (1 µg/ml, 1 ml per well) was added and cultured for another 12 h. Slides were incubated with either rabbit anti-recombinant ovine LGALS15 polyclonal IgG or rabbit IgG at a dilution of 1:100. Immunoreactive protein was taken up by oTr1 cells in protein rich cytoplasmc vesicles when exposed to exogenous LGALS15. Similar to trophectoderm cells *in vivo*, oTr1 cells do not produce endogenous LGALS15.

enJSRVs Env, *CSH1*, *PAG1* and genes associated with the Wnt signaling pathway (*FZD6, GSK3B, DKK1*). A major difference between the two lines was that *IFNT* was expressed in the oTr1, but not oTrF cell line. Moreover, the oTrF had a slightly more fibroblastic morphology whereas oTr1 had the traditional cobblestone morphology characteristic of other epithelial cell types. The major difference between the two established cell lines is that oTrF cells were developed from a Day 14 conceptus and cultured on a collagen substrate whereas oTr1 cells were developed from a Day 16 conceptus and cultured on tissue culture treated plastic. In sheep, the mononuclear trophectoderm cells secrete IFNT, the maternal recognition signal of pregnancy, between Days 10 and 21-25 of pregnancy with maximal expression occurring on Days 14 to 16 [53-55]. Thus both cell lines were established at the height of IFNT production, and the absence of IFNT expression and varied morphology of oTrF cell line is not well understood but may be due to the presence of collagen substrate on which the cells were grown. Despite differences in morphology and IFNT production, both cell lines behaved similarly when treated with exogenous LGALS15 in both cellular adhesion and migration assays [198] suggesting that at least some physiological characteristics of trophectoderm cells were maintained in both cell lines.

In ruminants, the conceptus remains unattached in the uterine luminal epithelium for up to two weeks [29] with superficial implantation beginning on Days 13 to 14, and continuing until Days 60 to 70 [9, 81]. Between Days 16 and 24 of pregnancy the uterine LE begins to fuse with BNCs to form trinuceate fetomaternal hybrid cells [111]. Continued fusion of BNCs with uterine LE gives rise to multinucleated syncytial plaques

with up to 25 nuclei that appear to be linked to surrounding cells by tight junctions [106, 112]. The trophectoderm will not invade further into the endometrium presumably due to inhibitory factors such as tissue inhibitors of metalloproteinases (TIMPs) [211]. However, if trophectoderm cells are transplanted to another part of the body individual cells will actively invade those tissues [212]. Invasion combines adhesion, migration and tissue remodeling and in the present study, a wound healing assay was used to ascertain migratory properties of oTr1 cells. Since epithelia form biological barriers in which individual cells must tightly associate with each other, typical epithelial cell migration *in vitro* is distinct from that of unattached/single cells due to the tendency of epithelial cells to migrate as a cohesive unit [213]. However, results of migration assays demonstrated that individual oTr1 cells can detach and migrate. Invasion of individual cells would be more efficient than forcing cell sheets into a highly organized preexisting tissue such as the uterine endometrium.

Development and characterization of the oTr1 primary cell line has shown them to be highly similar to conceptus trophectoderm in gene expression, migratory properties and morphology. Taken together these results indicate that the oTr1 primary cell line is an excellent model to study intracellular and extracellular roles of LGALS15 in conceptus trophectoderm. *LGALS15* mRNA is not expressed in the trophectoderm; however, LGALS15 protein secreted from the LE is present on the apical surface of trophectoderm cells, as well as internalized and packaged into crystalline structures within trophectoderm cells [173]. Support for this mechanism was obtained in the present study in which oTr1 cells were shown to accumulate exogenous LGALS15

protein *in vitro*. Interestingly, studies with breast carcinoma cell lines showed that LGALS3 can be absorbed from the medium and packaged into vesicles within the cytoplasm [214] suggesting that patterns of LGALS15 secretion and uptake are conserved across galectin superfamily members.

Historically, extracellular galectin functions have been the predominate subject of the literature leading to the classic definition of galectins as non-immunoglobulin, nonenzymatic carbohydrate-binding proteins [14]. However, recent studies have elucidated far more diverse roles of galectins than previously thought, including the discovery of distinct intracellular functions. With this increasing knowledge comes the difficulty of ascertaining the roles of different galectins and, in particular the role of galectins in complex tissues. However, by establishing primary cultures of individual cell types, such as ovine trophectoderm cells, specific assessments of the role of particular galectins in specific cells becomes possible, which may further clarify the importance of this superfamily of proteins.

CHAPTER IV

GALECTIN 15 (LGALS15) FUNCTIONS IN OVINE TROPHECTODERM CELL ATTACHMENT*

INTRODUCTION

Maternal support of blastocyst growth and development into an elongated conceptus (embryo/fetus and associated membranes) is critical for pregnancy recognition signaling and implantation in ruminants [215, 216]. In sheep, morula-stage embryos enter the uterus on Day 4 and form blastocysts by Day 6 that contains a blastocoele or central cavity surrounded by a monolayer of trophectoderm [102, 217]. After hatching from the zona pellucida (ZP) on Day 8, the blastocysts develop into a tubular form by Day 11 and then elongate between Days 12 and 16 to a filamentous conceptus of 10 cm or more in length. Blastocyst growth and elongation is crucial for pregnancy recognition signaling, which involves synthesis and secretion of IFNT from mononuclear trophectoderm cells of the elongating blastocyst that inhibits luteolysis [218, 219]. The factors supporting growth of peri-implantation blastocysts and elongating conceptuses are thought to be derived primarily from secretions of the uterus, collectively referred to as histotroph [220, 221]. This hypothesis is supported by failure of conceptus development in the uterine gland knockout ewe model [191, 222].

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The UGKO ewe experiences recurrent early pregnancy loss between Days 12 and 14 due to inadequate histotroph from the endometrial LE and GE [191, 222]. In order to understand the peri-implantation pregnancy defect, the UGKO ewe model was used in a gene expression profiling project based on an endometrial cDNA library from uteri of Day 14 pregnant ewes [173, 223]. Interestingly, approximately 1.4% of the expression sequence tags sequenced from the cDNA library were highly similar to *ovgal11*, a novel member of the galectin family of secreted animal lectins [196]. OVGAL11 was originally shown to be induced in gastrointestinal tissue and secreted into the intestinal lumen in response to inflammation and eosinophil infiltration after infection of sheep with the helminth, *Haemonchus contortus* [196]. The sequence of OVGAL11 protein displayed the highest similarity to human LGALS10 (also known as Charcot-Leyden Crystal protein) [224, 225] and human LGALS13 (also known as placental tissue protein 13 or PP13) [226]. Since *ovgal11* does not have a known orthologue, it was designated as a new member of the galectin superfamily and renamed LGALS15.

Galectins are proteins with a conserved CRD that bind beta-galactoside sugars, thereby cross-linking glycoproteins and glycolipid receptors on the surface of cells and initiating biological responses [13, 134, 227]. Functional studies of the extracellular and intracellular roles of galectins implicate them in cell adhesion, chemoattraction and migration as well as cell growth, differentiation and apoptosis [135, 228]. All of these biological roles are important for peri-implantation blastocyst growth and differentiation [102, 217].

In the ovine uterus, *LGALS15* mRNA is detected in the endometrial LE and superficial ductal GE after Day 10 of pregnancy, and *LGALS15* is induced by ovarian P4 and stimulated by conceptus IFNT [173, 197]. In the endometrium, LGALS15 protein has a nucleocytoplasmic distribution within the LE and sGE and is also concentrated near and on the apical surface. Secreted LGALS15 protein is abundant in histotroph recovered from the uterine lumen, where it exists in multimeric forms. It is localized to the apical surface of conceptus trophectoderm and within intracellular crystals [173, 199]. The ovine LGALS15 protein contains predicted CRD, LDV and RGD recognition sequences, which can bind and activate integrins [229]. The temporal and spatial alterations in abundance of *LGALS15* mRNA and protein in uterine endometrial epithelia and lumen during the peri-implantation period of early pregnancy, combined with the known biological activities of other galectins, make it a candidate mediator of conceptus-endometrial interactions during implantation [199, 230]. Recently, advanced growth and development of blastocysts in response to early P4 treatment of ewes was associated with induction of *LGALS15* in the endometrial epithelia [197].

The working hypothesis for the present study was that LGALS15, synthesized and secreted by endometrial LE and sGE into the uterine lumen, functionally binds and crosslinks beta-galactosides on glycoproteins and glycolipids using the CRD and integrins through the LDV and RGD recognition sequences, to function as a heterotypic adhesion molecule bridging conceptus trophectoderm and endometrial LE for successful blastocyst elongation and conceptus implantation [102, 231]. Experiments to test this working hypothesis, using recombinant ovine LGALS15 and ovine mononuclear

trophectoderm cells in functional assays, indicated that LGALS15 primarily stimulates trophectoderm attachment via its RGD integrin recognition sequence.

MATERIALS AND METHODS

Preparation of recombinant ovine LGALS15 and mutants

The entire coding sequence for ovine and caprine endometrial *LGALS15* mRNAs [173] was used to produce recombinant ovine and caprine LGALS15 (both LDV and LVV polymorphic forms) in bacteria. PCR reactions (50 µl) were conducted in Optimized Buffer F (Invitrogen, Carlsbad, CA) and contained 10 ng of ovine LGALS15 cDNA from Day 14 pregnant endometrium, 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 for affinity purification (**Figure 4.1**). The resulting plasmid was sequenced to ensure no mutations were present in the *LGALS15* sequence.

Mutation of the LDVRGD recognition sequence in LGALS15 to LAVRAD was conducted by PCR amplification using the ovine endometrial *LGALS15* cDNA as

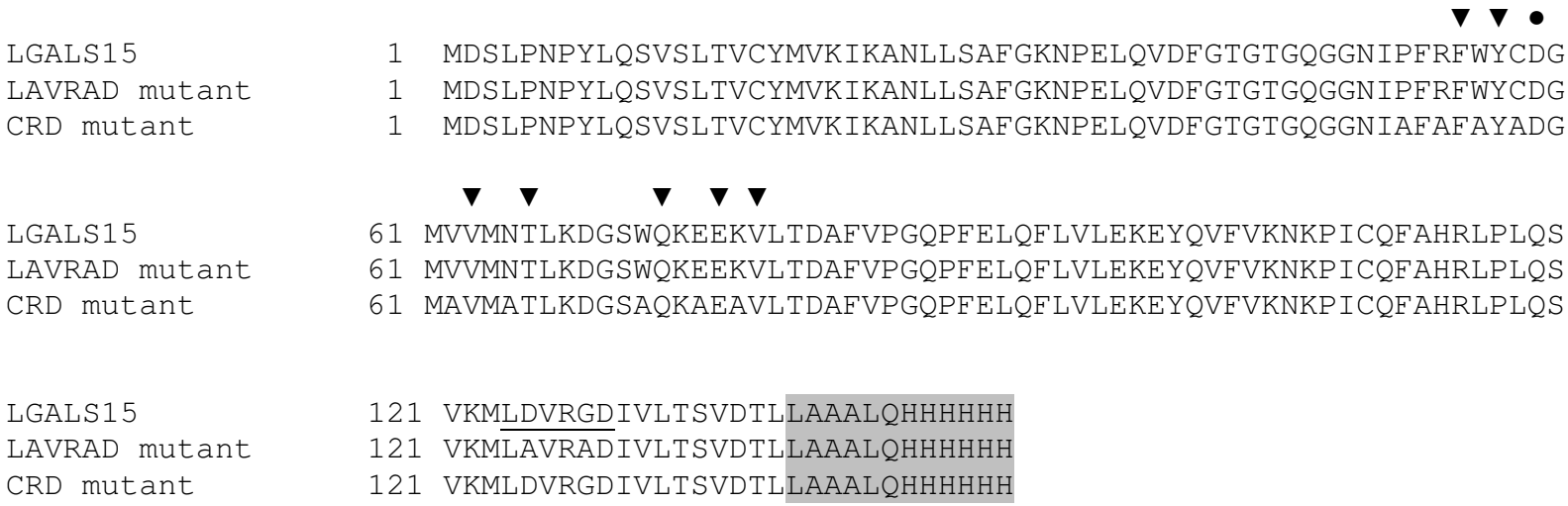

FIGURE 4.1
Alignment of the amino acid sequences of recombinant ovine LGALS15 and mutants. The underlined residues denote the conserved LDV and RGD recognition sequences for integrin binding near the C-terminus in ovine LGALS15. The arrows (▼) denote the conserved residues forming the carbohydrate recognition domain (CRD) in prototypical galectin family members. The circle (●) denotes a conserved C residue critical for mannose binding in LGALS10. The shaded sequence at the C-terminus contains the 6xHis tag used for affinity purification of the recombinant proteins.

described above, but with a different reverse primer (5'-CAG CAC GAT ATC TGC CCT CAC AGC CAG CAT TT-3') and the following modifications. PCR reactions were conducted with VENT polymerase buffer (New England BioLabs, Beverly, MA) and 1 U VENT Polymerase with conditions of: 1) 95°C for 2 min; 2) 95°C for 30 sec, 60°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 EcoRV enzymes and then directionally subcloned into the pET-28b(+) vector (Novagen, Madison, WI). The resulting plasmid was sequenced to ensure that targeted mutations translated into a LAVRAD mutant of the LDVRGD sequences in the C-terminus of wildtype LGALS15 (**Figure 4.1**).

Mutation of the predicted CRD in ovine endometrial LGALS15 was conducted using two sets of nested internal primers for PCR amplification. Set 1 mutated the first half of the CRD (forward 5'-CCA TTC GCT TTC GCG TAC GCC GAT GGC ATC GTG GCT ATG GCC ACT TTA AAG-3' and reverse 5'-CTT TAA AGT GGC CAT AGC CAC GAT GCC ATC GGC GTA CGC GAA AGC GAA TGG-3'). Set 2 mutated the second half of the CRD (forward 5'-GGG AGT GCG GGG AAG GCA CAG GCA CTG CAT ACT GAG GC-3' and reverse 5'-GCC TCA GTA TGC AGT GCC TGT GCC TTC CCC GCA CTC CC-3'). PCR reactions were conducted as described above to generate the LGALS15 LAVRAD mutant. Partial cDNAs were gel purified and then used in a PCR reaction with primers to amplify the full coding sequence. This cloning strategy mutated the predicted amino acids in the CRD to alanine in the LGALS15 and placed a His•Tag sequence at the C-terminus (**Figure 4.1**). The insert of the resulting

plasmid was sequenced to ensure the targeted mutations were present in the CRD of the LGALS15 sequence.

Wildtype and mutant forms of ovine endometrial LGALS15 protein were produced in BL21 bacteria according to the manufacturer's suggestions. Expression of His-glaectin15 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 (Invitrogen). 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 using a rabbit anti-ovine LGALS15 antibody. Fractions containing recombinant LGALS15 were dialyzed overnight in PBS (pH 7.2) at 4°C, concentrated using a spin column with a 3,500 MWCO (Vivaspin, Stonehouse, UK) and frozen in aliquots at -80^{o}C. Protein concentration was determined using a RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

Isolation and culture of mononuclear ovine trophectoderm (oTr) cells

All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A&M University. As described previously [204, 205], mature Suffolk-type ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 days). At estrus, ewes were mated to

intact rams. The conceptuses were recovered on Day 15 post-mating by flushing the uterus with 20 ml of sterile PBS (pH 7.2) supplemented with 100 U penicillin and 100 µg streptomycin per liter. The inner cell mass was removed from the conceptus whenever possible, and the remaining tissues were carefully minced, pooled, and placed in a trophoblast growth medium consisting of DMEM/F-12 supplemented with 10% fetal bovine serum, glutamine (2 mM), insulin (700 nM), pyruvate (1.0 mM), non-essential amino acids (0.1 mM), and antibiotics (50 U penicillin, 50 µg streptomycin). The tissues were maintained in a 5% CO_2 environment at 37°C. Two different ovine trophectoderm cell lines were established and designated as oTr1 and oTrF. The oTr1 cell line was established in tissue-culture treated plastic dishes, whereas the oTrF cell line was established on collagen-coated plastic dishes (Cohesion, Palo Alto, Ca). Fluid-filled trophoblastic vesicles, which spontaneously developed in culture, were physically ruptured with sterile 28-gauge needles to enhance the generation of a cellular monolayer. This primary culture was propagated on the same support by serial trypsinizations. All experiments were performed with both oTr1 and oTrF cell lines unless otherwise indicated.

Attachment assay

Attachment assays were adapted from published procedures [232, 233]. Cell suspension plates with 24 wells (Greiner Multiwell Tissue Culture Plates, PGC Scientific Co, Monroe, NC) were coated with either BSA (Bovine Serum Albumin Fraction V, Pierce, Rockford, IL) as a negative control, bovine FN (Fibronectin 0.1%

solution from bovine plasma, Sigma, St. Louis, MO) as a positive control, or recombinant ovine LGALS15 protein (wildtype or mutants) in triplicate and allowed to dry overnight in a sterile hood at room temperature. Wells were then blocked with 1 ml of BSA (10 mg/ml) in PBS for 1 h and rinsed three times with 1 ml serum and insulin-free DMEM per well. Equal numbers of freshly trypsinized oTr cells were seeded into each well and plates were incubated for 1.5 h. In some experiments, a cyclic blocking peptide (GRDGS and TLKDGS; Peptides International, Inc., Louisville, KY) and cyclic control peptide (GRADS) were added to the wells. Wells were washed three times with 1 ml of serum- and insulin-free media to remove unattached cells. Cell number was determined using a Janus Green assay. The entire experiment was independently repeated at least three times with different passages of oTr cells.

Janus green assay

Cell number was determined as described previously [234] for all attachment assays. Briefly, DMEM was removed from cells by vacuum aspiration and cells were fixed in 50% ethanol for 30 min followed by vacuum aspiration of the fixative. Fixed cells were stained with a Janus Green B in PBS (0.2% w/v) for 3 min at room temperature. The stain was removed using a vacuum aspirator, and the whole plate was sequentially dipped in water and destained by gentle agitation. The remaining water was removed by shaking. The stained cells were immediately lysed in 0.5N HCl and absorbance readings were taken at 595 nm using a microplate reader. As described previously [234], cell numbers were calculated from absorbance readings using the

formula [cell number = (absorbance-0.00462)/0.00006926]. The entire experiment was independently repeated at least three times with different passages of oTr cells.

Focal adhesion formation assays

The oTr cells were seeded into four-well Lab-Tek glass chamber slides (Nunc, Rochester, NY) coated with either recombinant LGALS15 (1 µg, 5 µg, 10 µg or 20 µg per well), bovine FN as a positive control, and either poly-L-lysine or no protein as negative controls. After 1.5 h, cells were fixed in cold methanol (-20^{o}C) for 10 min and air dried. Fixed cells were rehydrated at room temperature with 0.3% Tween 20 in 0.02 M PBS (rinse solution), blocked in antibody dilution buffer (2 parts 0.02 M PBS, 1.0% BSA, 0.3% Tween 20 [pH 8.0] and one part glycerol) containing 5% normal goat serum (v/v) for 1 h at RT, and incubated overnight at 4°C with a mouse monoclonal anti-talin antibody (1:1000) or mouse serum (1:1000) (Sigma T3287 Clone 8d4). Immunoreactive protein was then detected using an Alexa Fluor 488-conjugated secondary antibody for 1 h at RT. Slides were overlaid with Prolong antifade mounting reagent with DAPI (Invitrogen-Molecular Probes, Eugene, OR, USA) and affixed with a coverslip. The entire experiment was independently repeated at least three times with different passages of oTr cells.

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). 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). Percentage data were transformed by arcsin transformation before analysis. Independent variables included the LGALS15 treatments and replicate. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

RESULTS

LGALS15 mediates attachment of ovine trophectoderm cells

Several members of the galectin family are involved in heterologous cell-cell adhesion mediated by CRD binding of beta-galactosides on glycoproteins that include integrins [135, 233]. In addition to a predicted CRD, LGALS15 also contains predicted LDV and RGD integrin recognition sequences in the C-terminus (**Figure 4.1**). The RGD sequence is a well documented cell attachment site present in a large number of adhesive extracellular matrix, blood and cell surface proteins. Nearly half of the over 20 known integrins recognize this sequence in their adhesion protein ligands [229]. Integrins are heterodimeric cell surface receptors that mediate adhesion between cells and the ECM by binding to ligands with an exposed RGD sequence. These receptors also stimulate intracellular signaling and gene expression involved in cell growth, migration, and survival. Integrin binding and activation is an essential element of conceptus-endometrial interactions, blastocyst implantation and trophoblast

differentiation in many species [235, 236]. Therefore, a series of studies were conducted to explore the attachment functions of LGALS15 using oTr cells.

Recombinant ovine LGALS15 mediated attachment of oTr cells in a dose-dependent manner (**Figure 4.2 A and B**). Relative to wells coated with BSA as a negative control, there was an increase in oTr cell attachment in wells of non-adherent suspension plates coated with either 1 μg or 10 μg of recombinant ovine LGALS15. Moreover, the attachment function of LGALS15 was similar to that in response to bovine fibronectin (bFN), which was used as a positive control. BSA did not mediate attachment of oTr cells (data not shown). These results strongly support the hypothesis that LGALS15 contains an intrinsic attachment function and serves as a mediator of heterologous interactions between the conceptus trophectoderm and endometrial LE.

LGALS15 mediates attachment of ovine trophectoderm cells that is LDV-independent

LGALS15 is expressed in the uterus of pregnant sheep and goats at the time of implantation. In both species there is a common polymorphism in the integrin recognition sequence where the LDV sequence is converted to LVV. Ovine LGALS15 with the LDV sequence was shown to stimulate trophectoderm cell attachment. To investigate if LGALS15 with the LVV polymorphism affects LGALS15 attachment function, each form of the protein was expressed and purified and an attachment assay was conducted. BSA was used as a negative control for cellular attachment (data not shown) and bovine FN was used as a positive control. A dose-dependent increase ($P<0.01$) in oTr1 cell attachment was induced with both ovine and caprine LGALS15

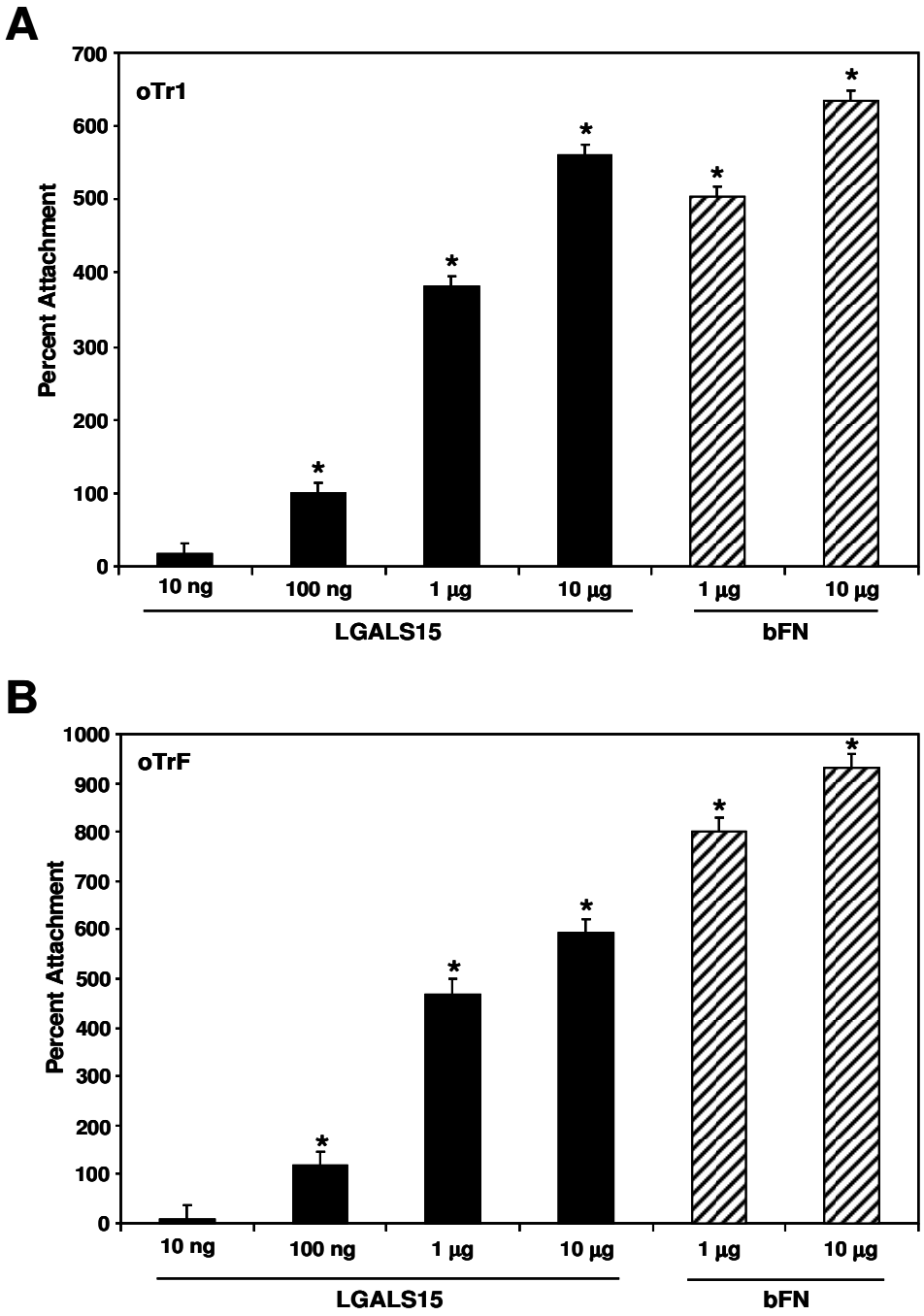

FIGURE 4.2
Effects of ovine LGALS15 on attachment of ovine trophectoderm cells. [A & B] Wells of suspension culture plates were precoated overnight with recombinant ovine LGALS15, BSA as a negative control, or bovine FN (bFN) as a positive control. Equal numbers of oTr cells were added to each well, and the number of attached cells determined after 1.5 h. Data is presented as percent attachment relative to BSA-coated wells. Note the dose-dependent increase in cell attachment mediated by LGALS15 that is similar to bFN.

indicating that the LDV sequence is not necessary for LGALS15 attachment function. LGALS15 and bovine FN induced similar increases in oTr1 cell attachment. (**Figure 4.3**)

LGALS15 mediates attachment of ovine trophectoderm cells that is RGD-dependent

The integrin-binding activity of adhesion proteins can be achieved using short synthetic peptides containing the RGD sequence. Such peptides promote cell adhesion when bound to the cell surface, and inhibit it when presented to cells in solution [229]. Inclusion of a cyclic GRGDS peptide inhibited ($P<0.01$) LGALS15-mediated oTr cell attachment, whereas the control peptide (GRADS), which contains the conservative substitution of alanine for glycine, had no detectable inhibitory activity (**Figure 4.4 A and B**). These results indicate that trophectoderm cells adhere to LGALS15 using receptors, such as integrins, that recognize a RGD sequence.

LAVRAD mutated LGALS15 does not stimulate attachment of ovine trophectoderm cells

The LDV and RGD recognition sequences in ovine LGALS15 were mutated to LAV and RAD using a PCR-based mutagenesis strategy, and the recombinant protein was used for oTr cell attachment assays. Wildtype LGALS15 increased attachment of oTr1 cells in a dose-dependent manner (**Figure 4.5 A and B**). There was no difference in attachment functions of LGALS15 and the LGALS15 LAVRAD mutants in wells precoated with either 100 ng or 1 μg of protein. However, there was a decrease ($P<0.01$) in oTr cell attachment in wells precoated with 10 μg LGALS15 LAVRAD mutant

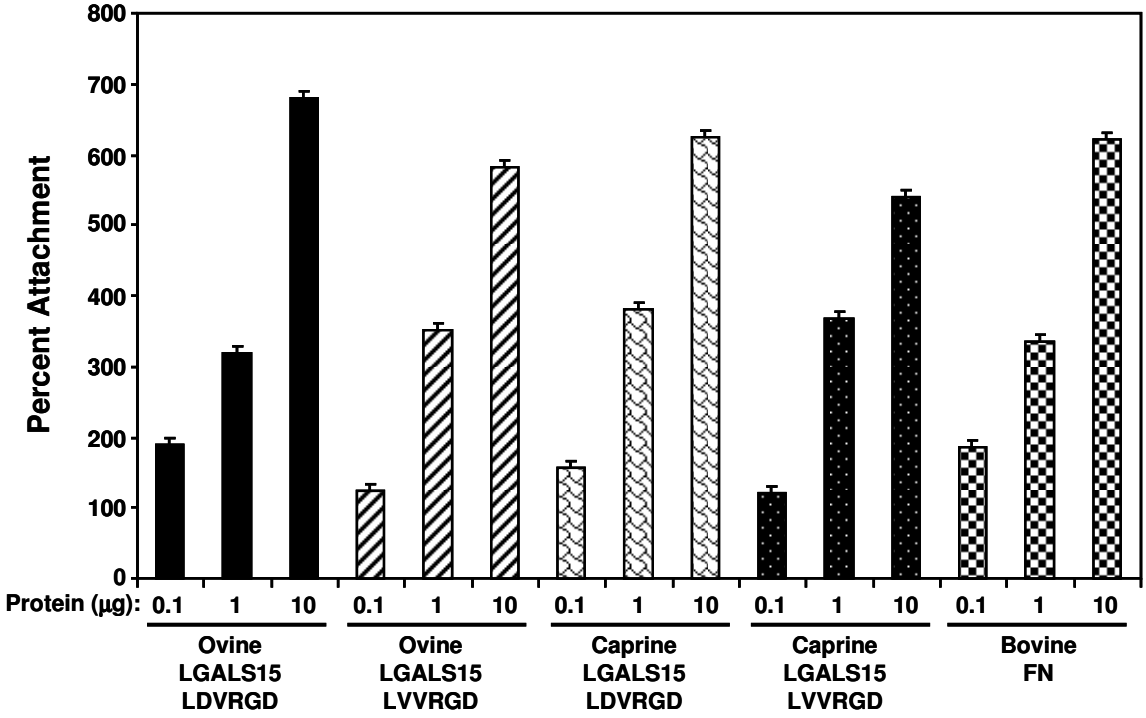

FIGURE 4.3
Attachment function assays of ovine and caprine LGALS15 using ovine trophectoderm cells. Wells of suspension culture plates were coated with increasing amounts (0.1, 1 or 10 µg) of recombinant ovine and caprine LGALS15, purified bovine fibronectin (bFN), or BSA. Freshly prepared oTr1 cells 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.

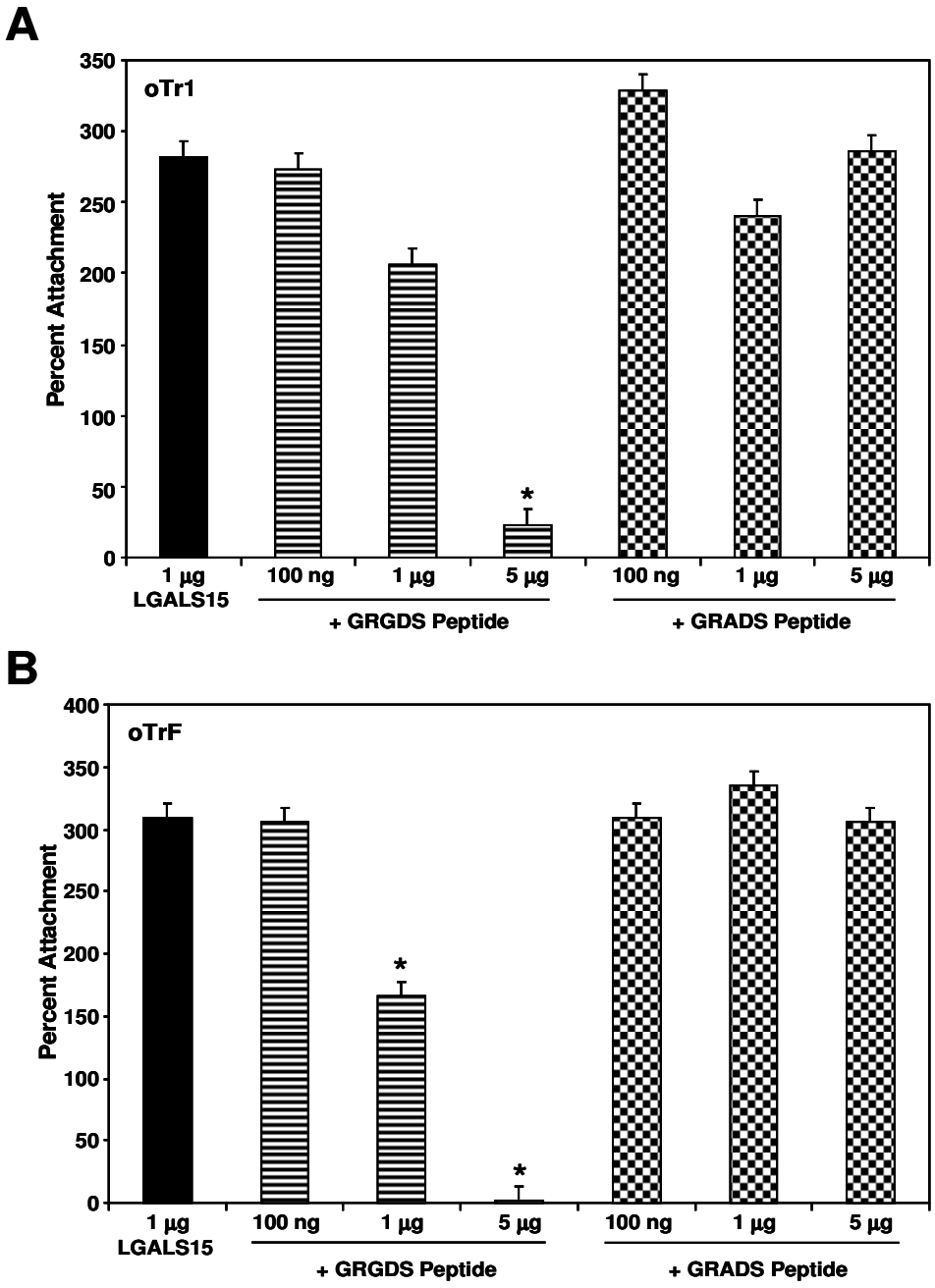

FIGURE 4.4
Effects of ovine LGALS15 on attachment of ovine trophectoderm cells. [A & B] Wells were precoated overnight with 1 µg of recombinant ovine LGALS15. An equal number of oTr cells were added to each well along with increasing amounts of synthetic cyclic GRGDS or GRADS peptides. The number of attached cells determined after 1.5 h. Data is presented as percent attachment relative to uncoated wells. Note the dose-dependent decrease in binding of oTr cells to LGALS15 elicited by the GRGDS but not the GRADS peptide.

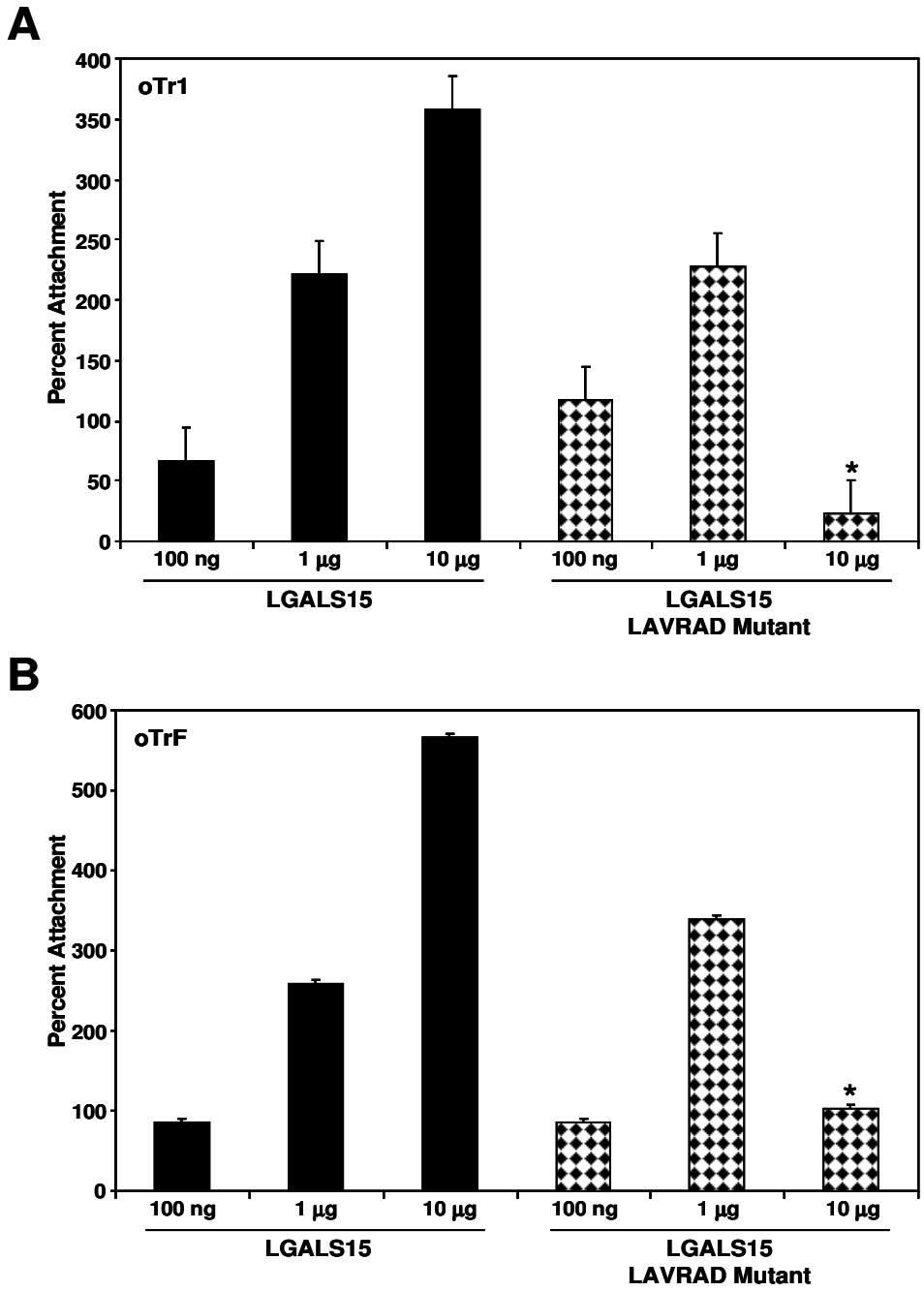

FIGURE 4.5
Effects of mutating the LDVRGD recognition sequence in ovine LGALS15 on attachment of ovine trophectoderm cells. [A & B] Wells of suspension culture plates were precoated overnight with recombinant ovine LGALS15 or the LAVRAD mutant of LGALS15. Equal numbers of oTr cells were added to each well, and the number of attached cells determined after 1.5 h. Data is presented as percent attachment relative to uncoated wells. Note the decrease in oTr cell attachment mediated by the 10 µg of the LAVRAD mutant of LGALS15 as compared to wildtype LGALS15.

compared to 10 μg wildtype LGALS15. Collectively, these results indicate that cell attachment function of LGALS15 is not entirely dependent on the RGD sequence in the C-terminus, meaning that another recognition sequence may exist within the protein that recognizes a sequence similar to the RGD recognition sequence.

LGALS15 mediates attachment of ovine trophectoderm cells that is CRD-independent

Other galectin family members that lack an RGD recognition sequence modulate cell adhesion by integrin binding via the CRD sequence [135]. Studies of other galectins have shown that ovine LGALS15 has a predicted CRD [173, 237]. The CRD is a consensus motif that consists of 13 amino acids [238], 8 of which (H.N.R..V.N..W..E.R) play a critical role in binding to sugars [239, 240]. Compared to the conserved CRD of other galectins, ovine endometrial LGALS15 has four residues that are identical (V62, N64, W71, E74) and three that are conservatively substituted (R54, W56, K76) (**Figure 4.1**). The C57 residue in ovine LGALS15 is different from prototypical galectins, but appears to allow binding of mannose in LGALS10 [241]. In order to determine if the CRD plays a role in the cell attachment function of LGALS15, each of the eight predicted residues forming the putative CRD in ovine LGALS15 were mutated to alanine using a PCR-based mutagenesis strategy. The recombinant protein was used for oTr cell attachment assays. In comparison to native LGALS15, there was an increase in oTr cell attachment in wells precoated with 100 ng LGALS15 CRD mutant, whereas there was a decrease in oTr cell attachment in wells precoated with 10 μg LGALS15 CRD mutant compared to wildtype LGALS15 (**Figure 4.6 A and B**). In order to

determine if the attachment function of the LGALS15 CRD mutant was dependent on integrin binding, competitive inhibition assays were conducted with a cyclic GRGDS peptide. The GRGDS peptide inhibited the ability of both the recombinant native ovine LGALS15 and the LGALS15 CRD mutant to mediate oTr cell attachment, whereas the control peptide (GRADS) had no detectable inhibitory activity (**Figure 4.6 C and D**). These studies implicate integrin binding via the RGD recognition sequence in the attachment function of LGALS15 for adhesion of trophectoderm cells and suggest that the sugar binding activity of LGALS15 CRD is not a primary determinant of its cell attachment function.

TLKDGS is not a candidate novel integrin recognition sequence responsible for LGALS15 attachment function

LGALS15 has two known functional domains - the CRD and LDVRGD integrin recognition sequences. Mutation of either domain does not fully inhibit the cell adhesion function of LGALS15. The LGALS15 amino acid sequence does not contain any other known integrin recognition sequences; however, the TLKDGS sequence may be novel. To investigate if this sequence participated in cell adhesion, a cyclic peptide was constructed against the TLKDGS sequence and introduced during cell seeding in an attachment assay using oTr1 cells. The potential inhibitory effects of the TLKDGS cyclic peptides were compared to all forms of roLGALS15, but there was no inhibitory effect on wild-type LGALS15 or the CRD mutant form of LGALS15. There was,

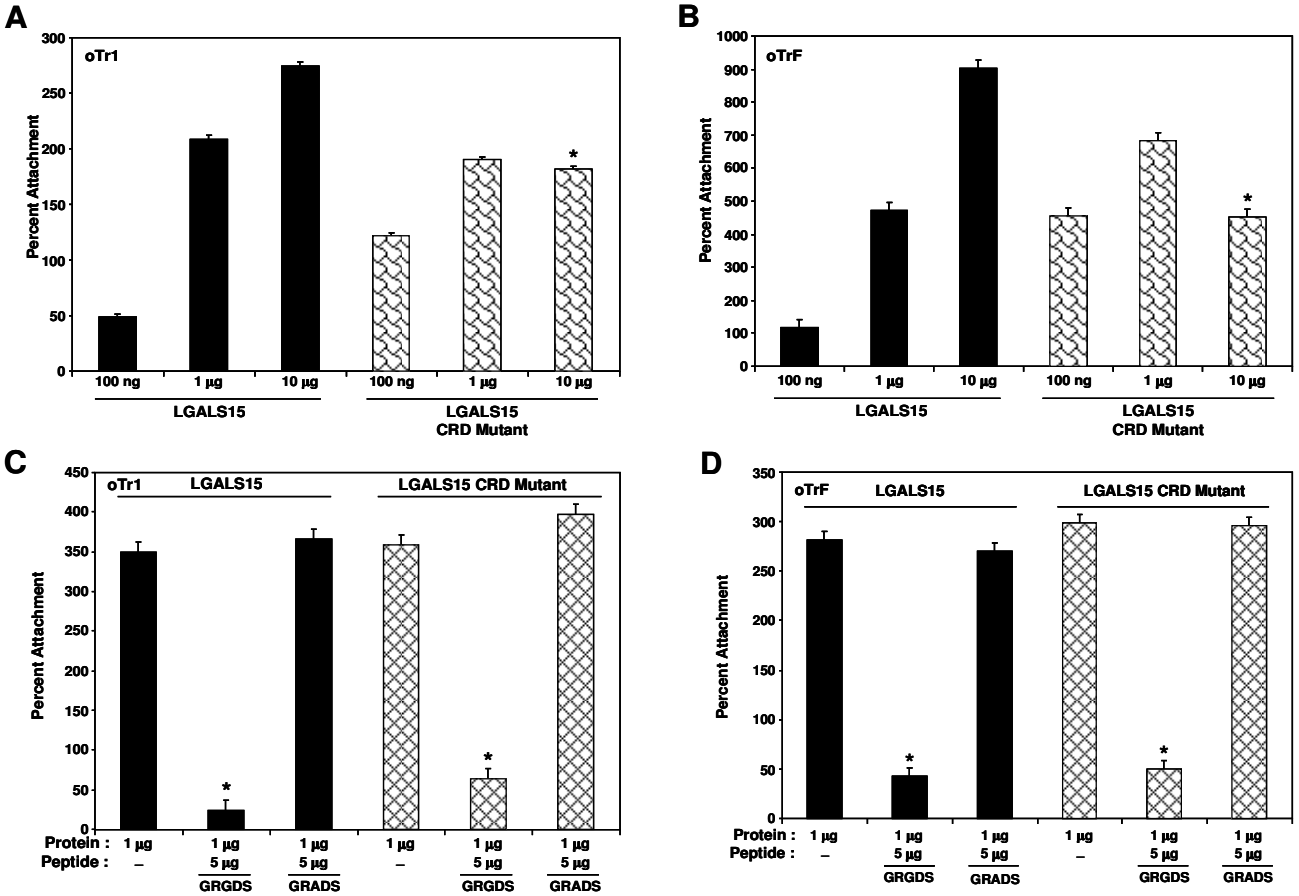

FIGURE 4.6

Effects of mutating the carbohydrate recognition domain (CRD) of ovine LGALS15 on attachment of ovine trophectoderm cells. [A & B] Wells of suspension culture pates were precoated overnight with recombinant ovine LGALS15 or the CRD mutant of LGALS15. Equal numbers of oTr cells were added to each well, and the number of attached cells determined after 1.5 h. Data is presented as percent attachment relative to BSA-coated wells. No difference in cell attachment function was noted between the CRD mutant of LGALS15 as compared to wildtype LGALS15. [C & D] Wells were precoated overnight with 1 µg of recombinant ovine LGALS15 or the LGALS15 CRD mutant. An equal number of oTr cells were added to each well along with 5 g of synthetic cyclic GRGDS or GRADS peptides. The number of attached cells determined after 1.5 h. Data is presented as percent attachment relative to uncoated wells. Note the inhibition of oTr cell attachment to both wildtype LGALS15 and the CRD mutant elicited by the GRGDS but not the GRADS peptide.

however, a decrease ($P<0.01$) in attachment in response to the LAVRAD mutated LGALS15 (**Figure 4.7**).

LGALS15-mediated trophectoderm cell attachment causes formation of focal adhesions

Results of the present studies indicate that LGALS15 possesses an intrinsic cell attachment function involving RGD-dependent binding of integrins on the trophectoderm. Activation of integrins in the trophectoderm by cell adhesion molecules with a RGD integrin recognition sequence, such as SPP1 and FN, elicits formation of focal adhesions [242]. A dose-dependent increase in oTr cell attachment occurred when glass slides were coated with recombinant ovine LGALS15, whereas no difference in oTr cell attachment occurred between control glass slides precoated with either nothing or BSA (**Figure 4.8 A**). Notably, there was an increase in oTr cell attachment and spreading on slides precoated with 10 μg compared to 1 μg LGALS15.

Next, the formation of focal adhesions was studied by visualizing talin, a focal adhesion protein that aggregates in response to integrin binding and activation essential for the stable linkage of aggregating integrins to the actin cytoskeleton, the organization of actin and the contractile apparatus, and integrin signaling [243]. Focal adhesions, visualized by punctuate aggregates of talin protein, were detected in oTr cells attaching to glass slides precoated with LGALS15 or bovine FN in a dose-dependent manner (**Figure 4.8 B**). Although some oTr cell attachment occurred on glass slides precoated with the LGALS15 LAVRAD mutant, the attached cells showed reduced spreading on the substrate and had few focal adhesions. Moreover, focal adhesions were not detected

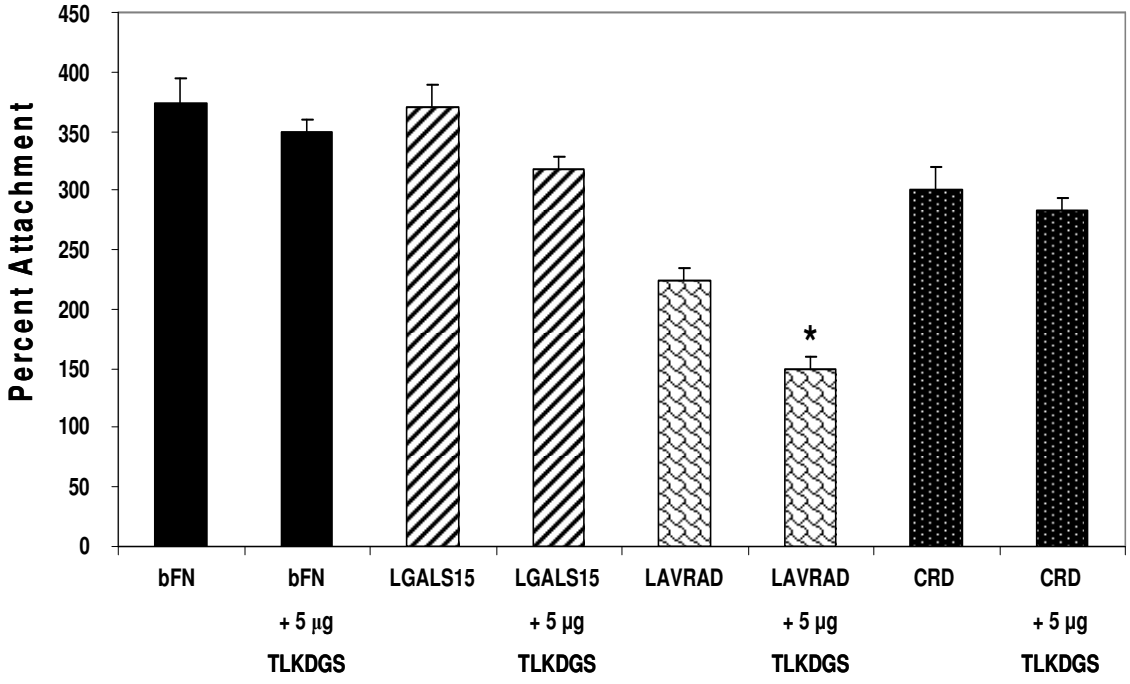

FIGURE 4.7
Effects of TLKDGS cyclic peptide on ovine LGALS15 trophectoderm cell attachment function. Wells were precoated overnight with 1 µg of recombinant ovine LGALS15. An equal number of oTr cells were added to each well along with increasing amounts of synthetic cyclic TLKDGS peptide. The number of attached cells was determined after 1.5 h. Data is presented as percent attachment relative to BSA coated wells (data not shown). Note the lack of binding inhibition of oTr cells to LGALS15 elicited by the TLKDGS peptide for all treatment groups except for LAVRAD mutated LGALS15.

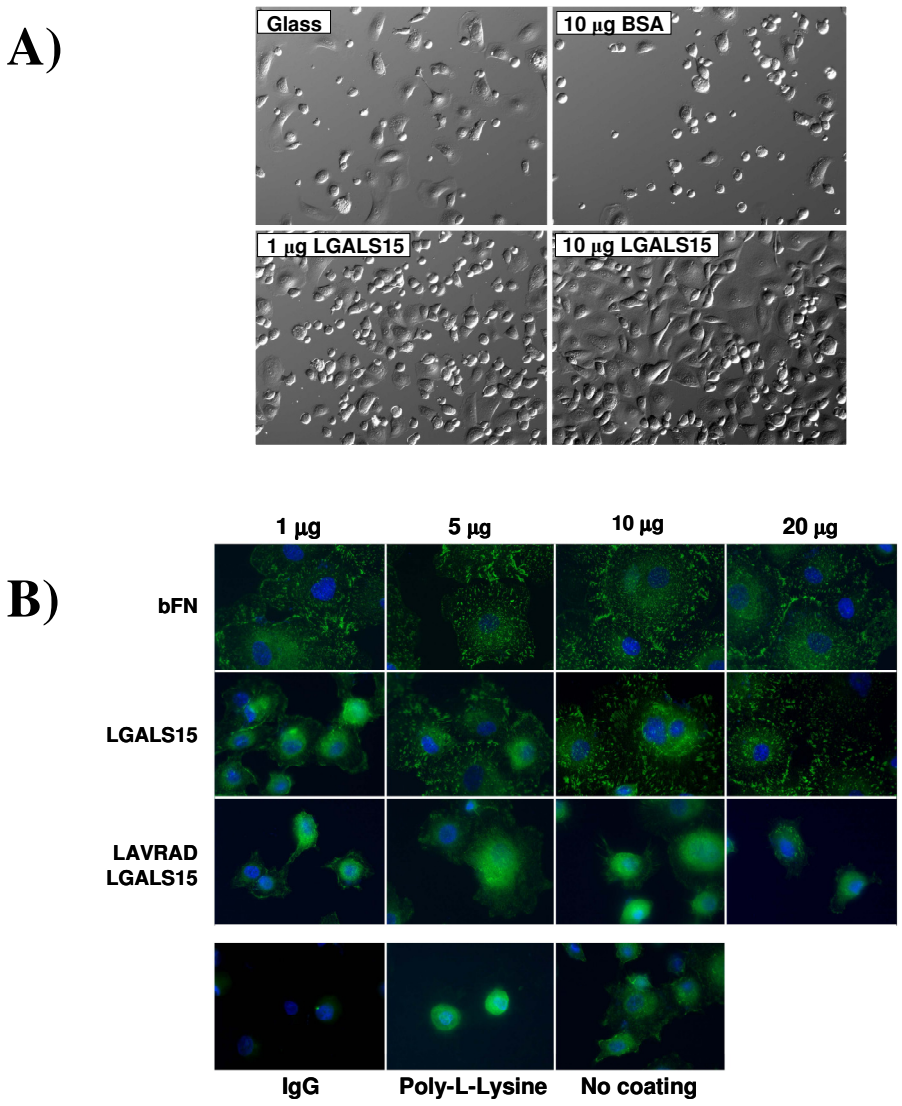

FIGURE 4.8
Effects of ovine LGALS15 on ovine trophectoderm cell attachment and formation of focal adhesions. [A] Glass slides were coated with recombinant ovine LGALS15 or BSA as a negative control. An equal number of oTr1 cells were added to each slide, and the slides were gently washed to remove unattached cells after 1.5 h. Note the increase in oTr1 cell attachment and spreading in wells coated with LGALS15 as compared to BSA. [B] Glass slides were coated with bovine FN (bFN) as a positive control, wildtype LGALS15, or the LAVRAD mutant of LGALS15. An equal number of oTr1 cells were added to each slide, and the slides were gently washed to remove unattached cells after 1.5h. Cells were then fixed, and immunoreactive talin visualized by immunoflourescence (green). Nuclei were stained with DAPI (blue) before visualization. Note that numerous aggregates of talin, an indicator of focal adhesion formation, was observed in cells attached to bFN and LGALS15, but not in cells attached to the LAVRAD mutant of LGALS15 or poly-L-lysine as a negative control.

in non-specifically attached oTr cells on glass slides coated with either nothing or poly-L-lysine as a negative control. The accumulation of talin indicates functional integrin activation as well as cytoskeletal reorganization in response to attachment of oTr cells to LGALS15 and recognition of the RGD sequence.

DISCUSSION

The results represent the first comprehensive analysis of the biological functions of LGALS15. Ruminant blastocysts will not fully elongate in culture, but will elongate if transferred to the uterus in domestic animals [244]. Parallel increases in proliferation, migration and attachment of trophectoderm cells are presumed requirements for blastocyst elongation in uteri of ruminants [217, 245-247]. The onset of blastocyst elongation on Day 12 in sheep is correlated with the induction of *LGALS15* in the endometrium by progesterone between Days 10 and 12. The onset of conceptus implantation is correlated with further increases in *LGALS15* by trophectoderm-derived IFNT and its presence within the uterine lumen between Days 14 and 16 [173]. Indeed, the total amount of LGALS15 protein recovered from the uterine lumen on Days 12 to 16 of pregnancy in sheep ranges from 1 to 20 μg (J.L. Farmer and T.E. Spencer, unpublished result). Results of the present study support the hypothesis that LGALS15 possesses an intrinsic ability to bind and activate integrins on trophectoderm cells that, in turn, stimulates their attachment. Further, the biological activities of LGALS15 are not mediated by the CRD sequence, but by integrin recognition sequences such as RGD in the C-terminus region of the protein.

In the present studies, LGALS15 mediated attachment of trophectoderm cells and formation of focal adhesions via binding and activation of integrins, which is an essential element of blastocyst implantation and trophoblast differentiation in many species [235, 236]. Integrins are proposed to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation in mammals [236, 248]. During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on apical surfaces of conceptus trophectoderm and endometrial LE [242]. Thus, conceptus implantation in sheep does not appear to involve temporal or spatial changes in integrin expression [242], but may depend primarily on changes in expression of secreted integrin ligands, such as LGALS15 and SPP1 [102, 249, 250]. Various adhesion molecules function sequentially, or in parallel, to stabilize adhesion of the trophectoderm to the endometrial LE [235, 242, 250].

LGALS15 is a candidate integrin bridging ligand in the uterine lumen during the peri-implantation period [173, 199, 223]. Although trophectoderm cells do not express *LGALS15* mRNA, LGALS15 protein accumulates in the uterine lumen and is present at the surface of these cells to act via integrin receptors [173, 199]. Within the uterine lumen, LGALS15 forms multimers on Days 14 and 16 of pregnancy, which could increase bridging of integrins expressed on endometrial LE and conceptus trophectoderm [199]. The cell attachment function of LGALS15 is due to sequences that mediate integrin recognition, such as the RGD, rather than the CRD. In the present studies, mutation of the CRD had little effect on the cell attachment function of LGALS15, which remained RGD-dependent in the CRD mutant. Other galectins, which

do not display a conserved RGD recognition sequence, can bind and activate integrins via their CRD sequence and bind fibronectin and laminin because these ECM proteins are modified with beta-galactoside sugars [13, 227]. Indeed, LGALS15 has little or no binding affinity for classical beta-galactosides (J.L. Farmer and T.E. Spencer, unpublished results). Thus, the cell attachment function of LGALS15 is most dependent on RGD recognition sequences because the cyclic GRGDS peptide inhibits its cell attachment function. Mutation of the LDVRGD recognition sequence in the C-terminus to LAVRAD did not affect LGALS15 cell attachment function when wells were coated with low amounts of LGALS15 protein, but did reduce trophectoderm attachment to wells coated with high amounts of LGALS15 protein. Further, the LDV recognition sequence of LGALS15 is not likely important because natural polymorphic variants of LGALS15 with LVV instead of LDV sequences in the C-termini of sheep and goat LGALS15 did not alter cell attachment functions [187].

One interpretation of results of the present studies is that LGALS15 has an integrin binding sequence(s) separate from the classical RGD sequence in the C-terminus, but recognizes the same site on integrins as the RGD sequence. This putative and unknown recognition sequence is functional in trophectoderm cell attachment assays using low amounts of LGALS15 LAVRAD mutant protein, but is inhibited when higher amounts of protein are used due to increased availability of the dysfunctional RAD sequence in the LGALS15 LAVRAD mutant. The cyclic GRGDS peptide inhibited cell attachment function of the LGALS15 LAVRAD mutant, and cell attachment functions of both wildtype LGALS15 and LGALS15 CRD mutant were inhibited by the GRGDS

peptide, but not the GRADS peptide. A candidate integrin binding site, TLKDGS, was investigated and cyclic peptides were made against this sequence, but addition of the cyclic peptides did not inhibit cell attachment. Residual attachment may be due to an unknown integrin recognition sequence located elsewhere in LGALS15. Although LGALS15 does not have another obvious conserved integrin recognition sequence, many cell adhesion molecules, such as SPP1 and FN [250, 251], have cryptic non-RGD integrin recognition sequences in addition to the conserved RGD recognition sequence [229].

Binding of integrins to ECM proteins promotes the aggregation of integrins and induces a hierarchical response leading to transmembrane accumulation of cytoskeletal proteins. Over 150 signal transduction molecules may be recruited to the β-integrin subunit cytoplasmic domain [131, 252] for assembly into well-developed aggregates composed of ECM proteins, integrins, and cytoskeletal proteins known as focal adhesions [252, 253]. Attachment of the c-Src substrates, tensin and focal adhesion kinase can result from integrin aggregation alone, but aggregation of cytoskeletal proteins including talin, α-actinin, vinculin and F-actin requires ligand occupancy and integrin aggregation [252]. Therefore, immunodetection of aggregated integrins, talin or α-actinin at focal adhesions, provides a sensitive functional index of integrin activation and outside-in signaling. The studies reported here exploited the ability of LGALS15 to induce focal adhesions by integrin-ECM interactions to demonstrate functional integrin activation and cytoskeletal reorganization in conceptus trophectoderm cells in response to LGALS15 binding. Accumulation of talin was detected at the interface between

LGALS15-coated slides and ovine trophectoderm cells. The focal adhesions resulted from RGD-integrin interactions because mutation of the LGALS15 RGD sequence clearly eliminated cytoskeletal aggregation of talin, although the identity of activated integrins remains unknown. Interestingly, αv and β3 integrin subunits that form the αvβ3 receptor capable of binding multiple matrix proteins, including SPP1, vitronectin and fibronectin, aggregate at sites of cell anchorage to the substrate in both LE and trophectoderm cells, suggesting the presence of this versatile receptor at focal adhesion sites during the peri-implantation period [242]. It is, therefore, reasonable to predict that in the pregnant ovine uterus, LGALS15 binding to integrin heterodimers induces focal adhesion sites that promote trophoblast elongation and stabilize attachment of trophectoderm to LE for implantation.

In summary, the temporal and spatial alterations in *LGALS15* mRNA and protein in endometrial LE and lumen of the ovine uterus during pregnancy, combined with the functional aspects of LGALS15 discovered in the present studies, support the hypothesis that LGALS15 functions as a heterotypic cell adhesion molecule bridging integrins in the endometrial LE and conceptus trophectoderm. These biological functions are undoubtedly required for growth and elongation of ruminant blastocysts/conceptuses prior to implantation *in utero*. Of particular note, the *LGALS15* gene is present in ruminants (cattle, sheep and goats) only, but is uniquely expressed in uterine endometria of members of the subfamily Caprinae (sheep and goats). Other galectin family members are expressed in the endometria and placentae of other mammals where they

may affect endometrial differentiation as well as blastocyst implantation and trophoblast differentiation [162, 254, 255].

CHAPTER V

GALECTIN 15 (LGALS15): A MULTIFUNCTIONAL PROTEIN

INTRODUCTION

A new member of the galectin superfamily, LGALS15, was recently discovered in the ovine intestinal epithelium as being induced in response to infection by *Haemonchus contortus*, a common nematode parasite which infects ruminants [196]. LGALS15 was later discovered to be a major component of histotroph in the ovine uterus which is a complex mixture of adhesion proteins, transport proteins, ions, growth factors, hormones, proteases, protease inhibitors, amino acids and other molecules [7, 173, 220]. Histotroph is necessary for conceptus (embryo/fetus and associated placental membranes) survival, growth and implantation in mammals as seen in experiments utilizing the UGKO ewe which cannot maintain pregnancy past Day 14 due to the absence of uterine glands, reduced luminal epithelium (LE) and insufficient histotroph [7, 92, 119, 191, 220, 222].

Expression of LGALS15 is detected after Day 10 of pregnancy in the endometrial LE and sGE and was induced by P4 and stimulated by IFNT, the pregnancy recognition signal produced by the conceptus [50, 256]. In the endometrium, LGALS15

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has a nucleocytoplasmic distribution within the LE and sGE and was also concentrated near and on the apical surface of those cells [173]. Further, secreted LGALS15 is abundant in histotroph recovered from the uterine lumen and is immunologically similar to the previously unknown 14 kDa protein that is a component of crystalline inclusions in endometrial epithelia and conceptus trophectoderm [23, 173].

Galectins are small proteins, ranging from 14 to 35 kDa, which bind beta-galactosides and functionally cross-link glycoprotein and glycolipid receptors on the surface of cells, to initiate biological responses that include cell proliferation, differentiation, motility, adhesion and apoptosis [13, 15, 134]. Most members of the galectin superfamily possess a CRD functional domain; however, ovine LGALS15 has a CRD and both LDV and RGD functional integrin recognition sequences [173]. The temporal and spatial expression patterns for LGALS15 mRNA and protein in uterine endometrial epithelia and in the uterine lumen during the peri-implantation period of early pregnancy, combined with known biological activities of other galectins, make it a strong candidate mediator of conceptus–endometrial interactions during implantation [173]. In fact, LGALS15 stimulates ovine trophectoderm (oTr) cell adhesion via its RGD integrin recognition sequence and increases in size and number of focal adhesions [198]. Integrin subunits that form the αvß3 receptor, which has a high affinity for the RGD sequence and is capable of binding multiple matrix proteins including SPP1, vitronectin, and fibronectin, aggregate at sites of cell anchorage to the substrate in both LE and trophectoderm, suggesting the presence of this versatile receptor at focal adhesion sites during the peri-implantation period [242]. Therefore, it is reasonable to

predict that in the pregnant ovine uterus, LGALS15 binding to integrin heterodimers induces focal adhesion sites that promote trophoblast elongation and stabilize attachment of trophectoderm to the LE for implantation [198].

While LGALS15 appears to function primarily as an adhesive protein in the ovine uterus at the time of conceptus implantation, it likely has other biological functions important to conceptus growth and survival since all galectins have a variety of affects on cells depending on the cell type and circumstances. For instance, galectin-1 (LGALS1) can either stimulate or inhibit cell proliferation [257-259] in addition to stimulating or inhibiting cell adhesion to extracellular matrix [148, 260]. Glectins can also simultaneously mediate distinct intracellular and extracellular functions. Indeed, over-expression of galectin-1 (LGALS1) in 293T cells increased membrane-associated Ras, Ras-GTP, and increased phosphorylation of ERK, resulting in cellular transformation [261]. Galectin 3 (LGALS3) also binds intracellular Ras to stimulate and/or inhibit ERK activation [261] which suggests that both Ras and ERK are potential targets of LGALS15. Both LGALS1 and galectin-3 (LGALS3) have been implicated in pre-mRNA splicing and regulation of cellular apoptosis [15, 140, 262] and recent studies have focused attention on possible effects of galectin on immune responses. For example, LGALS1 and galectin-9 (LGALS9) can induce apoptosis of activated T-cells by binding to cell surface oligosaccharides [263-267], LGALS3 can activate neutrophils [268, 269], and LGALS9 is a potent and specific chemoattractant for eosinophils [270]. Galectin-10 (LGALS10, also known as Charcot Leyden Crystal protein) and galectin-13 (LGALS13, also known as Placental Protein 13 and PP13), the closest family members

of LGALS15, have been implicated chemotaxis of immune cells to the uterus and placental development [164, 168]. Similar to LGALS15, LGALS10 exists in crystals in ovine uterine LE and trophectoderm [22, 95, 165].

The pleiotropic nature of members of the galectin family suggests that LGALS15 may stimulate oTr cell adhesion and have other functional roles. Results from the present studies indicate that LGALS15 affects oTr cell migration, proliferation, and apoptosis which are important for conceptus implantation, as well as hemagglutination, carbohydrate binding, and stimulation of differential gene expression; three functions common amongst galectin family members.

The current working hypothesis is that LGALS15 is secreted as a component of histotroph by LE and sGE throughout pregnancy in sheep where it functions to stimulate oTr cell migration, and proliferation, but inhibits oTr cell apoptosis in order to stimulate conceptus growth and elongation. Additional studies investigated functions common to other member of the galectin superfamily to determine their ability to regulate ERK activation and stimulate erythrocyte hemagglutination as a defense mechanism against parasite infection. In addition, possible roles of carbohydrate ligands were investigated through hybridization of recombinant LGALS15 protein to a glycan array and LGALS15 effects on differential gene expression was investigated using a bovine oligo DNA array.

MATERIALS AND METHODS

Preparation of recombinant ovine LGALS15

The entire coding sequence for ovine and caprine endometrial *LGALS15* mRNAs [173] was used to produce recombinant ovine and caprine LGALS15 (both LDV and LVV polymorphic forms) in bacteria. PCR reactions (50 µl) were conducted in Optimized Buffer F (Invitrogen, Carlsbad, CA) and contained 10 ng of ovine LGALS15 cDNA from Day 14 pregnant endometrium, 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 for affinity purification. The resulting plasmid was sequenced to ensure no mutations were present in the *LGALS15* sequence.

Recombinant ovine endometrial LGALS15 protein was produced in BL21 bacteria according to the manufacturer's suggestions. Expression of His-glaectin15 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 (Invitrogen). 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 using a rabbit anti-ovine LGALS15 antibody. Fractions containing recombinant LGALS15 were dialyzed overnight in PBS (pH 7.2) at 4°C, concentrated using a spin column with a 3,500 MWCO (Vivaspin, Stonehouse, UK) and frozen in aliquots at -80°C. Protein concentration was determined using a RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

Isolation and culture of mononuclear ovine trophectoderm (oTr) cells

All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A&M University. As described previously [204, 205], mature Suffolk-type ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 days). At estrus, ewes were mated to intact rams. The conceptuses were recovered on Day 15 post-mating by flushing the uterus with 20 ml of sterile PBS (pH 7.2) supplemented with 100 U penicillin and 100 µg streptomycin per liter. The inner cell mass was removed from the conceptus whenever possible, and the remaining tissues were carefully minced, pooled, and placed in a trophoblast growth medium consisting of DMEM/F-12 supplemented with 10% fetal bovine serum, glutamine (2 mM), insulin (700 nM), pyruvate (1.0 mM), non-essential amino acids (0.1 mM), and antibiotics (50 U penicillin, 50 µg streptomycin). The tissues

were maintained in a 5% CO_2 environment at 37°C. Two different ovine trophectoderm cell lines were established and designated as oTr1 and oTrF. The oTr1 cell line was established in tissue-culture treated plastic dishes, whereas the oTrF cell line was established on collagen-coated plastic dishes (Cohesion, Palo Alto, Ca). Fluid-filled trophoblastic vesicles, which spontaneously developed in culture, were physically ruptured with sterile 28-gauge needles to enhance the generation of a cellular monolayer. This primary culture was propagated on the same support by serial trypsinizations. All experiments were performed with both oTr1 and oTrF cell lines unless otherwise indicated.

Migration assay

Migration assays were conducted as described previously with minor modifications [271]. Briefly, oTr cells (50,000 cells per 100 µl serum and insulin-free trophoblast growth medium) were seeded on 8 µm pore Transwell inserts (Corning Costar #3422, Corning, NY). Treatments were then added to each well (n=3 wells per treatment) that included combinations of: (a) serum and insulin-free trophoblast growth medium (600 µl); (b) recombinant ovine LGALS15 at either 100 ng, 1 µg or 10 µg in serum and insulin-free trophoblast growth medium; (c) 1 µg/ml recombinant ovine LGALS15 with either 10, 50 or 100 µM 264 Y27632 (Rho-kinase inhibitor (ROCK) inhibitor, Catalog #688001, Calbiochem, San Diego, 265 CA) in serum and insulin-free trophoblast growth medium; (d) 1 µg/ml recombinant ovine LGALS15 with either 10, 50 or 100 µM cell permeable JNK (c-Jun N-terminal kinase) inhibitor (JNKI1; Catalog

#159-600, Alexis, San Diego, CA) in serum and insuli-free trophoblast growth medium; or (e) complete trophoblast growth medium as a positive control. After 12 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of cells that migrated onto the lower surface, inserts were fixed in 50% ethanol for 5 min. The Transwell membranes were then removed, placed on a glass slide with the side containing cells facing up, overlaid with Prolong antifade mounting reagent with DAPI, and overlaid with a cover slip (Invitrogen-Molecular Probes, Eugene, OR, USA). The migrated cells were systematically counted using a Zeiss Axioplan 2 fluorescence microscope with Axiocam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY). The entire experiment was repeated at least three times with different passages of oTr cells.

Proliferation assay

Trophectoderm proliferation assays were conducted as described previously with minor modifications [204]. Briefly, oTr cells were subcultured into 12-well plates (Corning Costar #3513, Corning, NY) to about 50% confluency in trophoblast growth medium for 6 to 8 h and then switched to serum and insulin-free trophoblast growth medium for 24 h. After 24 h, the wells (n=4 per treatment) were treated with either increasing amounts of recombinant LGALS15 (10 ng, 100 ng, 1 μg, or 10 μg) in serum and insulin-free trophoblast growth medium, complete trophoblast growth medium as a positive control, or serum and insulin-free trophoblast growth medium alone as a negative control. After 48 h of culture, cell numbers were determined as described

previously [234]. Briefly, medium was removed from cells by vacuum aspiration and cells were fixed in 50% (v/v) ethanol for 30 min followed by vacuum aspiration of the fixative. Fixed cells were stained with Janus Green B in PBS (0.2% [w/v]) for 3 min at room temperature. The stain was removed using a vacuum aspirator, and the whole plate was sequentially dipped into water and destained by gentle shaking. The remaining water was removed by shaking, and stained cells were immediately lysed in 0.5N HCl and absorbance readings taken at 595 nm using a microplate reader. As described previously [234], cell numbers were calculated from absorbance readings using the formula [cell number = (absorbance-0.00462)/0.00006926]. The entire experiment was repeated at least three times with different passages of oTr cells.

Apoptosis assays

Four-well chamber slides (Nunc, Rochester, NY) were seeded in triplicate with oTr cells at approximately 60% confluency in serum and insulin-free trophoblast growth medium and incubated for 24 h. Fresh serum and insulin-free trophoblast growth medium containing recombinant ovine LGALS15 (100 ng, 1 μg, or 10 μg) was added to each well and cultured for another 24 h. After 24 h, the medium was removed and fresh serum and insulin-free trophoblast growth medium containing staurosporine and original amounts of LGALS15 was added to each well (LC Laboratories, Woburn, MA) and cells cultured for another 24 h [272]. Cells were then fixed in 4% (w/v) paraformaldehyde in PBS and analyzed for apoptosis using the *In Situ* Cell Death Detection Kit (Roche, Nutley, NJ). Apoptotic nuclei were quantified using a Zeiss Axioplan 2 fluorescence

microscope with Axiocam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY). The entire experiment was repeated at least three times with different passages of oTr cells.

Hemagglutination assays

Hemagglutination assays were performed using 96-well conical microtiter plates as described previously [273, 274]. Briefly, blood samples from sheep, pig and mouse were collected into heparinized tubes. Erythrocytes were isolated by centrifugation at 16,000 x g for 10 min, serum removed, washed six times with sterilized phosphate buffered saline (PBS; pH 7.4) to remove traces of heparin, and resuspended in 1% (v/v). PBS. Recombinant LGALS15 (100 µl of 1 µg/µl stock) was diluted (two-fold) in PBS (pH 7.4) to create serial dilutions ranging from 1:2 to $1:2^{15}$. Erthyrocyte suspensions (100 µl) were then added to each well and incubated overnight at room temperature. Wells containing only saline and erythrocytes, but no LGALS15 were used as the negative control. Wells containing PBS, erythrocytes and 100 µl Concanavalin A (0.1% w/v), a well- established promoter of hemagglutination, served as the positive control.

Production of rabbit antibodies to ovine LGALS15

Rabbit antisera to ovine LGALS15 were commercially produced using recombinant ovine LGALS15 protein as the antigen. Rabbit anti-ovine LGALS15 IgG was purified from serum of immunized rabbits using an ImmunoPure (A/G) IgG Purification kit (PIERCE, Rockford, IL).

Western blot analyses

Ovine erythrocytes were incubated with LGALS15 (100 µl of 1 µg/µl stock) overnight at 4°C with gentle agitation. Whole cell extracts were prepared as described previously [275]. Briefly, cells were rinsed with cold PBS and lysed by incubation in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.2 mM phenylmethylsulfonylfluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min at 4°C. Cell lysates were passed through a 26-gauge needle and clarified by centrifugation (16,000 x g, 15 min, 4°C). The protein content was determined using the RC/DC protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using SDS-PAGE, and transferred to nitrocellulose. Blots were blocked for 1 h at room temperature with either 5% BSA-TBST (5% wt/vol BSA, Tris-buffered saline, 0.1% Tween-20) for phospho-specific antibodies or 5% nonfat milk-TBST for all other antibodies. Primary antibodies were diluted in 2.5% nonfat milk-TBST and incubated at 4°C overnight. Primary antibodies included rabbit anti-mouse ERK 1/2 polyclonal IgG (Santa Cruz, CA) rabbit anti-mouse phospho-ERK1/2 monoclonal IgG (Santa Cruz, CA) at a 1:200 dilution and rabbit anti-ovine LGALS15 IgG at a 1:10,000 dilution. Western blot analyses were performed as described previously [276] using enhanced chemiluminescence detection (Super Signal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to manufacturer's recommendations. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Western blots were

quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA).

Carbohydrate array

Recombinant LGALS15 (1 ml of 1 mg/ml stock) was sent to the Protein-Carbohydrate Interaction Core at Emory University School of Medicine for analysis in with a carbohydrate array. The Protein-Glycan Interaction Core (H) utilizes Consortium Printed Array Slides (Schott Cat. No. 1070936B) with 406 different glycans. Glycan spotting concentration was at 100 μM per glycan with six replicate spots per glycan. Slides were printed at the Consortium Carbohydrate Synthesis/Protein Expression Core (D) located at the Scripps Research Institute. Preprinted slides are soaked in deionized water for 5 min at room temperature and dried under a stream of nitrogen. The galectin sample was diluted to assay concentration in binding buffer before 50 μl of FITC labeled lectin was applied to the printed surface and coverslipped. Slides were protected from light in a humidified chamber for 1 h at room temperature, washed and dried. The binding image was read in a Perkin Elmer Microscanarray XL4000 scanner and image analysis performed using Imagene (V.6) image analysis software.

LGALS15-induced gene expression in suspended versus attached oTr cells

Microarray analyses were conducted using trophectoderm cells that were either in suspension at the time of LGALS15 treatment or adhered at time of treatment. For

treatment of cells in suspension, oTr1 cells were incubated in sterile 50 ml glass bottles containing 35 ml trophoblast growth medium with either LGALS15 (1 µg/ml) or BSA (1µg/ml), as a negative control, for 12 h. Cells were kept in suspension with gentle stirring until pelleted by centrifugation at 13,000 x g for 5 min at room temperature and isolation of total RNA as described previously. The quantity and quality of total RNA was determined by spectrometry and denaturing formaldehyde-agarose gel electrophoresis, respectively. For treatment of adhered cells, equal numbers of oTr cells were plated in 100 mm dishes with trophoblast growth medium and allowed to attach over night. The following day the medium was removed and replaced with serum and insulin free trophoblast growth medium and incubated under cell culture conditions for an additional 24 h. The medium was replaced with fresh serum and insulin free trophoblast growth medium supplemented with either 1 µg/ml recombinant LGALS15 or 1 µg/ml BSA as a negative control and incubated under cell culture conditions for an additional 12 h. Total cellular RNA was extracted as stated previously. RNA extracted from oTr cells was reverse transcribed and used to screen a bovine oligo DNA array (University of Missouri) according to manufacturer's instructions (Genishpere Inc, Hatfield, PA) and using methods described previously [223].

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). 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). Percentage data were subjected to arcsin transformation before analysis. Independent variables included LGALS15 treatments and replicate. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

RESULTS

LGALS15 increases migration of trophectoderm cells via JNK

Several members of the galectin family stimulate migration of cells [227], and trophectoderm migration occurs during elongation of ruminant conceptuses [217]. Therefore, effects of LGALS15 on migration of oTr cells were determined. Recombinant ovine LGALS15 dose-dependently increased ($P<0.001$) migration of both oTr1 and oTrF cells in serum- and insulin-free media (**Figures 5.1 A and B**). Cell movement and migration can be stimulated by the planar cell polarity pathway involving activation of Rho-ROCK and JNK-JUN pathways [277]. Treatment of oTr cells with a ROCK (Rho-kinase) inhibitor, and JNK (JUN N-terminal kinase) inhibitor did not ($P>0.10$) affect basal rates of oTr1 or oTrF cell migration in the absence of LGALS15 (data not shown). However, the JNK inhibitor, but not the ROCK inhibitor, reduced ($P<0.001$) LGALS15-stimulated oTr1 and oTrF cell migration in a dose-dependent manner (**Figures 5.1 C and D**). These results support the hypothesis that LGALS15

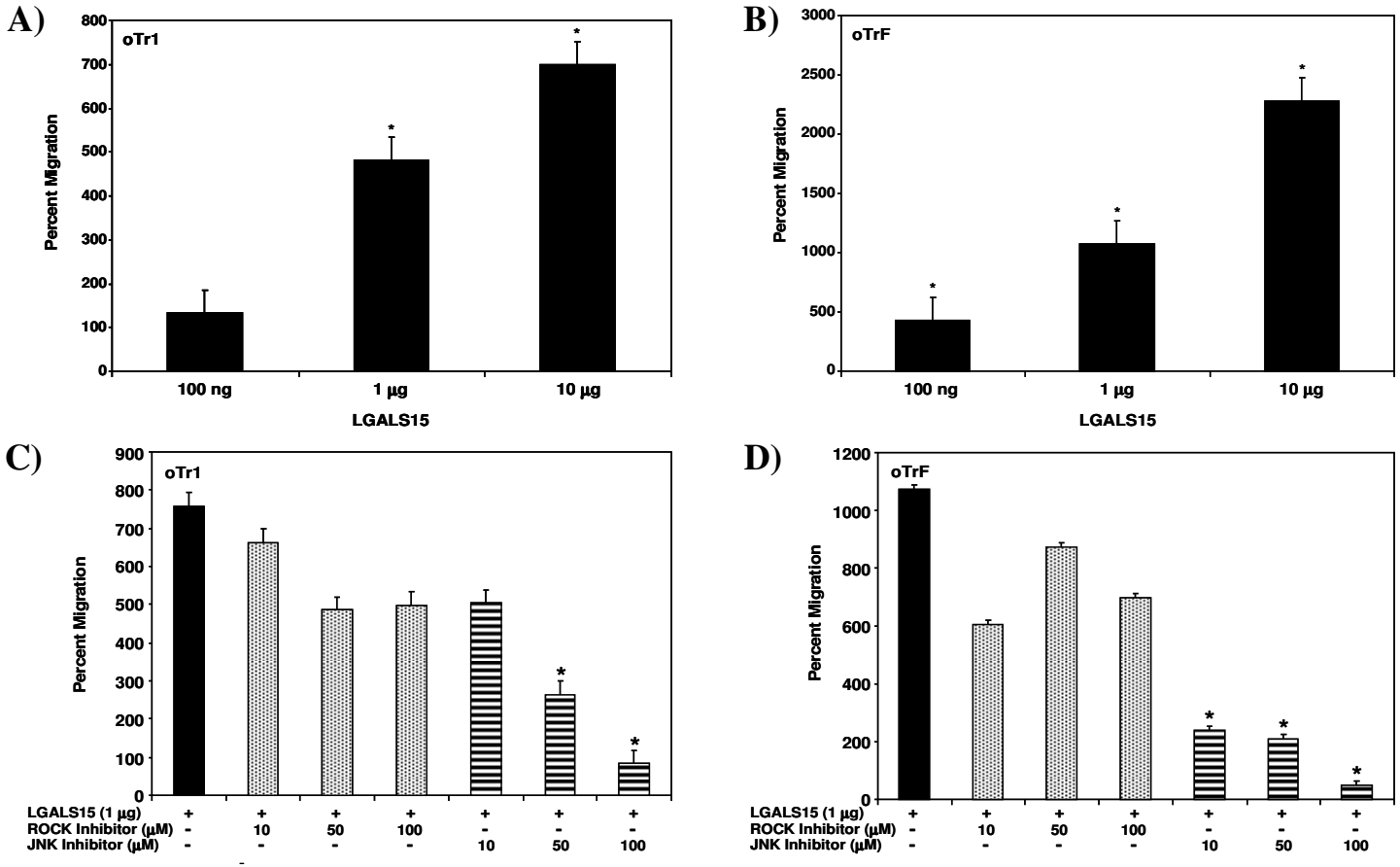

FIGURE 5.1

Effects of ovine LGALS15 on migration of ovine trophectoderm cells. [A] Cells were cultured in a Transwell plate in serum- and insulin-free media and treated with recombinant ovine LGALS15. Cell migration was determined after 8 h of treatment and presented as percent migration relative to BSA controls. An increase in oTr1 cell migration was observed at all doses of recombinant ovine LGALS15. [B] The oTr cells were cultured in a Transwell plate in serum- and insulin-free media and treated with recombinant ovine LGALS15, Y27632 (Rho-kinase (ROCK) inhibitor), a cell permeable JNK inhibitor (JNKI1), or their combination. Cells grown in serum- and insulin-containing media served as a positive control. Cell migration was determined after 8 h of treatment and expressed as percent migration relative to ovine LGALS15. Statistically significant ($P<0.001$) differences in cell migration due to treatment with LGALS15 and inhibition of cell migration by the JNKI are noted.

from the endometrium acts in a paracrine manner on ovine conceptus to stimulate trophectoderm cell migration via activation of a signaling pathway involving JNK.

Changes in ERK abundance in oTr1 cells in response to LGALS15

Treatment of oTr1 cells with LGALS15 for 20 min had a dose-dependent effect to increase ERK 1/2 protein abundance. However, treatment with as much as 10 μg LGALS15 did not affect phospho-ERK 1/2 protein abundance (**Figure 5.2**).

LGALS15 has moderate effects on trophectoderm proliferation

Several members of the galectin family affect proliferation of cells [227], and proliferation of trophectoderm cells is involved in elongation and differentiation of peri-implantation ruminant conceptuses [217]. This study determined effects of LGALS15 on oTr1 and oTrF cell proliferation in serum- and insulin-free media (**Figure 5.3**). Recombinant ovine LGALS15 did not affect ($P>0.10$) proliferation of oTr1 cells, but did stimulate oTrF cell proliferation. A 24% increase in oTrF cell numbers was detected at 100 ng LGALS15, but not at other amounts (cubic effect, $P<0.03$). In both types of oTr cells, BSA did not affect ($P>0.10$) cell number, whereas there was a 215% and 368% increase ($P<0.01$) in oTr1 and oTrF cell numbers, respectively, in response to serum- and insulin-containing trophoblast growth medium (data not shown). These results suggest that effects of LGALS15 on trophectoderm cell proliferation were dose- and cell-type-dependent.

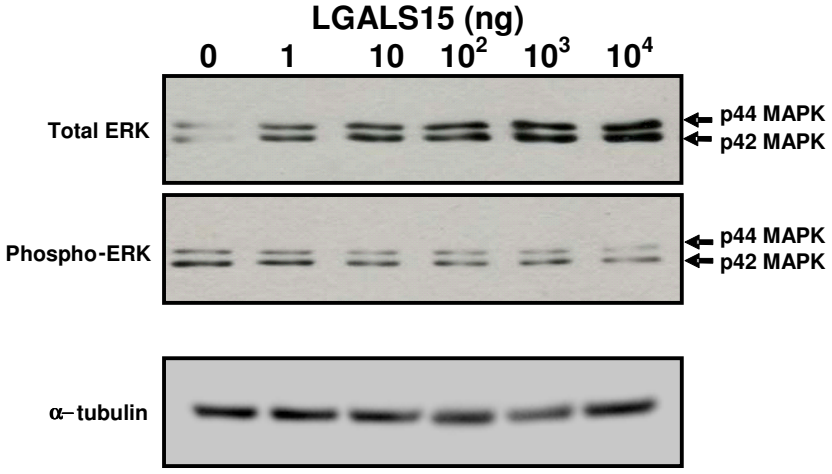

FIGURE 5.2
Effects of ovine LGALS15 treatment on total and phosphorylated ERK1/2 protein expression in ovine trophectoderm cells. oTr cells cultured for 72 hours in serum free media with increasing amounts of LGALS15 prior to protein extraction. Protein extracts were subjected to western blot analysis for total and phosphorylated 42/44 mitogen activated protein kinase.

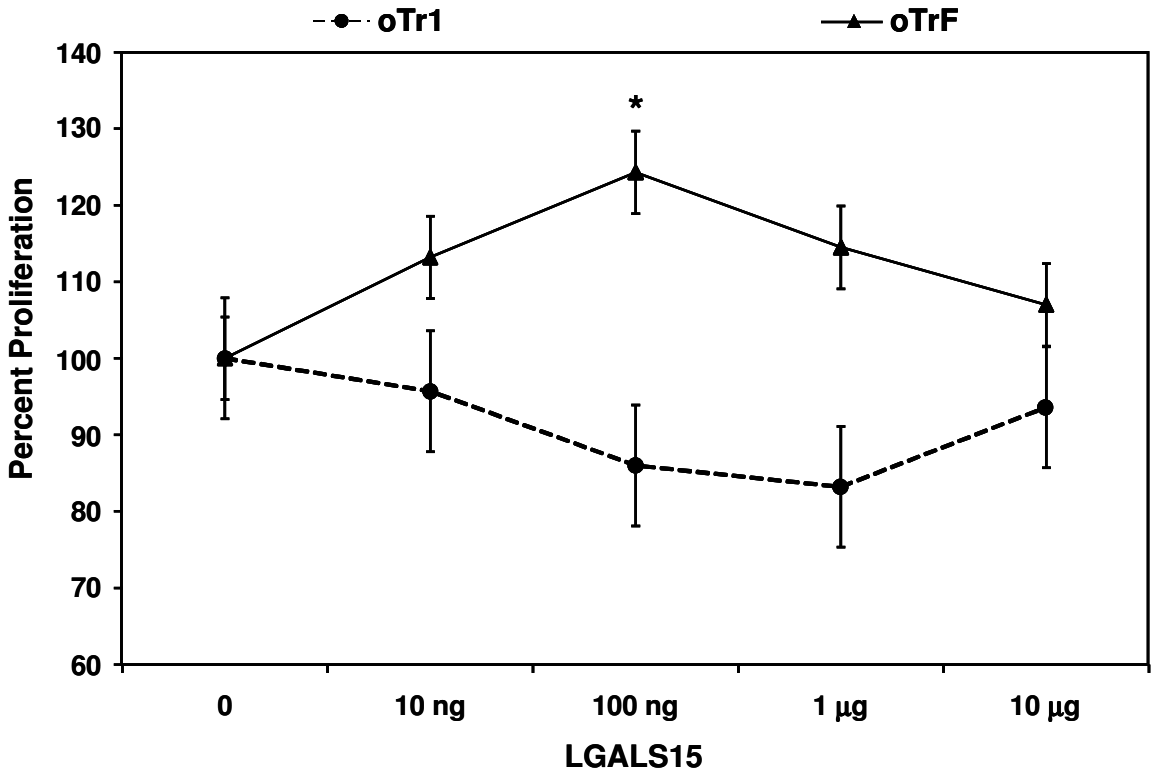

FIGURE 5.3
Effects of ovine LGALS15 on proliferation of ovine trophectoderm cells. Ovine trophectoderm (oTr) cells were treated with increasing amounts of recombinant ovine LGALS15 in serum- and insulin-free media. Cell numbers were determined after 48 h, and data are expressed relative to untreated controls (100%).

LGALS15 does not induce apoptosis, but reduces staurosporine-induced apoptosis in trophectoderm cells

Several members of the galectin family have either negative or positive effects on cell apoptosis [227]. This study examined effects of LGALS15 on apoptosis in oTr1 cells and determined that LGALS15 alone had no effect ($P>0.10$) on oTr1 cell apoptosis regardless of dose (data not shown). Next, staurosporine (*Streptomyces staurospores*), a relatively non-selective protein kinase inhibitor, was used to induce apoptosis [278]. Staurosporine induced apoptosis of oTr1 cells in a dose-dependent manner (**Figure 5.4**). Almost 100% of oTr1 cells were apoptotic at $3x10^{-4}$ M and 85% of oTr cells were apoptotic at $3x10^{-5}$ M, whereas only ~60% were apoptotic at $3x10^{-6}$ M staurosporine. At high levels of staurosporine-induced apoptosis, preincubation of oTr1 cells with recombinant ovine LGALS15 had no effect ($P>0.10$). In contrast, 10 μg LGALS15 decreased ($P<0.03$) apoptosis by ~30% in oTr1 cells incubated with $3x10^{-6}$ M staurosporine. Lower amounts of LGALS15 (100 ng or 1 μg) had no effect ($P>0.10$) on staurosporine-induced apoptosis. These results suggest that LGALS15 alone does not cause apoptosis, but LGALS15 can inhibit trophectoderm cell apoptosis induced by staurosporine in a dose-dependent manner.

Treatment of ovine and porcine erythrocytes with LGALS15 does not induce hemagglutination

LGALS15 was unable to induce hemagglutination of either ovine, porcine or murine erythrocytes. To determine if the lack of hemagluttination by LGALS15 was

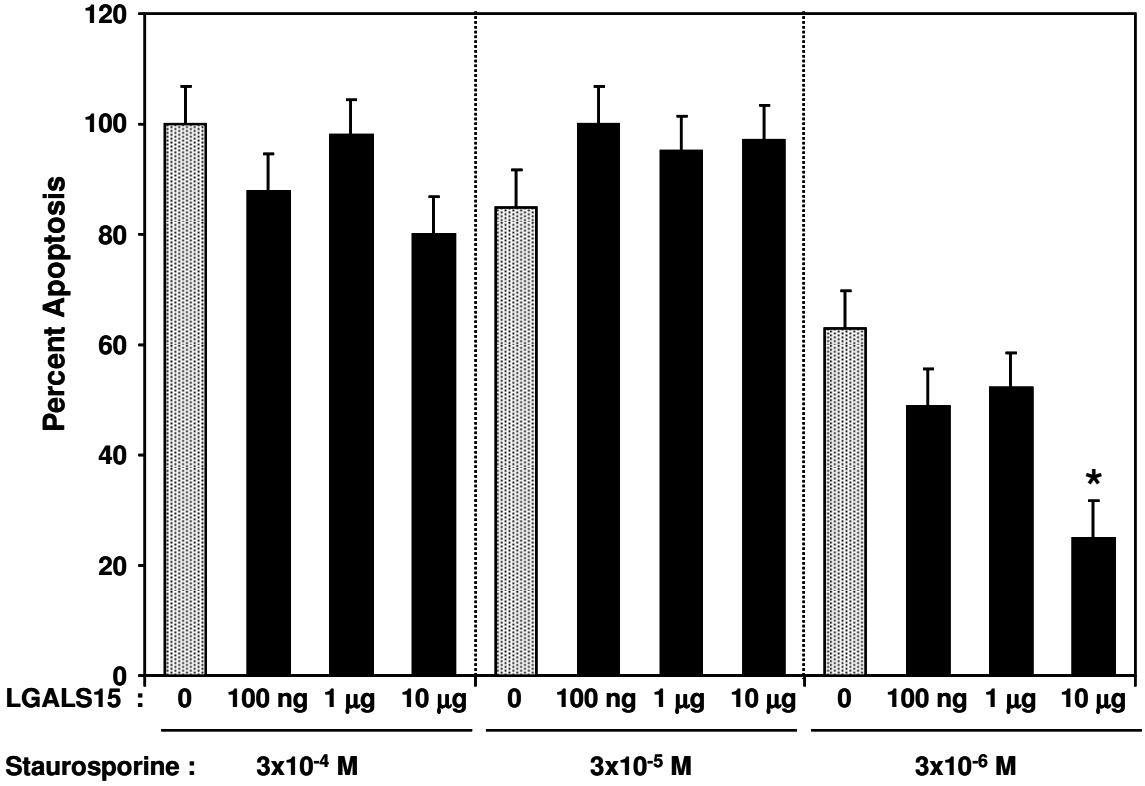

FIGURE 5.4
Effects of ovine LGALS15 on staurosporine-induced apoptosis of ovine trophectoderm cells. The oTr1 cells were exposed increasing amounts of recombinant ovine LGALS15 and then exposed to staurosporine, which is an inducer of apoptosis. High amounts of staurosporine ($3x10^{-4}$ and $3x10^{-5}$ M) induced considerable cell apoptotis that could not be inhibited by LGALS15. At the $3x10^{-6}$ M concentration of staursporine, oTr cell apoptosis was reduced and considerably decreased in cells treated with 10 µg of LGALS15, but not 100 ng or 1 µg of LGALS15.

due to its inability to either bind or cross-link ovine erythrocytes, a 1% erythrocyte solution was incubated with LGALS15 (1 μg/μl) in TBS for 2 h at room temperature. Erythrocytes were then centrifuged (16,000 x g) and the supernatants collected. Cell pellets were washed three times and protein was extracted as previously described [275]**.** Western blot analysis revealed the presence of LGALS15 in the supernatant, but not erthyrocyte protein extracts, confirming that LGALS15 was unable to bind to ovine erythrocytes (**Figures 5.5 A and B**).

LGALS15 binds multiple non-beta-galactoside carbohydrate ligands

Recombinant LGALS15 glycan binding affinity was analyzed against 406 possible carbohydrate ligands. The ligands for which roLGALS15 showed the highest affinity are listed in **Table 5.1**. LGALS15, unlike many galectin family members, does not have high affinity for beta-galactosides, but does bind mannose based sugars, a characteristic of LGALS10. Sugars of particular interest that bound to LGALS15 include Fucα1-3GlcNAcβ, manose hybrid sugars (Mana1-2Mana1-2Mana1-3Mana-Sp9), melibiose (Gala1-6Glcb-Sp8), transferrin, and ceruloplasmin. Transferrin, ceruloplasmin, and mannose hybrid sugars play important roles in iron transport in the uterus during pregnancy. Melibiose is associated with microorganism infection [279]. Fucα1-3GlcNAcβ associates with Lewis antigens present on the surface of uterine LE in sheep and goats [144]. Many sugars that bound to LGALS15, including Fucα1-3GlcNAcβ, are polyfucosylated glycans that represent novel blood group active glycopeptides unique to the small intestine as compared with glycopeptides of other

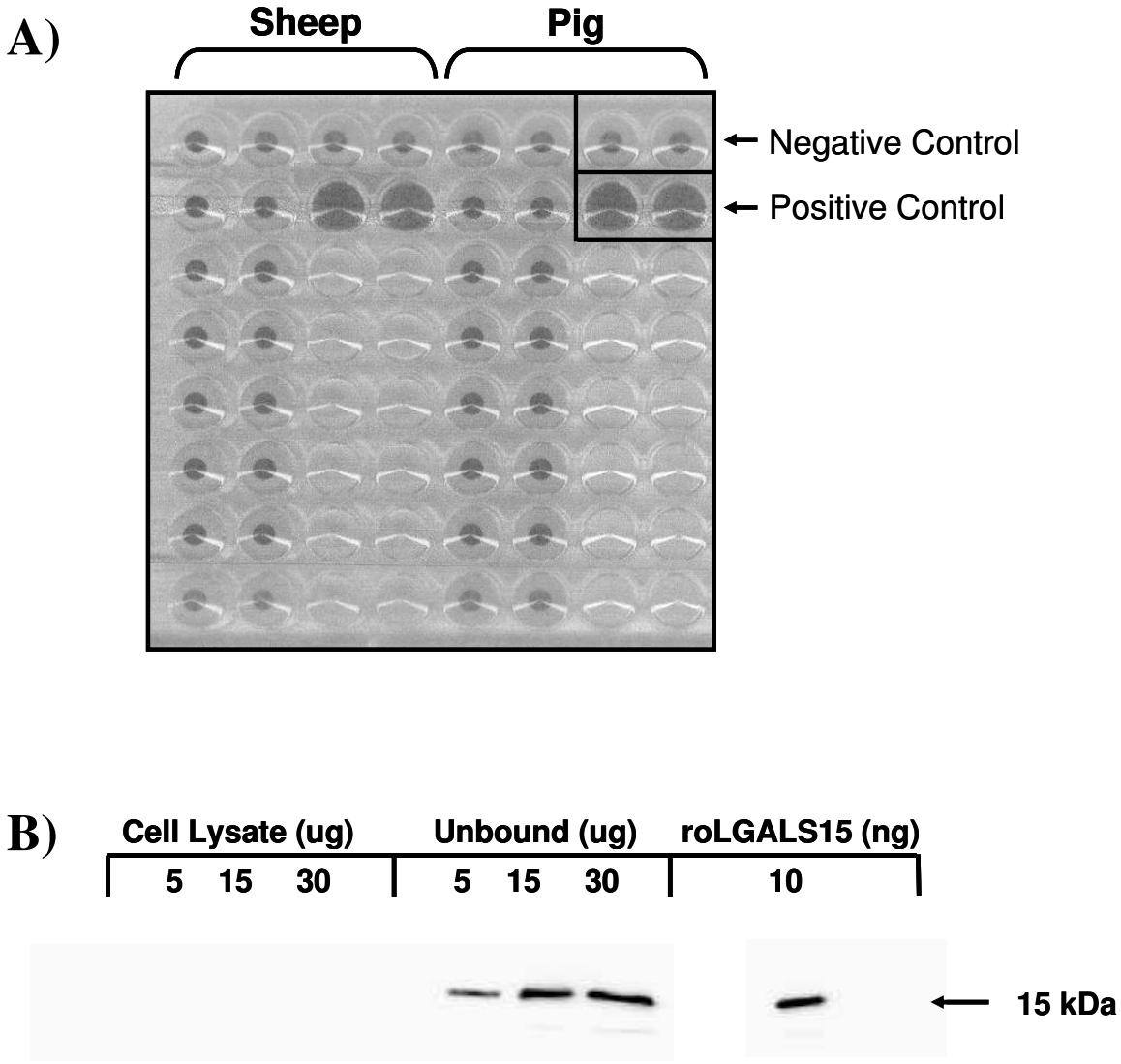

FIGURE 5.5
Effects of ovine LGALS15 on erythrocyte hemagglutination. [A] LGALS15 (100 µl of 1 µg/µl stock) was incubated overnight at room temperature with erythrocytes from sheep and pig. In both cases LGALS15 was unable to stimulate hemagglutination. [B] Sheep erythrocytes were incubated with LGALS15 (100 µl of 1 µg/µl stock) and western blot analyses performed on cellular proteins and supernatants to determine the location of LGALS15 protein.

TABLE 5.1
Binding affinities of different ligands to ovine LGALS15. Ligands with the highest affinity for LGALS15 as measured by relative fluorescent units from FITC labeled LGALS15 are shown. Standard deviation (STDEV), standard error of the mean (SEM) and the coefficient of variation (%CV) are shown.

Glycan No.	Glycan Name	Ave. RFU[1]	STDEV	SEM	%CV
76	Fucα1-3GlcNAcβ–Sp8	43118	16036	6547	37
189	Mana1-2Mana1-2Mana1-3Mana-Sp9	42258	14008	5719	33
113	Gala1-6Glcb-Sp8	26337	12732	5198	48
6	Transferrin	24695	9790	3997	40
4	Ceruloplasmin	24626	12285	5015	50
196	Mana1-3(Mana1-2Mana1-2Mana1-6)Mana-Sp9	22981	13052	5328	57
55	Fuca1-2Galb1-3GalNAcb1-3Gala-Sp9	22613	10707	4371	47
81	GalNAca1-3(Fuca1-2)Galb1-4GlcNAcb-Sp0	21707	4408	1800	20
82	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ–Sp8	19826	6802	2777	34
168	GlcNAcb1-4MDPLys (bacterial cell wall)	19215	4193	1712	22
140	Galβ1-4[6OSO3]Glcβ–Sp8	17543	17865	7293	102
80	GalNAca1-3(Fuca1-2)Galb1-4(Fuca1-3)GlcNAcb-Sp0	16879	5547	2265	33
83	GalNAca1-3(Fuca1-2)Galb1-4Glcb-Sp0	15865	9581	3911	60
84	GalNAcα1-3(Fucα1-2)Galβ–Sp8	15643	4142	1691	26
1	Alpha1-acid glycoprotein (AGP)	13632	1881	768	14
79	GalNAca1-3(Fuca1-2)Galb1-3GlcNAcb-Sp0	13252	6587	2689	50
3	AGP-B (AGP ConA bound)	12951	9593	3916	74
182	G-ol-amine	12129	19315	7885	159
2	AGP-A (AGP ConA flowthrough)	11925	4665	1905	39
26	[3OSO3][6OSO3]Galb1-4[6OSO3]GlcNAcb-Sp0	11650	9495	3876	81

[1]Relative Fluorescence Units

tissue sources and may be correlated with specialized functions of epithelial cells in the small intestine [280].

LGALS15 gene regulation in suspended and adhered oTr1 cells

A bovine DNA array detected differences in gene expression in suspended oTr1 cells compared to oTr1 cells adhered to a substrate following treatment with 1µg/ml LGALS15 (**Table 5.2**). Overall, LGALS15 affected a greater number of genes in suspended rather than adhered cells. Of the genes affected by LGALS15, a majority were increased several fold above baseline and were associated with vascular and immune function, adhesion and nutrient transport.

DISCUSSION

A wide range of biological functions have been described for galectins, including regulation of cell adhesion, migration, cell growth, apoptosis, and pre-mRNA splicing. Extracellularly, they typically bind to beta-galactosides containing glycoconjugates of ECM components and cell surface adhesion molecules. Interestingly, research over the past decade has identified a novel role for galectins as versatile regulators of cell-cell and cell-matrix interactions implicating galectins as a class of matricellular proteins [281, 282]. Matricellular proteins do not serve primarily structural roles as integral components of physical entities such as basement membranes or fibers. Rather, they function contextually as adapters and modulators of cell- extracellular matrix interactions [283]. Based on established functions of galectins and functions of

TABLE 5.2

Differential gene expression profiles for ovine trophectoderm cells exposed to exogenous ovine LGALS15 while in suspension [A] or while adhered to a substrate [B]. Total number of genes differentially expressed per treatment [C].

A)

Description	Group	Fold Increase or Decrease	Accession #
Thrombin receptor	Vascular	13.49 ↑	P25116
Heparin-binding growth factor 1	Vascular	6.51 ↑	P05230
Integrin alpha 2 precursor	Adhesion	6.09 ↑	P17301
Rho Kinase 2	Adhesion	3.97 ↑	O75116
Integrin alpha 5 precursor	Adhesion	3.72 ↑	P08648
Homeoboxprotein EMX2	Transcription	5.00 ↑	Q04743
TNF– stimulated gene 6 protein	Transcription	6.25 ↓	P98066
Homeoboxprotein Hox C-11	Transcription	3.38 ↓	O43248
IL11 Chemokine	Immune	4.66 ↑	Q9Y4X3
Immunoglobulinmu binding protein 2	Immune	4.25 ↑	P38935
IL6 receptor	Immune	4.22 ↑	P08887
CX3C chemokinereceptor	Immune	3.42 ↑	P08648
IL8 precursor	Immune	3.38 ↑	P10145
Cathepsin H	Protease	3.79 ↑	P09668
Parathyrin	Nutrient Levels	3.73 ↑	P01270
Gastric inhibitory polypeptide	Nutrient Levels	3.43 ↑	P09681

B)

Description	Group	Fold Increase or Decrease	Accession #
Parathyrin	Nutrient Transport	5.81 ↑	P01270
Sodium / Glucose Co-transporter 1	Nutrient Transport	5.26 ↑	P13866
Regulatory associated protein of mTOR	Cell growth and maintenance	3.61 ↑	Q8N122
G1/S– specificcyclin E2	Cell Clycle	5.70 ↑	O96020
Early growth response protein 2	Transcription	4.02 ↑	P11161
Homeobox protein Hox-A5	Transcription	3.98 ↑	P20719
LIF receptor	Implantation	4.93 ↑	P42702
Endothelial cell–selective adhesion molecule precursor	Cell Junctions	3.46 ↑	Q96AP7

C)

	Up - Regulated Genes	Down - Regulated Genes
Suspension	728	491
Attached	271	234

LGALS15 based on the present studies, LGALS15 may be classified as a matricellular protein. LGALS15 is a potent stimulator of oTr cell adhesion via its RGD integrin recognition sequence [198] and results of studies presented here focused on its agonistic/antagonistic roles in oTr cell migration, proliferation and apoptosis which influence trophectoderm survival and successful implantation. The ability of LGALS15 to stimulate hemagglutination, bind to numerous carbohydrates and regulate gene expression, as characteristic of the galectin superfamily members, was also investigated to understand pleiotropic functions of LGALS15.

In contrast to humans and rodents, blastocysts of domestic ruminants must elongate prior to implantation, a process requiring cellular proliferation and migration [102, 217]. The noncanonical or planar cell polarity pathway is involved in cell movement and proliferation via activation of Rho-ROCK and JNK-JUN pathways [277]. The JNK-JUN pathway is involved in a number of cellular processes including epithelial sheet migration [284]. Results of the present studies indicate that LGALS15 stimulation of migration of oTr cells is dependent on JNK, but not ROCK cell signaling. Indeed, JNK is a downstream target of integrin activation [285] involved in human trophoblast responses to placental growth factor [286]. Further, phosphorylated JNK and JUN proteins are present in ovine trophoblast cells (K. Hayashi and T.E. Spencer, unpublished result). Future studies will focus on roles of the JNK-JUN cell signaling pathways in the ovine trophoblast. Results of the present studies indicate that endometrial-derived LGALS15 acts in a paracrine manner to stimulate motility and migration of oTr cells. Trophectoderm elongation involves cell migration and

proliferation and is required for formation of the conceptus and developmentally-regulated production of IFNT for pregnancy recognition [217, 287]. In sheep, the approximately 200 μm spherical blastocyst on Day 7, begins elongation on Days 12 to 13 to form a 19-20 cm filamentous conceptus by Days 16-18 that covers the entire length of the lumen of the uterine horn ipsilateral to the corpus luteum and extends through the common uterine body into the contralateral uterine horn. Thus, blastocyst elongation undoubtedly requires an extraordinary amount of trophectoderm cell motility and migration. In fact, blastocyst elongation is compromised in the uterine gland knockout ewe model which lacks endometrial glands and has a reduced amount of LE, little or no sGE and no LGALS15 [173, 191, 223]. Collectively, available results link the induction and increase in LGALS15 from the endometrial LE and sGE to stimulation of trophectoderm migration needed for peri-implantation blastocyst elongation and formation of a filamentous conceptus.

Intracellular effects of LGLAS1 and LGALS3 are to regulate many cell signaling pathways including the p42/44 mitogen activated protein kinases (*ERK1/2*), *Ras*, tumor necrosis factor (*TNF*), nuclear factor kappa B (*NFkB*), and Wnt signaling cascades [261]. In the present study, treatment of oTr1 cells with LGAS15 increased the abundace of ERK 1/2, but not phospho-ERK 1/2. However, persistent activation of the p42/44 mitogen activated protein kinases was detected after 72 h of serum starvation suggesting some level of constitutive activation of this protein in oTr1 cells. At present, the mechanism of this activation is unclear.

Death and lysis of some trophoblast cells occurs in ovine blastocysts from Days 12 to 16 [288] and, interestingly, several members of the galectin family have both negative and positive effects on cellular apoptosis [227]. In the present study, LGALS15 alone did not stimulate apoptosis, but did partially inhibit induction of apoptosis of trophectoderm cells induced by staurosporine. Interestingly, focal adhesion kinase (FAK) dephosphorylation and focal adhesion disassembly is a very early event mediating the onset of staurosporine-induced endothelial cell apoptosis [278]. Thus, the ability of LGALS15 to counteract staursporine-induced apoptosis may be mediated its ability to bind and activate integrins involved in formation of focal adhesions [198]. Although LGALS15 is abundant in the uterine lumen during early pregnancy [173, 199, 223], it does not appear to inhibit other mechanisms leading to apoptosis of trophectoderm cells which is a common feature of conceptus development that depends on cell remodeling and/or removal of genetically deficient cells [288].

LGALS15, unlike many galectin family members, does not have high affinity for beta-galactosides. Analyses of binding affinity of LGALS15 for over 400 glycans revealed that highest affinity was for Fucα1-3GlcNAcβ, manose hybrid sugars (Mana1-2Mana1-2Mana1-3Mana-Sp9), melibiose (Gala1-6Glcb-Sp8), transferrin, and ceruloplasmin. Fucα1-3GlcNAcβ is a carbohydrate present on the surface of various parasites, such as the helminth *Schistosoma mansoni*, and is one component of the Lewis X antigen (Gal beta 1-4(Fucα1-3)GlcNAc) and Lewis A antigen (Gal beta 1-4(Fuc alpha 1-3)GlcNAc) [144, 289]. Lewis X is expressed in the LE on Days 15-17 of pregnancy and Lewis A is expressed in the endometrial stroma from Days 5 to 25 of pregnancy in

goats [144]. While it has not been shown that *Haemonchus contortus* expresses Fucα1-3GlcNAcβ, one may hypothesize that the affinity of LGALS15 for Fucα1-3GlcNAcβ affects functional roles of LGALS15 in the intestine during parasite infection and as a ligand in the uterus during pregnancy. Melibiose (Gala1-6Glcb-Sp8) is a disaccharide consisting of one galactose and one glucose moiety in an alpha (1-6) glycosidic linkage. Melibiose is a component of microorganisms known to infect the intestine and mammary glands of cattle [279]. This corresponds to possible roles for LGALS15 in the intestine of sheep infected with the helminth *Haemonchus contortus* where it was first discovered [196].

Ceruloplasmin is expressed in the liver, hypothalamus, spleen and uterus of sheep [290] and by uterine endometrium and placenta in humans and rats [291]. It has six atoms of copper in its structure and assists in copper-dependent oxidase activity, which is associated with oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron), thereby assisting in iron transports in plasma by transferrin which requires iron in the ferric state [292]. Elevated levels of ceruloplasmin are associated with infection, pregnancy and postpartum events in cows and ewes [293]. Mannose hybrid sugars (Mana1-2Mana1-2Mana1-3Mana-Sp9) are associated with transferrin receptors in sheep reticulocytes [294]. Transferrin is produced by developing embryos, most likely the rapidly disappearing yolk sac, and is detectable in uterine flushings and allantoic fluid on Day 17 of pregnancy [295]. Transferrin is an iron-binding transport protein important for cell function, differentiation, and proliferation. The yolk sac synthesizes transferrin from Day 15 of pregnancy in sheep, just before vasculogenesis, hematopoiesis and

differentiation and growth of the allantois, which demonstrates the importance of the yolk sac as a supplementary source for transferrin when the demand for iron is high [295]. LGALS15 also accumulates in allantoic fluid [173] and the C57 residue in the CRD sequence of LGALS15 CRD is also found in LGALS10 and thought to be responsible for mannose binding. Bovine trophoblast primary cell lines cultured in medium supplemented with transferrin showed an enhanced rate of growth and were less likely to change morphologically from a polygonal cell to a spindle shaped cytokeratin-positive cell [296].

LGALS15 is abundantly expressed both before and after implantation. Thus, trophectoderm cells are exposed to LGALS15 under two very different sets of circumstances; while they are in "suspension," that is during the pre-implantation period, and while they are "adhered" to a substrate during the post-implantation period. Therefore, we examined whether or not exposure of oTr1 cells to LGALS15 under these two different conditions *in vitro* resulted in different patterns of gene expression. Although there were differences in gene expression between LGALS15- versus BSA-treated cells in both culture conditions, cells cultured in suspension were more sensitive to LGALS15 treatment. In particular, cells in suspension showed an increase in integrin α2 and α5 monomeric proteins that play important roles in LGALS15 extracellular adhesion functions where it is hypothesized to function as a bridging ligand via integrins present on uterine LE and trophectoderm [198]. Another gene up-regulated in suspended cells include *Rho Kinase 2*. Wnt5a stimulates oTr1 cell movement and migration via the Rho-ROCK pathway [205]. Interestingly, LGALS15 stimulated oTr

cell migration via the JNK signaling pathway, but it may also affect other mechanisms to promote oTr1 cell migration. Overall, LGALS15 stimulated approximately 700 genes in suspended oTr1 cells with most associated with immune response, vasculogenesis, cell adhesion, transcription, proteases, and nutrient production which are very important for establishing a hospitable uterine environment for implantation. On the other hand, LSGAL15 stimulated an increase in approximately 270 genes in adhered oTr cells that were associated with nutrient transport, cell growth and maintenance, cell cycle progression, transcription, implantation, and cell junction formation. Examples of up-regulated genes associate with LGALS15 treatment of adhered cells include sodium/glucose co-transporter 1, regulatory associated protein of mTOR, and LIF receptor. Recent experiments with rodent fibroblast and epithelial cells revealed that signaling through the Akt-mTOR pathway is important for exogenous JSRV Env-induced transformation [297]. The enJSRVs act on mononuclear trophectoderm cell to stimulate outgrowth and differentiation of trophoblast giant BNCs during the peri-implantation period of pregnancy [204, 206]. These results suggest that during and after implantation the conceptus uses LGALS15 to stimulate expression of genes for nutrient exchange, cell growth and implantation. Overall, genes stimulated by LGALS15 in oTr1 cells correspond to specific requirements for a particular cellular environment, such as preparing for implantation, as necessary for cells in suspension, as well as growth and differentiation, which are requisite for trophectoderm cells after implantation.

LGALS15 had a slight effect on proliferation of only oTrF cells at a dose of 100 ng which suggests that this is not its main function. LGALS15 also failed to stimulate

hemagglutination of sheep, pig, or mouse erythrocytes as characteristic of other members of the galectin superfamily. Galectins produced by the helmith *Haemonchus contortus* induce hemagglutination of erythrocytes of mice, dogs, and chickens but not sheep [273]. Indeed, the absence of this function in LGALS15 may be beneficial in the uterus where hematopoeitic nutrient exchange is vital and erythrocyte agglutination would lead to clotating and reductions in blood flow.

In conclusion, results of these and previous studies clearly demonstrate that LGALS15 is involved in a number of critical processes including stimulation of cellular adhesion and migration, as well as inhibition of apoptosis. Further, pleiotropic functions of LGALS15, based on potential effects on gene expression, include iron transport and immune functions. Taken together, these findings provide strong support for LGALS15 serving as an important intermediary in many physiological processes essential for ovine blastocyst growth, elongation and implantation *in utero*.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY

Galectins are involved in cellular adhesion, growth, migration, apoptosis, and survival of several cell types and tissues. *LGALS15* is abundantly expressed by endometrial LE and sGE of the ovine uterus and is hypothesized to play an integral role in the growth and implantation of the conceptus. The described experiments were conducted to test this hypothesis and specifically to define the biological functions of LGALS15 in the ovine uterus during the peri-implantation stage of early pregnancy. The findings of these experiments advance our understanding of the biological roles of *LGALS15* within the context of the overall relationship between the conceptus and maternal endometrium during early pregnancy.

Previous studies indicated that endometrial secretions, collectively termed histotroph, are produced during pregnancy and are required to support ovine conceptus survival, growth and development beyond Day 14 of pregnancy [5, 7, 191, 220, 222]. Additional studies revealed that *LGALS15* is highly expressed by the uterine LE and sGE during the peri-implantation period where it is synthesized and secreted as a component of histotroph [23]. Secreted LGALS15 forms multimers and was discovered to be the previously unknown 14 kDa protein in intracytoplasmic crystalline inclusions in LE and trophectoderm cells [22, 23, 95, 173] (**Figure 6.1**). *LGALS15* is induced by P4 and further stimulated by IFNT and expression increases between Days 14 and 16 of

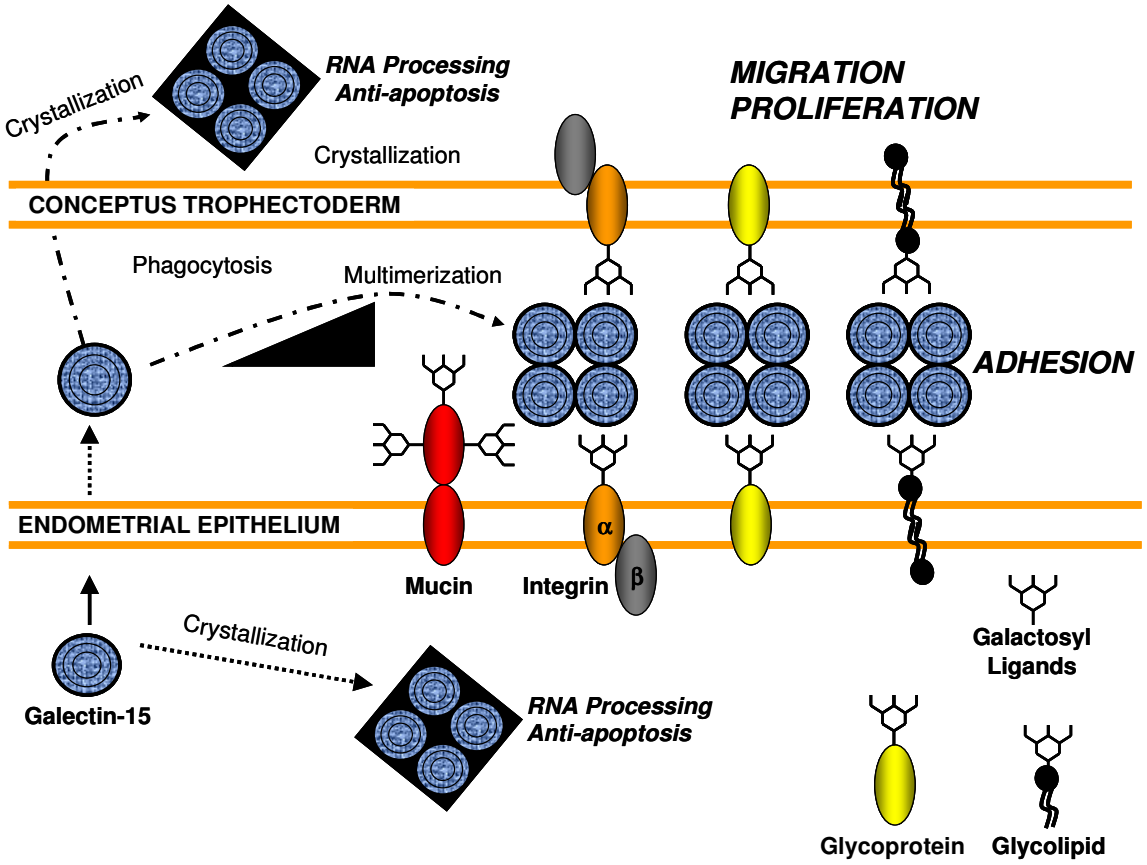

FIGURE 6.1
Proposed mechanism for secreted ovine LGALS15 on the ovine endometrial LE and trophectoderm during the implantation period.

pregnancy in association with conceptus adhesion and implantation [173]. Pregnant ewes treated with exogenous P4 prior to onset of endogenous P4 production showed accelerated growth of the conceptus and IFNT secretion as well as increased *LGALS15* expression [179]. In the present studies, the tested hypothesis was that LGALS15 acts on conceptus trophectoderm to stimulate proliferation, migration, growth, adhesion and survival of conceptus trophectoderm.

Chapter III focused on the development of a primary trophectoderm cell line that was used to determine LGALS15 function *in vitro*. The oTr cell lines displayed typical trophectoderm epithelial cell morphology with both mononuclear cells and a small population of BNC which persisted for more than 20 passages. Furthermore, oTr cells were shown to express genetic markers of embryonic trophectoderm cells *in vivo*, such as *IFNT*, enJSRVs *env* and *gag*, *HYAL2* receptor for enJSRVs Env, *CSH1*, *PAG1* and genes associated with the WNT signaling pathway (*FZD6, GSK3B, DKK1*). Studies presented in this chapter showed that oTrF did not express *IFNT* like the oTr1 cells. Because both cell lines were developed from conceptus tissue at the height of IFNT production these differences presumably resulted from oTrF cells being cultured on a collagen substrate whereas oTr1 cells were developed and cultivated on tissue-culture treated plastic.

Migration of oTr1 cells described in Chapter III further demonstrated that individual cells are capable of detaching and migrating, indicating an invasive property. Sheep have a synepitheliochorial placentation with limited invasion, although the conceptus is invasive when it is transplanted outside of the uterus. The present studies

also indicated that oTr1 cells were capable of accumulating exogenous LGALS15 protein *in vitro* in a similar pattern as LGALS3 in breast carcinoma cell lines [214] suggesting that the observed patterns of LGALS15 secretion and uptake are conserved across galectin superfamily members. Despite the differences in morphology and *IFNT* production both cell lines behaved similarly when treated with exogenous LGALS15 in both cellular adhesion and migration assays (Chapters IV and V), indicating that at least some physiological characteristics of trophectoderm cells were maintained in both cell lines. Taken together these results indicate that the oTr primary cell lines are good *in vitro* models to study intracellular and extracellular roles of LGALS15 on the conceptus trophectoderm.

Chapter IV investigated LGALS15 function in trophectoderm cell adhesion. It was found that a dose-dependent increase in oTr cell attachment to LGALS15 was observed, which is inhibited by cyclic GRGD, but not GRAD, peptides. Mutation of the LDVRGD integrin binding sequence of *LGALS15* to LADRAD decreased its ability to promote oTr cell attachment, whereas mutation of the CRD had little effect. Further, the LDV recognition sequence of *LGALS15* is not likely to be important because natural polymorphic variants of *LGALS15* with LVV instead of LDV sequences in the C-terminus in sheep and goat *LGALS15* do not alter their cell attachment function [187]. LGALS15 induced formation of robust focal adhesions in oTr cells that was abolished by mutation of the LDVRGD sequence again indicating the importance of the integrin recognition sequence to its function. LGALS15 was also found to mediate attachment of trophectoderm cells and formation of focal adhesions via binding and activation of

integrins, which is an essential element of blastocyst implantation and trophoblast differentiation in many species [235, 236].

Chapter V tested the hypothesis that *LGALS15* is a secreted regulator of blastocyst development and gene expression, as well as growth, migration, and apoptosis of the trophoblast. LGALS15 had a minimal effect on oTr cell proliferation with only the oTrF cell line showing an increase in proliferation following LGALS15 treatment suggesting that if LGALS15 functions to increase trophectoderm proliferation, it is likely not the main function. LGALS15 also failed to stimulate hemagglutination of sheep, pig, or mouse erythrocytes. The absence of this function for LGALS15 may be beneficial in the uterus where hematopoeitic nutrient exchange is vital and erythrocyte agglutination would function as a clot and prevent adequate blood flow. It was also shown that LGALS15 stimulated migration of mononuclear trophectoderm cells in a manner dependent on the JUN-JNK signaling pathway but not the Rho-ROCK pathway utilized by WNT5a, another known stimulator of oTr cell migration.

LGALS15 did partially inhibit the induction of trophectoderm cell apoptosis by staurosporine presumably by counteracting staurosporine's break down of focal adhesions. Although LGALS15 is abundant in the uterine lumen during early pregnancy [173, 199, 223], it does not appear to inhibit other mechanisms for trophectoderm apoptosis as it is a common feature of conceptus development that may be required for cell remodeling or removal of genetically deficient cells [288].

Binding studies indicated that LGALS15, unlike many galectin family members, does not have high affinity for beta-galactosides. Binding studies with LGALS15 using

over 400 glycans showed that Fucα1-3GlcNAcβ, manose hybrid sugars (Mana1-2Mana1-2Mana1-3Mana-Sp9), melibiose (Gala1-6Glcb-Sp8), transferrin, and ceruloplasmin had the highest affinties indicating a role for LGALS15 in iron transport and immune function. Furthermore, genes stimulated by LGALS15 treatment of oTr1 cells are consistent with its purported function such as preparing for implantation, as is necessary for cells in suspension, as well as growth and differentiation, which is requisite of trophectoderm cells after implantation. Taken together, these findings support the idea that LGALS15 is an important intermediary in many of the physiological processes essential for ovine blastocyst growth and elongation prior to implantation *in utero*.

CONCLUSIONS

In conclusion, the temporal and spatial alterations in *LGALS15* mRNA and protein in endometrial LE and lumen of the ovine uterus during pregnancy, combined with the functional aspects of LGALS15 discovered in the present studies, support the hypothesis that LGALS15 functions as a heterotypic cell adhesion molecule. LGALS15 functions by bridging integrins in the endometrial LE and conceptus trophectoderm to induce focal adhesions by integrin-ECM interactions and demonstrates functional integrin activation and cytoskeletal reorganization in conceptus trophectoderm cells in response to LGALS15 binding via its RGD site (**Figure 6.2**), as well as possible roles in cell migration, iron transport, and differential gene expression. These biological

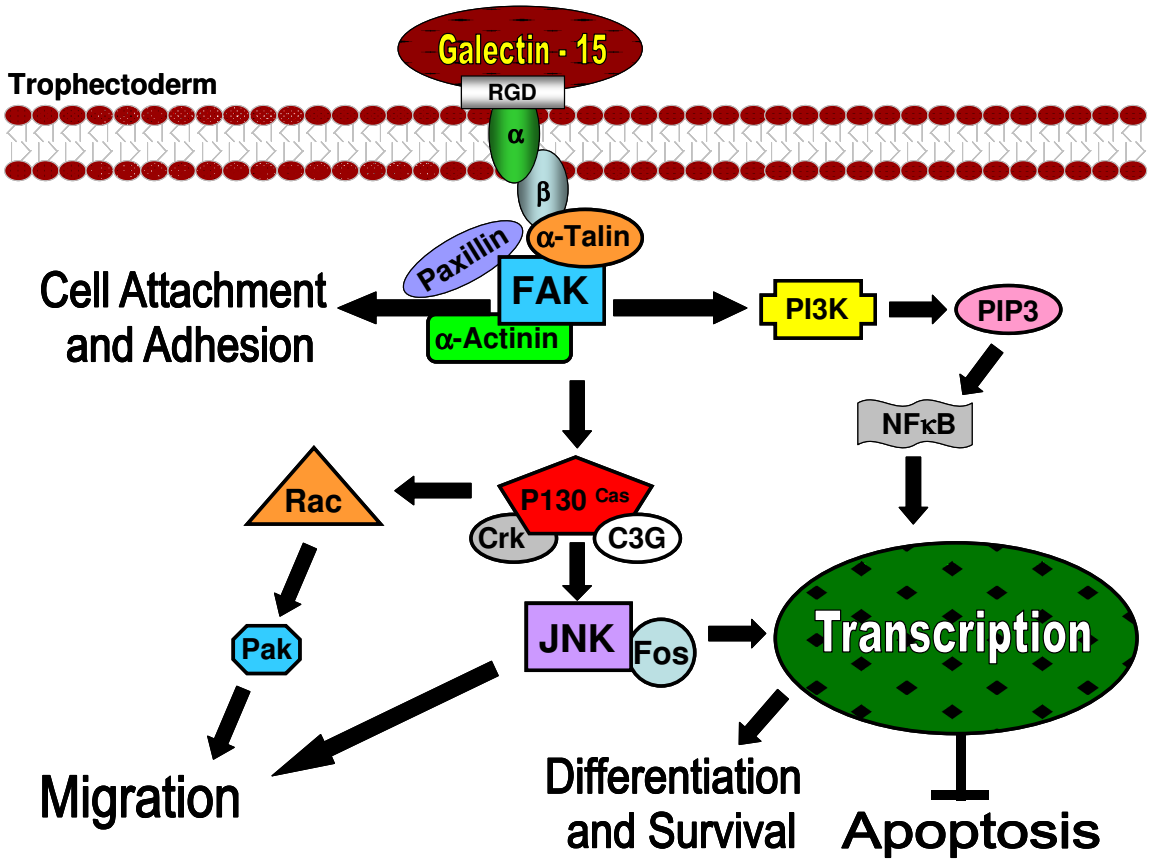

FIGURE 6.2
Proposed signaling cascade for ovine LGALS15 via integrin binding by its RGD integrin recognition sequence in ovine trophectoderm cells during the implantation period.

functions are undoubtedly required for ruminant blastocyst growth and elongation prior to implantation *in utero*.

Of particular note, it was recently determined that the *LGALS15* gene is present in ruminants (cattle, sheep and goats) only, but is uniquely expressed in uterine endometria of ruminants in the subfamily Caprinae (sheep and goats). However, other galectin family members are expressed during implantation in many mammals. *LGALS9* has recently been discovered to be an endometrial marker for the mid- and late-secretory phases of the menstrual cycle and decidual phases during pregnancy in humans [298]. *LGALS9* along with *LGALS1* and *LGALS3* have been implicated in uterine receptivity and decidualization in mice and humans where they are expressed in uterine stroma, epithelium and decidua [254, 298]. *LGALS3* is expressed in trophoblast of cows, mice and humans [299] suggesting a role in embryo implantation and cell–cell and cell–matrix interactions of trophoblast during placentation [300]. *LGALS5* is also present in blastocysts at the time of implantation in the mouse [162]. Thus, galectin family members are commonly expressed in the endometria and placentae of many mammals, where they may also function in endometrial differentiation as well as blastocyst implantation, immune modulation and trophoblast differentiation [162, 254, 255].

LGALS15 was first discovered in sheep abomasal tissue infected with the nematode parasite, *Haemonchus contortus*, and was shown to have both nuclear and cytoplasmic localization and is abundantly secreted into the surrounding mucus [196]. It is unclear if LGALS15 expression is induced by the presence of the parasite or if it is induced due to the tissue damage and inflammation that occurs with infection.

Interestingly, the embryo functions in much the same way as a parasite during pregnancy, causing alterations in tissue structure, stimulating inflammatory responses, and siphoning nutrients away from its host. There are apparent similarities between implantation and immune cell infiltration including cellular apposition, adhesion and invasion of the embryo at the implantation site and rolling, adhesion and extravasation of immune cells at the site of inflammation. It is well documented that galectins have many immune modulatory properties. Perhaps *LGALS15* evolved in the uterus as a way for the maternal system to cope with the embryo semi-allograft, thus linking galectin immune and reproductive functions.

Experiments in this dissertation have contributed toward the fundamental knowledge of LGALS15 function in the ovine uterus during implantation. These analyses have determined the effectiveness of LGALS15 to stimulate trophectoderm cell adhesion, migration, survival, and development *in vitro* and provide an opportunity for future studies in the sheep. Future experiments must be directed toward determining the cellular and molecular mechanisms regulating successful conceptus survival and implantation involving this dynamic protein. These experiments include: (1) characterizing spatial and temporal expression for genes stimulated by LGALS15; (2) determining the role of LGALS15 in trophoblast immune evasion; (3) inactivating *LGALS15* RNA in the LE and determining the consequences on conceptus development; (4) infusing recombinant LGALS15 into the uterus of UGKO ewes and the effect on conceptus elongation; (5) investigations into the role of LGALS15 crystals in the LE and conceptus; and (6) determining the function of LGALS15 in the allantoic fluid throughout gestation, whether related to prostaglandin production, metabolic regulation, iron transport or carbohydrate binding.

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