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Review

Novel pathways for implantation and establishment and maintenance of pregnancy in mammals

Fuller W. Bazer¹,²,⁵, Guoyao Wu¹, Thomas E. Spencer¹, Greg A. Johnson³, Robert C. Burghardt³, and Kayla Bayless⁴

¹Departments of Animal Science, Texas A&M Health Sciences Center, College Station, TX 77843, USA ²WCU Biomodulation Major, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea ³Departments of Veterinary Integrative Biosciences, Texas A&M Health Sciences Center, College Station, TX 77843, USA ⁴Texas A&M University and Department of Molecular and Cellular Medicine, Texas A&M Health Sciences Center, College Station, TX 77843, USA

Correspondence address. E-mail: fbazer@cvm.tamu.edu

ABSTRACT: Uterine receptivity to implantation varies among species, and involves changes in expression of genes that are coordinate with attachment of trophectoderm to uterine luminal and superficial glandular epithelia, modification of phenotype of uterine stromal cells, silencing of receptors for progesterone and estrogen, suppression of genes for immune recognition, alterations in membrane permeability to enhance conceptus-maternal exchange of factors, angiogenesis and vasculogenesis, increased vascularity of the endometrium, activation of genes for transport of nutrients into the uterine lumen, and enhanced signaling for pregnancy recognition. Differential expression of genes by uterine epithelial and stromal cells in response to progesterone, glucocorticoids, prostaglandins and interferons may influence uterine receptivity to implantation in mammals. Uterine receptivity to implantation is progesterone-dependent; however, implantation is preceded by loss of expression of receptors for progesterone (PGR) so that progesterone most likely acts via PGR-positive stromal cells throughout pregnancy. Endogenous retroviruses expressed by the uterus and/or blastocyst also affect implantation and placentation in various species. Understanding the roles of the variety of hormones, growth factors and endogenous retroviral proteins in uterine receptivity for implantation is essential to enhancing reproductive health and fertility in humans and domestic animals.

Key words: implantation / placentation / pregnancy / uterus / conceptus development

Introduction

Strategies for implantation

Implantation involves attachment of conceptus trophectoderm (Tr) of the developing conceptus (the embryo and its associated extraembryonic membranes) to uterine luminal epithelium (LE) in a highly synchronized series of events requiring reciprocal secretory and physical interactions during a restricted period known as the ‘window of receptivity’ (Carson et al., 2000; Dey et al., 2004). The ‘window of receptivity to implantation’ is established by actions of progesterone and, in some species, estrogen (E2) that regulate locally produced cytokines, growth factors, homeobox transcription factors and cyclooxygenase-derived prostaglandins through autocrine and paracrine pathways (Paria et al., 2002). A paradox is the role of progesterone to sequentially down-regulate expression of progesterone receptors (PGR) in uterine LE, as well as superficial (sGE) and mid-to deep-glandular (GE) epithelia as a prerequisite for endometrial receptivity to implantation; however, PGR continue to be expressed in stromal and myometrial cells of the uterus. Subsequent effects of progesterone on PGR-negative uterine epithelia are likely mediated by stromal cell-derived growth factors known as ‘progestamedins’ (Spencer and Bazer, 2002; Cunha et al., 2004).

Implantation may be non-invasive (central) or invasive (interstitial or eccentric) depending on whether or not conceptus Tr invades through uterine LE into the stroma. Implantation in domestic animals differs from that of rodents and primates where the conceptus enters a receptive uterus and almost immediately attaches to uterine LE. Domestic animals have a protracted preimplantation period (the prereceptive phase) during which the developing conceptus migrates throughout the uterine lumen. Equine blastocysts remain spherical and contained within a capsule prior to attachment, whereas pig and ruminant conceptuses shed the zona pellucida (hatch) and transform morphologically from spherical to tubular and filamentous forms. Pre-attachment conceptus development is accompanied by growth...
and differentiation of the Tr that secretes a pregnancy recognition signal.

In all mammals, initial conceptus attachment requires alteration in the expression of anti-adhesive components, mainly mucins, contained in the glycocalyx of LE that sterically inhibit attachment (Brazman et al., 2004). The mucin, MUC1, exists as both an intrinsic transmembrane mucin and an alternatively spliced, secreted variant. Both forms are localized to the apical uterine LE to provide a barrier to attachment, but are generally reduced during the receptive phase (mice, pig, sheep) or locally at the site of blastocyst attachment (human, rabbit) due to activation of cell surface proteases (Carson et al., 2006).

Unmasking adhesion molecules on the surface of uterine LE permits initial contacts with Tr that progressively develop into more stable adhesion through interactions between Tr and maternal extracellular matrix (ECM), as well as stromal cells encountered following intrusion beyond the uterine LE during invasive implantation (Burghardt et al., 2002). Initial adhesion or attachment is mediated by molecules that contribute specific carbohydrate ligand binding including selectins and galectins, as well as heparan sulfate proteoglycan, heparin binding epidermal growth factor (EGF)-like growth factors, cadherins and CD44 (Carson et al., 2000). Low-affinity interactions are followed by stable adhesion involving integrins expressed on Tr and uterine LE and their ECM bridging ligands that also have roles in adhesion, migration, invasion, cytoskeletal organization and bidirectional signaling (Burghardt et al., 2002). In humans, expression of \( \alpha v \beta 3 \) and \( \alpha v \beta 1 \) integrins increase in uterine LE during the window of implantation (Lessey et al., 1996). These and other integrins at both maternal and conceptus interfaces along with integrin-binding matrix proteins such as fibronectin, oncoketal fibronectin, vitronectin, secreted phosphoprotein 1 (SPP1 or osteopontin), laminin, and the latency associated peptide linked to transforming growth factor-beta (TGF\(\beta\)) are critical in species having non-invasive and invasive implantation (Burghardt et al., 2002). These and other ECM constituents such as insulin-like growth factor binding protein-1 (IGFBP1) (Simmons et al., 2009a) and galectin-15 (LGALS15) (Farmer et al., 2008) may function as bridging ligands to promote blastocyst expansion initially followed by stable adhesion between apically expressed maternal and fetal integrins. Other genes reported to be markers of uterine receptivity to implantation include laminin \( \beta 3 \), microfibril-associated protein 5, angiopoietin-like 1, endocrine gland-derived vascular endothelial growth factor (VEGF) and nuclear localized factor 2 that, in a general thematic context, are associated with angiogenesis and vascularization of tissues (Haouzi et al., 2009).

During the initial stages of implantation conceptus/maternal interactions differ between domestic animals (non-invasive implantation) and rodents, carnivores and primates (invasive implantation) (Bazer et al., 2009b). Differences among species exist in the extent of interactions between Tr (gives rise to chorion) and maternal uterus at the interface between maternal and fetal cells giving rise to placental structures. For example, intimate contact between chorion derived from Tr and an intact LE is maintained in pigs throughout pregnancy (epitheliochorial placenta). Ruminant conceptuses form binucleate Tr cells which migrate and fuse with uterine LE and each other to form plaques of multinucleated syncytiotrophoblastic syncytial placentas. Binucleate Tr cells and the syncytia derived from binucleate cells migration and fusion are the source of placental lactogen as well as other hormones such as progesterone (Wooding et al., 1992). In both epitheliochorial and synepitheliochorial placentation, the conceptus remains within the uterine lumen throughout gestation. In ruminants, contact between the chorioallantois and caruncles, discrete sites on the endometrial mucosa devoid of uterine glands, leads to development of opposing highly vascularized cotyledons of the chorioallantois that form placentomes. The placentomes are critical for exchange of nutrients and gases across the placenta (Reynolds et al., 2005).

Carnivores, rodents, and primates exhibit invasive implantation where the blastocyst invades and implants deeply into the endometrial stroma and then the uterine LE is restored over the site of implantation. During initial contact, the Tr is highly proliferative and undergoes syncytial formation to form a syncytiotrophoblastic cell layer which develops stable adhesion with uterine LE followed by penetration of syncytiotrophoblasts into the uterine wall to establish extensive contacts with the maternal vasculature. Loss of maternal vascular endothelial cells results in the formation of maternal blood sinuoids in hemochorial placentae of higher primates and rodents, whereas the hemochorial placenta of carnivores retains the endothelial layer. Mononuclear cytotrophoblasts underlie syncytiotrophoblasts and migrate out of the trophoblast layer as well as fuse together to maintain the syncytiotum.

Global gene profiling comparing endometrial tissue between proliferative and secretory phases of human endometria identified many differentially expressed genes including cell surface proteins/receptors, ECM molecules, secretory proteins, immune modulators and cytokines, cytoskeletal proteins, transport proteins and transcription factors as well as proteins involved in cholesterol trafficking, prostaglandin biosynthesis, detoxification, cell cycle regulation, signal transduction, transport and metabolism of nutrients, coagulation cascades, chemotaxis, phagocyte recruitment, angiogenesis and other cellular functions (Carson et al., 2002; Horcajadas et al., 2007; Savaris et al., 2008; Tseng et al., 2009). About 20% of the changes were attributed to genes encoding cell surface receptors, adhesion and ECM proteins and growth factors, including markers of uterine receptivity in humans such as glycodelin and SPP1, stromal cell-specific insulin growth factor binding proteins-1 and -2 (IGFBP1, IGFBP2), prostaglandin E\(2\) receptor (EP2), interleukin-15 (IL15) and TGF\(\beta\) type II receptor for which expression increased. Notably, expression of SPP1 by uterine GE increased 12-fold during the receptive phase in women (Carson et al., 2002) and up to 60-fold during pregnancy in rats (Girotti and Zingg, 2003), suggesting a direct role in conceptus–uterine interactions. SPP1 is an abundantly secreted ECM protein up-regulated in uteri during early pregnancy in humans, mice, rabbits, goats, sheep and pigs (Johnson et al., 2003a). SPP1 contains an Arg-Gly-Asp (RGD) sequence that mediates binding to cell surface integrin receptors, including \( \alpha v \beta 3 \), \( \alpha 5 \beta 1 \), \( \alpha v \beta 5 \), \( \alpha v \beta 6 \) and \( \alpha 8 \beta 1 \), as well as alternative binding sequences for interactions with \( \alpha v \beta 1 \), \( \alpha 9 \beta 1 \) and \( \alpha 4 \beta 7 \). Binding of SPP1 to these various receptors elicits diverse effects including cell-to-cell and cell-to-ECM adhesion, chemotaxis of leukocytes, smooth muscle cells and endothelial cells, endothelial and epithelial cell survival, and migration of fibroblasts, macrophages and tumor cells. Similar microarray studies have addressed changes in uterine gene expression during early pregnancy in ruminants (Spencer et al., 2008).
Cytoskeletal changes mediate blastocyst expansion and elongation of conceptus Tr in non-invasive implantation and trophoblast outgrowth and invasion in invasive implantation

The mechanisms responsible for elongation of pig conceptuses are likely common to conceptuses of other livestock species that undergo rapid elongation during the peri-implantation period of pregnancy, i.e. a reduction in diameter and a rapid increase in length of Tr. Pig conceptus Tr cells in the elongation zone are columnar compared with cuboidal in areas peripheral to the elongation zone and that this structural modification is associated with changes in length and orientation of microfilaments (Geisert et al., 1982). That is, orientation of microfilaments in pig Tr cells change from horizontal to parallel relative to the lateral cell borders suggesting that elongation is initially through migration or condensation of Tr cells into the region of the embryonic pole forming the elongation zone in 10 mm diameter blastocysts. Within the elongation zone, alterations in microfilaments and junctional complexes of Tr cells and extension of filopodia from extraembryonic endodermal cells allow movement and redistribution of cells toward the ends of tubular blastocysts. There are also changes in the actin cytoskeleton in Tr during the transition from spherical to tubular and filamentous forms as follows: (i) early cleavage stage embryos have filamentous actin concentrated at sites of contact between blastomeres; (ii) compacting morulae accumulate actin at the margins of blastomeres; and (iii) Tr cells of expanding blastocysts initially exhibit pericellular distribution of actin that later forms continuous actin-rich lateral borders and stress fibers along their basal surface (Albertini et al., 1987; Mattson et al., 1990). The actin cytoskeleton is essential for force generation for conceptus elongation as constricted regions along the length of filamentous conceptuses contain polarized Tr cells with a distinct F-actin array. Focal adhesions are macromolecular complexes comprised of heterodimeric transmembrane integrin receptors that connect ECM to the actin cytoskeleton to regulate cell growth, proliferation, survival, migration, gene expression and cell morphology (Erikson et al., 2009). Recently, SPPI was shown to bind directly to the integrin heterodimers αVβ6 on Tr and αVβ3 on uterine LE to induce assembly of focal adhesions that promote migration and attachment of Tr to LE that may be critical to conceptus elongation and implantation (Erikson et al., 2009).

Living cells use tensegrity (tensional integrity) architecture to control shape and structure of tissues and cells through changes in stability of cytoskeletal structures that include: (i) microfilaments, self-assembling actin polymers that form relatively rigid but flexible networks that self-assemble into cross-linked bundles, or when associated with myosin II, form ‘contractile microfilaments’ that generate tension; (ii) intermediate filaments, polymers composed of cytokeratins in epithelial cells that form flexible cables extending from the cell surface to the nucleus to distribute force; and (iii) microtubules, larger hollow polymers of tubulin that extend across the cytoplasm to the cell periphery (Zaidel-Bar et al., 2007; Ingber, 2008). Integrins and associated proteins are regarded as ‘tensegrity structures’ because molecular connections between ECM, integrins, cytoskeletal filaments and nuclear scaffolds provide a discrete path for transfer of mechanical signals through cells as well as a mechanism for producing integrated changes in cell and nuclear structure. The resulting focal adhesion complex or ‘integrin adhesome’ (Zaidel-Bar et al., 2007) physically links integrins to the ends of contractile microfilament bundles (‘stress fibers’) to form a molecular bridge between ECM and cytoskeleton. Focal adhesions increase in size as tension increases across transmembrane integrin receptors (Riveline et al., 2001). Cells are therefore able to respond to both internally generated or externally applied forces and can sense the rigidity and anisotropy of the ECM (Geiger et al., 2009). Pulling on ECM tugs on integrins and associated focal adhesion proteins to deform mechanosensory molecules that elicit biochemical signals which change intracellular metabolism and gene expression as our model proposes for elongating ovine conceptus Tr.

Integrin and growth factor associated cell signaling from the extracellular space into the cell (outside-in signaling) regulates multiple cellular processes including survival, proliferation, shape, polarity, adhesion, migration and differentiation of cells (Hehlgans et al., 2007). Ligand binding to the extracellular integrin domain induces conformational changes and integrin clustering for activation of signaling cascades and recruitment of multiprotein complexes to focal adhesions. More than 150 different proteins have been identified that either physically reside within these adhesion sites or interact with the adhesion components and affect their activity (Zaidel-Bar et al., 2007). Many of these components link integrin-mediated signals with other signaling pathways to promote extensive cross-talk with growth factors, cytokines, G-protein coupled receptors and nutrient signaling pathways.

In mice, leucine or arginine is required for expanded blastocysts to exhibit motility and outgrowth of Tr required for implantation (Lessey et al., 1994; Zeng et al., 2008). These amino acids regulate motility and outgrowth of Tr through activation of serine/threonine kinase mTOR (FRAP1) cell signaling which activates Rac-1, a member of the Rho GTPase family. Increased FRAP1 signaling also stimulates protein synthesis and expression of IGF2, nitric oxide synthase (NOS) and ornithine decarboxylase (ODC) mRNAs (Martin and Sutherland, 2001; Martin et al., 2003). Implantation of the human embryo and migration of human extravillous Tr requires multiple Rho GTPase family members in both Tr cells and the endometrial stromal cells into which they invade (Grewal et al., 2008; Nicola et al., 2008). Rho GTPases including RhoA, Rac1 and Cdc42 are ubiquitous proteins that control cytoskeletal changes by forming actin-containing stress fibers and projecting filopodia and lamellipodia during cell migration through linking ECM molecules with the actin cytoskeleton by forming focal adhesions. Therefore, activation of GTPases are also thought to be controlled by integrin activation, but the mechanism(s) whereby ECM favors activation of individual molecules is not known (Symons and Segall, 2009).

The mTOR signaling pathway has been linked to elongation of conceptus Tr in sheep. For ovine conceptus development during implantation and placentation, integrin activation by SPPI binding and arginine are proposed to stimulate remodeling of Tr for elongation and adherence to LE/stGSE via cytoskeletal reorganization that facilitates cell motility, stabilizes adhesion and collectively activates mTOR signaling pathways mediated by AKT1, TSC1/2 and mTORC1 (cell proliferation and mRNA translation), as well as mTORC2 (cell migration, cell survival and cytoskeletal organization).
in Tr cells. For ovine Tr cells, SPP1 binds αvβ3 and α5β1 integrins to induce focal adhesion assembly, a prerequisite for adhesion and migration of Tr, through activation of: (i) RPS6K via crosstalk between FRAP1/mTOR and mitogen-activated protein kinase (MAPK) pathways; (ii) mTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate Tr cell migration; and (iii) focal adhesion assembly and myosin II motor activity to induce migration of Tr cells. These cell signaling pathways, acting in concert, mediate adhesion, migration and cytoskeletal remodeling of ovine Tr cells essential for expansion and elongation of conceptuses and attachment to uterine LE for implantation (J. Kim et al., unpublished results).

Decidualization
Penetration of the LE barrier by invasive Tr cells triggers a series a stromal responses collectively termed decidualization (Salamonsen, 1999). During decidualization, hyperplasia and hypertrophy transforms small spindle-like endometrial stromal cells into enlarged polygonal epithelial-like cells with extensive cell–cell contacts (Salamonsen et al., 1995; Afonso et al., 1997). As they differentiate, these cells express additional or different arrays of cytoskeletal proteins (Gard and Lazarides, 1980) and exhibit marked accumulation of filamentous proteins which include microtubules, microfilaments, intermediate filaments and the microtubular lattice (Sananes et al., 1978). Two cytoskeletal proteins characteristic of decidual cells are desmin (Oliveira et al., 2000; Glasser and Julian, 1986) and α-smooth muscle actin (Christensen et al., 1995). These cytoskeletal proteins are believed to be physically involved with changes in growth, shape and protein secretion by stromal cells during the decidualization process (Rao and Cohen, 1991). Functionally, decidualized stromal cells secrete prolactin (Maslar et al., 1986; Tessier et al., 2000) and IGFBP1 (Bell, 1991; Kim et al., 1999) which likely function in complex gene networks that restrain trophoblast invasion (Bell, 1979; Irwin and Giudice, 1998), as well as many other endocrine and paracrine factors (Popovic et al., 2000; Brar et al., 2001; Salamonsen et al., 2002). Decidual cells also accumulate ECM proteins including SPP1, laminin and fibronectin. For SPP1, expression is by decidual natural killer cells in mice (White et al., 2005), but by stromal cells in humans (von Wolff et al., 2004), and SPP1 may be involved in angiogenesis within the decidua. The end result is the formation of a morphologically and functionally distinct tissue that produces hormones, promotes nutrition of conceptuses, prevents fetal allograft rejection and regulates placentation by limiting Tr invasion through generation of a local cytokine environment which promotes Tr attachment over invasion (Lee et al., 1997; Irwin and Giudice, 1999). The decidua constitutes the maternal side of the maternal-fetal interface involved in exchange of molecules between these tissues necessary for successful completion of gestation. It is increasingly apparent that varying degrees of decidualization-like differentiation of stromal cells is common to all implanting species with most extensive transformation accompanying invasive implantation in rodents and primates, moderate transformation with synepithelochorial placentation, and very minor changes with epitheliochorial placentation (Johnson et al., 2003b, 2009).

Pregnancy recognition signaling
Establishment and maintenance of pregnancy in mammals requires that a functional corpus luteum (CL) be maintained beyond its normal cyclic lifespan for continued production of progesterone required for secretory functions of the endometrium essential for embryonic development, implantation and placentation. The maternal recognition of pregnancy signals from the conceptus may be luteotropic, if it directly promotes luteal function, or anti-luteotropic, if it prevents uterine release of luteolytic prostaglandin F2α (PGF) which would cause regression of the CL (Bazer et al., 2008). Chorionic gonadotrophin (CG), the luteotropic signal in primates, acts directly on the CL as does prolactin released from the anterior pituitary in response to mating in rodents. In domestic animals, anti-luteolytic signals from the conceptus include estrogen and prolactin in pigs, interferon-τ (IFNT) in ruminants, and an undetermined factor(s), perhaps estrogen and interferon delta (IFND), in horses (Bazer et al., 2008).

Pregnancy recognition signaling by IFNT in ruminants
IFNT, the pregnancy recognition signal in ruminants, suppresses transcription of ESR1 and, therefore, estrogen-induced expression of the oxytocin receptor (OXTR) gene in uterine LE/sGE to abrogate development of the endometrial luteolytic mechanism involving oxytocin-induced luteolytic pulses of PGF (Bazer et al., 2008). However, basal production of PGF is higher in pregnant than cyclic ewes due to continued expression of prostaglandin endoperoxide synthase 2 (PTGS2). IFNT silencing of ESR1 expression also prevents estrogens from inducing PGR in endometrial epithelia, which is critical as the absence of PGR in uterine epithelia is required for expression of progesterone-induced and IFNT-stimulated genes in ovine uterine LE/sGE (Bazer et al., 2009a, b).

Pregnancy recognition signaling in pigs
The pregnancy recognition signal is estrogen secreted by pig conceptuses on Days 11 and 12 of pregnancy to redirect PGF secretion from the uterine vasculature to the uterine lumen. The theory of estrogen-induced maternal recognition of pregnancy in pigs is based on evidence that: (i) the uterine endometrium secretes luteolytic PGF; (ii) pig conceptuses secrete estrogens which are anti-luteolytic; (iii) PGF is secreted toward the uterine vasculature (endocrine) in cyclic gilts to induce luteolysis; and (iv) secretion of PGF in pregnant gilts is into the uterine lumen (exocrine) where it is sequestered from the corpora lutea and/or metabolized to prevent luteolysis (Bazer and Thatcher, 1977). In addition, PGE2, as well as lysosphosphatidic acid (LPA) have proposed roles in pregnancy recognition signaling. Expression of PGE2 synthase by trophoblast and endometrium decreases production of PGF in favor of PGE2 to support CL maintenance (Ziecik et al., 2008). In addition LPA increases in uterine luminal fluids of pigs, and its receptor, EDG7, is expressed by pig conceptuses, and its expression is increased by estrogen in endometrial epithelia during early pregnancy (Seo et al., 2008). Indeed, LPA3 is critical for migration of blastocysts as they space themselves equally throughout the two uterine horns. Pig conceptus Tr also secretes interleukin-1 beta (IL1B) during this period, but its role is not known (Ross et al., 2003). Pig conceptus Tr is unique in secreting both IFNΔ, a Type I IFN, and interferon gamma (IFNG), a Type II IFN, during the peri-implantation period (Cenci et al., 2003). Abundant IFNG mRNA is detectable in porcine conceptus Tr between Days 13 and 20 of pregnancy, whereas IFND mRNA is detectable in Day 14 conceptuses only by RT–PCR analysis (Joyce et al., 2007). On Day 15 of pregnancy,
IFNG and IFND proteins are co-localized to peri-nuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus, as well as cytoplasmic vesicles within clusters of Tr cells along the endometrial LE. This expression is characterized by de novo appearance of zona occludens one, a component of epithelial tight junctions, on their basal aspect, suggesting changes in endometrial polarity (Cencić et al., 2003). Although pig conceptus IFNs have no known anti-luteolytic effects for pregnancy recognition, their receptors are expressed on uterine epithelial cells (Cencić et al., 2003). Increased secretion of PGE2, expression of several known IFN-responsive genes, and modulation of uterine stromal and GE gene expression in response to IFNs have been demonstrated in preparations of pig conceptus secretory proteins (Johnson et al., 2009).

**Pregnancy recognition signaling in primates**
Pregnancy recognition signaling in primates extends CL function at least until the time of the luteal-placental shift when production of progesterone by the placenta is adequate to support pregnancy (Fazleabas et al., 2004; Afshar et al., 2007). Primate blastocysts begin implantation following attachment to uterine LE on Days 7–9 post-ovulation in macaques and humans or Days 11–12 in marmoset monkeys. Syncytiotrophoblast cells of human conceptuses secrete CG from Days 8 to 10 for pregnancy recognition and implantation begins on Days 7–9 post-ovulation. CG produced by primate blastocysts signals maternal recognition of pregnancy through its luteotropic actions via LHCGR on luteal cells. Circulating concentrations of CG, first detected around the time of implantation in all primates, increase to peak values in the first trimester and then decrease during late gestation in humans. Production of CG by the human conceptus may be regulated by gonadotropin-releasing hormone one from the uterus as gonadotrophin-releasing hormone receptors are detectable in placental tissue. Importantly, gonadotrophin releasing hormone receptor agonists enhance and antagonists suppress CG secretion. In many primates, CG production decreases at the time of the luteal-placental shift in progesterone production. Further, exogenous CG increases progesterone production and extends CL lifespan in both women and monkeys.

**Pregnancy recognition signaling in rodents**
In rodents, mating induces release of prolactin from the anterior pituitary and it is the initial luteotropic signal for CL formation and production of progesterone to about Day 12 of pregnancy, and then lactogenic hormones from conceptuses and uterine decidua act on luteal cells to maintain their function and secretion of progesterone (Soares, 2004). The gestation period for rats, mice and hamsters is 20–22 days, and functional CL must produce progesterone through Day 17 (Soares, 2004). Thus, maintenance of functional CL and production of progesterone, requires two endocrine events in rodents: (i) mating-induced diurnal and nocturnal surges of PRL increase LH receptors on luteal cells for formation of CL and suppress 20α-hydroxysteroid dehydrogenase activity to prevent conversion of progesterone to 20α-hydroxy progesterone that will not support pregnancy; and (ii) production of lactogenic hormones by uterine decidua and placenta act via receptors for prolactin on CL to maintain production of progesterone throughout gestation (Soares, 2004).

**Uterine biology and conceptus development during the peri-implantation period**

**Progestamedins, estramedins, corticoids and prostaglandins**

Uterine receptivity to implantation is dependent on progesterone which is permissive to actions of interferons, CG, prolactin and placental lactogen (Soares, 2004; Fazleabas, 2007; Joyce et al., 2007; Slayden and Keator, 2007; Bazer et al., 2009a). The paradox is that cessation of expression of PGR and ESR1 by uterine epithelia is a prerequisite for uterine receptivity to implantation, expression of genes by uterine epithelia and selective transport of molecules into the uterine lumen that support conceptus development. In the ewe, we demonstrated that down-regulation of PGR in uterine GE is required for induction of secretory gene expression by progesterone in the same epithelia; however, co-administration of estrogen and progesterone resulted in up-regulation of expression of PGR and decreased expression of serine protease inhibitors and SPP1 mRNAs and protein in uterine GE (Spencer et al., 1999). Down-regulation of PGR is also associated with down-regulation of MUC1 on uterine LE which is a prerequisite for uterine receptivity to implantation, as well as up-regulation of galectin 15, SPP1 and IGFBP1 in uterine LE/sGE that stimulate migration and attachment of Tr cells. Further, silencing expression of PGR in uterine epithelia allows progesterone to act on PGR-positive uterine stromal cells to increase expression of progestamedins, e.g. fibroblast growth factor-10 (FGF10) and hepatocyte growth factor (HGF) in sheep uteri (Satterfield et al., 2008) or FGF7 and HGF in primates (Slayden and Keator, 2007). These progestamedins exert paracrine effects on uterine epithelia and conceptus Tr that express receptors for FGF7 and FGF10 (FGFR2IIb) and HGF (MET; protooncogene Met). In sheep, many genes are progesterone-induced and IFN-stimulated; however, a fundamental unanswered question is whether actions of progestamedins and IFNs on uterine epithelia or other uterine cell types involve novel non-classical cell signaling pathways, independent of PGR and STAT1 (Bazer et al., 2009b). There is evidence that both progestamedins and IFNT can signal via MAPK and phosphoinositide-3 kinase (PI3K) to affect gene expression and uterine receptivity to implantation (Platanias, 2005). Interestingly, Type I IFNs bind the same receptor, but activate novel cell-specific signaling pathways to differentially affect gene expression in uterine LE/sGE versus GE and stromal cells. Cell-specific gene expression in the ovine uterus is due, at least in part, to expression of interferon regulatory factor 2 (IRF2), a potent inhibitor of transcription, by uterine LE/sGE (Choi et al., 2001; Spencer and Bazer, 2002).

**Estramedins in pigs**
Pig conceptuses secrete estrogens between Days 10 and 15 for pregnancy recognition, but also to increase expression of genes within the uterine LE, which act on conceptus Tr and uterine LE to stimulate proliferation, migration, adhesion and gene expression that supports implantation and development of the conceptus (Bazer et al., 2008). The limited number of estrogen-stimulated genes localized in endometrial of pigs include: AKR1B1, B2M, CD24, FGF7, IRF2, MX1, NMB, SLAs 1, 2, 3, 6, 7, 8, spp1, STC1 and EDG7 (Johnson et al.,
IGF1 is expressed by uterine glands of cyclic and pregnant pigs and IGF1 receptors are expressed by cells of the endometrium and conceptuses, suggesting paracrine and autocrine actions of IGF1 (Letcher et al., 1989). FGF7, an established stromal cell derived paracrine mediator of hormone-regulated epithelial growth and differentiation (Ka et al., 2007). However, FGF7 expression is novel in pigs as it is expressed by uterine LE between Days 12 and 15 of the estrous cycle and pregnancy. FGF7 binds to and activates FGFRIIib expressed by uterine epithelia and conceptus Tr. Estrogen increases FGF7 expression only after progesterone suppresses expression of PGR by uterine epithelia. The FGF7, in turn, increases cell proliferation, phosphorylated FGFR2Iib, the mitogen-activated protein kinase cascade and expression of urokinase-type plasminogen activator, a marker for Tr cell differentiation (Ka et al., 2007). From about Day 20 of pregnancy, FGF7 is expressed by uterine GE in pigs in response to progesterone and may continue to affect uterine epithelia and conceptus development (G.A. Johnson et al., unpublished results).

In addition to the increase in secretion of estrogens between Days 11 and 15 of pregnancy for maternal recognition of pregnancy, increases in estrogens from the placenta between Days 20 and 30 increase expression of endometrial receptors for prolactin, uterine secretory activity and uterine blood (Bazer et al., 2008). Corticoids

Corticoids have positive effects that promote pregnancy. These include stimulation of secretion of CG, suppression of uterine natural killer cells and promotion of trophoblast growth/invasion. There are also potential negative effects that might compromise pregnancy by inhibiting cytokine-prostaglandin signaling, restriction of trophoblast invasion, induction of apoptosis, and inhibition of embryonic and placental growth (Michael and Papageorghiou, 2008). A dialogue initiated by cell surface signaling molecules on conceptus Tr and uterine LE includes integrins and fibronectin that glucocorticoids could suppress to adversely affect implantation (Ryu et al., 1999). However, effects of glucocorticoids on fibronectin expression are tissue-specific with dexamethasone suppressing fibronectin in term human cytotrophoblasts and amnion, but acting in synergy with TGFβ to up-regulate fibronectin expression in matched samples of chorion and placental mesenchymal cells. Also, during the peri-implantation period of pregnancy, events mediated by pro-inflammatory cytokines, such as IL1B, TNFA and prostaglandins, are modulated by anti-inflammatory effects of glucocorticoids which could otherwise impair the cytokine-prostaglandin signaling required for implantation. Both IL1B and TNFA increase expression and activity of hydroxysteroid dehydrogenase (HSD)1 IB1 whereas suppressing expression of HSD1 IB2 in term human chorionic trophoblasts. The result is an increase in conversion of corticosterone to active cortisol and creation of a negative feedback loop at the uterine–conceptus interface between glucocorticoids and cytokines.

In most tissues, the anti-inflammatory effect of glucocorticoids is to inhibit synthesis of prostaglandins and thromboxanes by decreasing the expression and/or activity of phospholipase A2 (PLA2) and liberation of arachidonic acid as substrate for prostaglandin-endoperoxide synthase 1 (PTGS1) and PTGS2 (Barnes et al., 2006). However, in the placenta, glucocorticoids increase PLA2, PTGS2 and prostaglandin synthases (Zhang et al., 2006) and decrease expression of 15-alpha hydroxyprostaglandin dehydrogenase (HPGD) that converts prostaglandins to their inactive forms (Patel et al., 2003). Prostaglandins increase expression and activity of HSD1 IB1 (Alfaidy et al., 2001) and decrease activity of HSD1 IB2 to increase synthesis of cortisol (Hardy et al., 2001). Glucocorticoids have been reported to stimulate trophoblast growth and up-regulate expression of pro-matrix metalloproteinase (proMMP)-2 (Mandl et al., 2006), but other reports indicate that they inhibit expression of MMP-9 and migration (invasiveness) of cytotrophoblast cells (Librach et al., 1994). Further, glucocorticoids affect degradation of ECM during trophoblast invasion by increasing urokinase-type plasminogen activator (uPA) that leads to plasmin-associated degradation of ECM and tissue-type enzyme (tPA) plasmin-dependent breakdown of fibrin for establishment of an efficient vascular exchange in the placenta (Loskutoff et al., 1993). The activities of both uPA and tPA are inhibited by plasminogen activator inhibitor (PAI1) secreted by trophoblast and decidual cells (Hofmann et al., 1994). Both cortisol and dexamethasone increase expression of PAI1 (Ma et al., 2002) which may result in poor placental exchange of nutrients and gases and lead to pre-eclampsia and intrauterine growth retardation (IUGR) (Grancha et al., 1996).

Establishment of pregnancy in sheep requires elongation of the conceptus and production of IFNT for pregnancy recognition signaling as discussed previously. Expression of HSD1 IB1 may be stimulated by progesterone, prostaglandins and/or cortisol which is consistent with findings that HSD1 IB1 mRNA is more abundant in ovine uterine LE/sGE between Days 12 and 16 of pregnancy than the estrous cycle and that expression of both HSD1 IB1 and PTGS2 by uterine LE/sGE is coordinate with conceptus elongation in sheep (Simmons et al., 2009b). Physiological levels of cortisol are potent stimulators of both arginase and ornithine decarboxylase expression in cells to increase the synthesis of polyamines essential for cell proliferation and differentiation (Flynn et al., 2009; Rhoads and Wu, 2009). Although HSD1 IB1 is abundant in the uterine LE/sGE, its expression is barely detectable in the conceptus, whereas HSD1 IB2 expression is barely detectable in uterine endometria, but abundant in the conceptus. Expression of HSD1 IB1 is induced by progesterone and further stimulated by IFNT in uterine LE/sGE. The glucocorticoid receptor, NR3C1, is present in all ovine uterine cell types. Therefore, HSD1 IB1 expression in ovine uterine LE/sGE of early pregnancy is regulated by progesterone, IFNT and prostaglandins that generate cortisol that may then act via NR3C1 to regulate ovine endometrial functions during early pregnancy. Prostaglandins such as PGE2, acting via PGE receptors (PTGER1-PTGER3), may also activate p38 MAPK cell signaling (Minamizaki et al., 2009). In bovine uterus, IFNT stimulates expression of PTGS2 and PGE synthase to increase the relative abundance of PGE, but also increases expression of EP2 in uterine epithelia (Arosh et al., 2004). Therefore, in uterine epithelia, IFNT, prostaglandins and prostaglandins may act additively or synergistically via common or complimentary cell signaling pathways to stimulate gene expression in support of elongation, development and implantation of the conceptus.

In rodents, critical spatial and temporal changes in expression of ESR1 and PGR occur in the uterus during the peri-implantation period, i.e. Days 1 to 8 of pregnancy (Tan et al., 1999). On Days 1–2, ESR1 are primarily in uterine LE and GE, also in stromal cells by Days 3–4 and, on Day 5 following implantation, ESR1 is in LE and GE, but much lower in stratum compactum stroma. On Days
6–8, ESR1 is primarily in the secondary decidual zone, particularly in the subepithelial cells at the mesometral pole, but undetectable in the primary decidual zone, whereas the undifferentiated stroma is ESR1 positive. PGR are at very low levels in murine endometrial on Day 1, modest in LE and GE on Day 2, and in LE, GE and stromal cells on Days 3–4. However, PGR are lost from LE and restricted to stromal cells by Days 5–8 and are particularly abundant in the decidua. As for primates and livestock species, cell-specific expression of ESR1 and PGR inform about coordinated effects of estrogen and progesterone in preparation of the uterus for implantation and decidualization during pregnancy.

In an elegant study by Simon et al. (2009), uteri from wild-type and PGR null mice were used to produce tissue recombinants in which PGR was present in stroma and/or epithelia or absent in either compartment. Only tissue recombinants involving wild-type stroma and wild-type epithelium responded to progesterone with an increase in the expression of Indian hedgehog (Ihh) comparable to that in intact uteri. These results, in conjunction with earlier studies of regulation of expression of Ihh, Ptc1 and Nr2f2 mRNAs and proteins indicate that progesterone binds PGR in stromal cells to induce epithelial Ihh which then induces Ptc1 and Nr2f2 in stromal cells to initiate a cell signaling cascade critical to expression of genes necessary for implantation and decidualization. As noted by Simon et al. (2009), a central remaining question is related to the mechanism whereby progesterone stimulates Ihh mRNA expression, i.e. does this require an autocrine or paracrine action of a progesteromedin?

Progesterone may also signal in the endometrium through a mechanism(s) that is independent of the classical PGR pathway (Pru, 2009). Indeed, studies of Pgr-null mice revealed that some actions of progesterone are not accounted for by activation of PGRs (Losel et al., 2003). For instance, it is accepted that classical PGRs mediate genomic responses to progesterone in the uterus, but Ihh, a gene essential for uterine receptivity and fertility in mice, is transiently up-regulated in endometrial epithelia of PGR-deficient female mice in response to progesterone treatment in vivo, indicating that even progesterone-dependent genomic responses in the uterus are not exclusively coordinated by classical PGRs (Matsumoto et al., 2002). There are two families of non-classical progesterin receptors in the uterus which may play a role in non-classical progesterone signaling that is complimentary to or independent of classical effects of progesterone mediated via PGR (Fernandes et al., 2005; Zhang et al., 2008).

### Progesterone, estrogen and interferons

A common feature of the peri-implantation period of pregnancy in domestic animals, rodents and primates is production of a Type I and/or a Type II IFN by conceptus Tr that induces and/or stimulates expression of IFN-stimulated genes (ISGs) in the uterus in a temporal and cell-specific manner. Although IFNT is the only known IFN to act as the pregnancy recognition signal, other IFNs may affect uterine receptivity to implantation, decidualization and placental growth and development in primates, ruminants, pigs and rodents (Bazer et al., 2008, 2009a). All Type I IFNs bind a common receptor composed of two subunits, IFNAR1 and IFNAR2, to induce cell signaling via the janus activated kinases (JAKs) and tyrosine kinase 2 (TYK2) pathways, respectively (Darnell et al., 1994). Signaling by Type II IFNG involves activation of JAK1 and JAK2 associated with IFNGR1 and IFNGR2 subunits of Type II IFNR, respectively. IFNG stimulates autophosphorylation and subsequent tyrosine phosphorylation and homodimerization of STAT1 which translocates to the nucleus and bind GAS elements in promoter regions of IFNG-regulated genes (Leanza et al., 2007). IFNs are expressed by human placenta (IFNA, IFNB, IFNG), decidua (IFNA, IFNB and IFNG) and fetal membranes (IFNA, IFNG), as well as conceptus Tr of sheep (IFNT), pig (IFND and IFNG), horse (IFND) and rodent uter and/or conceptuses (IFNA, IFNB) (Bazer et al., 2008, 2009b). These IFNs have classical antiviral, anti-proliferative and immunosuppressive effects, as well as unique biological activities.

It is now clear that Type I IFNs activate unique cell-specific signaling pathways to differentially affect gene expression in LE/sGE, GE and stromal cells of the uterus (Spencer et al., 2008; Bazer et al., 2009b). In the ovine uterus limited expression of IFNT-stimulate genes is due, at least in part, to expression of IRF2, a potent inhibitor of transcription, in uterine LE/sGE (Choi et al., 2001). In spite of expression of IRF2 in ovine uterine LE/sGE, many ISGs are progesterone-induced and further stimulated by IFNT in these cells, perhaps due to both prostegastemides and IFNT activating MAPK and PI3K cell signaling (Platianis, 2005; Spencer et al., 2007; Bazer et al., 2008, 2009b). These genes include those for glucose transport (SLC2A1 and SLC5A1) and amino acid transport (SLC7A1 and SLC7A2) that increase abundance of glucose, leucine and arginine in that uterine lumen. These nutrients can then stimulate proliferation of Tr cells by activating the glutamine:fructose-6-phosphate amidotransferase (GFAT)-mediated FK506 binding protein 12-rapamycin associated protein 1 (FRAP1, mTOR or MTOR) signaling pathway (Wen et al., 2005). Arginine is also essential for fetal-placental growth and development through effects on synthesis of nitric oxide (NO) and polyamines that stimulate vascular functions and DNA and protein synthesis for proliferation and differentiation of cells, respectively (Wu et al., 2004). Other genes induced by progesterone and further stimulated by IFNT in ovine uterine LE/sGE include LGALS15, cathepsin L (CSTL), cystatin C (CST3), hypoxia inducible factors 2A (HIF2A), gastrin-releasing peptide (GRP), HSD11B1 and IGFBP1. In contrast, major histocompatibility complex class I molecules and β2-microglobulin that regulate immune rejection responses are silenced in LE/sGE, perhaps to protect the conceptus allograft. Details on regulation of expression and potential functions of these genes have been reviewed (Spencer et al., 2007; Bazer et al., 2008, 2009b).

Estrogens and IFNs regulate endometrial genes that affect conceptuses during pregnancy in pigs (Johnson et al., 2009). Estrogens secreted by pig conceptuses induce SPP1 expression in uterine LE, whereas stromal induction of STAT1 is coordinate with secretion of IFNG and IFND by pig conceptus Tr. Indeed, estradiol induces SPP1 mRNA in endometrial LE (White et al., 2005), although intrauterine delivery of conceptus secretory proteins containing IFND and IFNG in cyclic pigs treated with exogenous estrogen increases expression of STAT1 (Joyce et al., 2007). Up-regulation of SPP1 within uterine LE and STAT1 within stroma and GE is unique to uterine LE in close proximity to a conceptus which implies paracrine regulation of these genes by conceptus estrogens and IFNs. In contrast, initial increases in expression of STAT1 in stromal cells are restricted to sites of contact between the conceptus and uterus although IFNG synthesis...
and secretion by pig conceptuses is similar in magnitude to IFNT production by sheep conceptuses (Joyce et al., 2007). Indeed, STAT1 expression increases universally in the stroma and GE of pregnant ewes, presumably due to secretion of abundant amounts of IFNT by conceptus Tr (Spencer et al., 2007). Perhaps the spatial pattern of STAT1 expression in the pig uterus requires that IFND and IFNG act synergistically to up-regulate expression of ISGs. Interactions between Type I and Type II IFNs on cell signaling (Decker et al., 1989) may allow IFNG to act on uterine stroma and GE to increase intracellular IFN-stimulated gene factor 3 and permit low amounts of IFND to up-regulate STAT1 expression in close proximity to implanting pig conceptuses. Both estrogen- and IFN-stimulated genes have been localized in pig endometrium (Johnson et al., 2009). Type I and Type II interferons each induce expression of largely non-overlapping sets of genes and they may also have synergistic interactions to affect physiological responses (Levy et al., 1990). Although IFNG may enhance uterine receptivity to implantation in pigs, highly localized and abundant expression of IFNG, TNFA, IL1B and IL1R has been linked to arrested conceptus development between Days 15 and 23 of pregnancy (Wessels et al., 2007).

**Nutrients, nutrient sensing pathways, growth factors and ECM Molecules affecting growth and development of the conceptus**

Conceptus growth and development requires amino acids, glucose, fatty acids, vitamins and minerals. Before placentaion, these nutrients are transported from maternal plasma into the uterine lumen. Thereafter, they are supplied to the fetus through the umbilical circulation. Both amino acids and glucose are major sources of energy for the embryo/fetus and amino acids are building blocks of proteins and some of them (e.g. branched-chain amino acids, glutamate, serine and proline) undergo extensive catabolism in the placenta (Wu et al., 2009). Glutamine is the most abundant amino acid in fetal plasma and is present at exceedingly high concentrations in fetal fluids (2–20 mM) (Wu et al., 1996). Interestingly, the placenta of sheep, pigs and rats have a limited ability to degrade glutamine due to the lack of glutaminase and use arginine in a species-dependent manner. For example, the ovine placenta actively degrades arginine by arginase, but the porcine placenta lacks this pathway (Wu et al., 2004). Notably, the ovine conceptus uses citrulline as an effective precursor of arginine to support fetal growth (Lassala et al., 2009).

mTOR/FRAP1 cell signaling pathway is an evolutionarily conserved serine/threonine kinase located downstream of PI3K that is central to control of cell growth and proliferation through regulation of mRNA translation for protein synthesis and cell proliferation (Wullschleger et al., 2006). Cellular events directly controlled by the mTOR pathway include mRNA translation, ribosome synthesis, expression of metabolism-related genes, autophagy and cytoskeletal reorganization (Kim et al., 2002). During embryonic development, molecules that stimulate mTOR activity may also stimulate translation of mRNAs critical to blastocyst/conceptus development, including IGF2 and actions of selected amino acids as mTOR is a ‘nutrient sensing system’ (Martin and Sutherland, 2001). Cell signaling via mTOR stimulates cell migration and invasion, as well as cell growth and proliferation in different cell types (Liu et al., 2008). In fact, mTOR/Frap1 null mice die shortly after implantation due to impaired cell proliferation and hypertrophy in both the embryonic disc and trophoblast (Murakami et al., 2004).

Nutrients are essential components of histotroph required for development and survival of conceptuses during pregnancy (Bazer et al., 2008, 2009b; Wu et al., 2009). A systematic study of temporal and cell-specific changes in expression of transporters for glucose and amino acids, their regulation by progesterone and/or IFNT, changes in expression of NOS isoforms, ODC and related proteins, as well as components of mTORC1 and mTORC2 cell signaling in ovine uteri and conceptuses revealed that: (i) total recoverable glucose, Arg, Leu, Gln, glutathione, calcium and sodium are more abundant in uterine fluids of pregnant than cyclic ewes between Days 10 and 16 after onset of estrus or mating; (ii) uteri and conceptuses express tissue and cell-specific facilitative and sodium-dependent transporters for glucose, as well as for cationic, acidic and neutral amino acids, some of which are regulated by progesterone or progesterone and IFNT; (iii) transport of Arg into the uterine lumen and uptake by conceptuses is by System y+ (SLC7A1, 2 and 3) cationic amino acid transporters; (iv) NOS1 and ODC1 are most abundant in uterine LE/sGE although NOS3 is most abundant in Tr and endometrial conceptuses; (v) expression of GCH1, the key enzyme for synthesis of tetrahydrobiopterin, a cofactor for NO production, ODC1 and NOS1 is more abundant in conceptuses than endometrial cells; and (vi) progesterone stimulates expression of NOS1 and GTP cyclohydrolase (GCH1), although IFNT inhibits expression of NOS1. Further, components of both the mTORC1 and mTORC2 cell signaling pathways (FRAP1, LST8, MAPKAP1, RAPTOR, RICTOR, TSC1, TSC2, RHEB and EIF4EBP1) are localized to uterine LE/sGE, GE and stromal cells, as well as Tr and endometrial conceptuses between Days 13 and 18 of pregnancy. The abundance of FRAP1, RAPTOR, RICTOR, TSC1 and TSC2 mRNAs in endometria was not affected by pregnancy status or day of the estrous cycle or pregnancy; however, increased expression of LST8, MAPKAP1, RHEB and EIF4EBP1 mRNAs only occurred in endometria during early pregnancy. Further, progesterone and IFNT stimulate expression of RHEB and EIF4EBP1 in uterine endometria. Importantly, FRAP1 was abundant in cytoplasm and phosphorylated FRAP1 was very abundant in nuclei of ovine Tr cells and endoderm, and increases in abundance of RICTOR, RHEB and EIF4EBP1, as well as RHEB protein in endometria were coordinate with rapid conceptus growth and development during the peri-implantation period. These results suggest differential effects of mTORC1 and mTORC2 on elongation of ovine conceptuses.

In related studies (Satterfield et al., 2008, 2009b), an early increase in circulating levels of progesterone accelerates blastocyst growth and development in ewes that is associated with increases in total recoverable glucose, aspartate (acidic amino acid), Arg and lysine (basic amino acids), and citrulline, asparagine, serine, Gln, beta-alanine and alanine (neutral amino acids) in uterine flushings on Day 9 of pregnancy compared with control ewes. However, on Day 12 of pregnancy, only Arg and lysine were more abundant in uterine flushings from progesterone-treated ewes as were transporters for glucose (SLC2A1 and SLC5A1) and Arg (SLC7A2B) in uterine LE/sGE on both Days 9 and 12. These novel results indicate that progesterone-induced advances in transport of select nutrients, particularly Arg and glucose, into the uterine lumen on Days 9 and 12 of pregnancy are coordinate with advanced conceptus development.
These findings are supported by unpublished results (J. Kim, G. Wu, G. Johnson, T.E. Spencer and F.W. Bazer) from in vitro studies with ovine Tr1 cells indicating novel cell signaling whereby: (i) Arg activates mTOR cell signaling and phosphorylation of ribosomal protein kinase (RPSK); (ii) Arg, Leu and glucose increase phosphorylation of AKT1, GSK3B, FRAP1 and RPS6K proteins; (iii) Arg increases the abundance of pRPS6K and pRPS6 in the cytoplasm of oTr cells; and (iv) cell proliferation was independent of NOS, but dependent on production of polyamines. Both NO and polyamines are critical for implantation and development of conceptuses. Our studies of pathways for their production revealed effects of the estrous cycle, pregnancy, progesterone and IFNT on expression of NO synthases (NOS1, NOS2, NOS3), GTP cyclohydrolase (GCH1, the key enzyme in de novo synthesis of BH4, a cofactor for NO production), and ODC1 in uterine endometria from cyclic and pregnant ewes. NOS1 and ODC are most abundant in uterine LE/sGE whereas NOS3 is abundant in Tr and endoderm of ovine conceptuses, as are total NOS1 and NOS3 proteins, inhibitory phosphorylated (p) p-NOS1 protein and stimulatory p-NOS3 protein. GCH1 is abundant in Tr and endoderm of conceptuses between Days 13 and 15 of pregnancy, but decreases whereas ODC1 abundance increases between Days 13 and 18 of pregnancy. Progesterone stimulates NOS1 and GCH1 expression in ovine uterine LE/sGE and GE, although IFNT inhibits NOS1 expression in these cell types. Thus, bio-synthesis of NO and polyamines in ovine uterine endometria and conceptuses is regulated at transcriptional, translational and post-translational levels to favor conceptus development and implantation.

Insulin-like Growth Factor 2 (IGF2), an imprinted and paternally expressed gene in the fetus and placenta of mice, humans and sheep, regulates fetal and placental growth and differentiation, extravesicular trophoblast migration/invasion, and nutrient transfer through placental exchange mechanisms (Kim et al., 2008). In mice, deletion of Igf2 in the labyrinthine trophoblast decreased fetal-placental growth and placental exchange of nutrients (Constância et al., 2002). Further, altered expression of IGF2 in human trophoblast cells during early pregnancy is associated with intrauterine growth restriction, premature delivery and pre-eclampsia (Smith et al., 2002).

In pregnant ewes, IGF2 mRNA is most abundant in caruncular endometrial stroma; however, in intercaruncular endometrium, its expression transitions from uterine stroma to LE between Days 14 and 20 of pregnancy (Kim et al., 2008). IGF2 is present in all cells of the conceptus, but particularly abundant in primitive endoderm and yolk sac early in pregnancy and in the choioallantois and LE of ewes during mid- to late-pregnancy. Abundant amounts of IGF2 at the interface between cotyledonal and caruncular sides of placenomes suggest a role in conceptus development and fetal-placental development. For example, IGF2 regulates nutrient transport (glucose transporters and amino acid transporters) by the placenta to meet fetal demands, perhaps via a positive regulatory feedback loop between IGF2 and mTOR (Gingras et al., 2004). In support of this, IGF2 increases abundance of p-PDK1, p-AKT1, p-GSK3B, p-FRAP1 and p-RPS6K proteins in ovine Tr cells that is coordinate with rapid increases in p-ERK1/2 and p-P38 MAPK proteins, as well as proliferation and migration of ovine Tr1 cells (Kim et al., 2008). Thus, IGF2 may coordinately activate multiple cell signaling pathways critical to survival, growth and differentiation of mammalian conceptuses during early pregnancy. Further, available evidence suggests novel converging pathways whereby Arg, SPP1 and IGF2 may activate both mTORC1 (cell proliferation and mRNA translation) and mTORC2 (cytoskeletal alterations, cell migration and cell survival) in ovine Tr cells (Bazer et al., 2009b).

Transport of nutrients into the uterine lumen and conceptus
Research with domestic and laboratory animals has revealed that many components of uterine luminal fluid are required for conceptus development. In fact, conceptuses fail to develop beyond Day 14 of pregnancy in ewes that lack uterine glands and their secretions (Gray et al., 2002) that include nutrients (amino acids, glucose, essential fatty acids, vitamins and minerals), proteases, protease inhibitors, transport proteins for nutrients and minerals, cytokines, lymphokines and growth factors (Bazer, 1975; Roberts and Bazer, 1988; Mahan and Vallet, 1997; Spencer et al., 2008; Satterfield et al., 2009a). Vitamins and/or their transport proteins identified in uterine secretions, allantonic fluid and blood of fetuses include those for riboflavin, thiamin, niacin, biotin, cobalamin, retinol and retinoic acid, Vitamin E, ascorbic acid, folic acid, vitamin D and vitamin K (Mahan and Vallet, 1997; Vallet et al., 1998). Folic acid has received considerable attention as it is important in reproductive health of humans and animals by participating in the metabolism of one-carbon units and amino acids including homocysteine, methionine, glycine, serine and histidine (Tamura and Picciano, 2006; Forges et al., 2007). Folic acid is a required cofactor in the transfer of methyl groups and metabolites of folic acid are required for synthesis of the purine ring, methionine (from homocysteine) and thymidine essential for cell division, fetal growth and erythropoiesis (Vallet et al., 1998; Kim and Vallet, 2004). In pigs, both secreted and placental membrane forms of folate-binding protein are expressed in the intrauterine environment during pregnancy (Kim and Vallet, 2004). Deficiencies in folic acid are associated with birth defects. Mice in which the folate binding protein has been deleted die in utero during the peri-implantation period and are resorbed (Spiegelstein et al., 2004). Treatment of dams deficient in folate binding protein before and throughout gestation with folic acid (N5-formyl-tetrahydrofolate) prevented premature death of most embryos. For folate binding protein null embryos, maternal supplementation with various forms of folic acid could rescue the phenotype to some extent, but surviving fetuses had a high frequency of neural tube defects, as well as malformations of craniofacial structures, eyes and abdominal wall.

Conceptus Tr development and endogenous retroviruses: cellular and molecular regulators of mononuclear Tr proliferation and differentiation into trophoblast binucleate cells
The endogenous retroviruses (ERVs) are now implicated in development and differentiation of conceptus Tr in humans, rodents and sheep (Spencer et al., 2007). During the course of evolution, all vertebrates have been exposed to multiple waves of cross-species infection by exogenous retroviruses. Some of those viruses infected germ cells and are inherited in an integrated, proviral form (Boeke and Stoye, 1997). These ERVs have undergone further amplification and now make up a greater fraction of our DNA than do normal coding sequences (Jern and Coffin, 2008). Although once considered junk
DNA, it is now clear that ERVs have important biological roles in protection against retroviral infection (Best et al., 1997) and placental development (Harris, 1998; Rawn and Cross, 2008).

Syncytin-1 and -2, a product of the two human ERV envelope (env) genes that entered the primate lineage 25–40 million years ago, was discovered in 2000 as a captive retroviral protein expressed in human placental cells (Knerr et al., 2004). Both of the human ERV env genes encode highly fusogenic retroviral Env proteins (syncytin-1 and -2), possibly involved in the formation of the placenta syncytiotrophoblast layer generated by trophoblast cell fusion (Blond et al., 2000; Mi et al., 2000). Similarly, mice have two ERVs, syncytin-A and -B, that are expressed in syncytiotrophoblast and also elicit cell-cell fusion in vitro (Dupressoir et al., 2005). Syncytin-A plays an important biological role in syncytiotrophoblast development, because syncytin-A null mice die in utero, apparently as a result of the failure of trophoblast cells to fuse and form one of the two syncytiotrophoblast layers present in the placenta (Dupressoir et al., 2009). The syncytiotrophoblast layers of mammalian placentae play a key role in transport of nutrients for the developing conceptus (Watson and Cross, 2005).

The ERVs also appear to play an essential role in placental development in sheep. Jaagsiekte sheep retrovirus (JSRV) is a pathogenic exogenous retrovirus and the causative agent of ovine pulmonary adenocarcinoma (Dunlap et al., 2006a; Arnaud et al., 2008). The sheep genome contains at least 27 copies of endogenous retroviruses (enJSRVs) highly related to JSRV. The earliest hints that enJSRVs could participate in some aspect of uteroplacental biology came from the observation that their RNA was particularly abundant in organs of the reproductive tract (Palmarini et al., 2004). The highest levels of enJSRVs RNA are expressed in uterine LE and GE, as well as in the epithelia of the oviducts and cervix. Lower levels of enJSRVs RNA are also detected in vaginal epithelia. In the conceptus, enJSRVs RNA is detectable in mononuclear Tr, but more abundant in trophoblast giant binucleate cells (BNCs) and multinucleated syncyta that form the fetal part of placentomes for nutrition of the conceptus (Dunlap et al., 2006b). Of particular note, hyaluronidase 2 (HYAL2), a cell-surface receptor for the exogenous JSRV and enJSRVs envelope proteins is expressed by BNC and syncytial plaques in sheep placentae, but not uterine epithelia, stroma or myometrium. Expression of enJSRV env in conceptus Tr begins on Day 12 of pregnancy which is coincident with the onset of conceptus elongation and production of IFNt for pregnancy recognition signaling (Dunlap et al., 2006a). Most interestingly, inhibition of enJSRVs Env production by morpholino antisense oligonucleotides in utero retards conceptus growth and elongation and inhibits trophoblast giant BNC differentiation, which culminates in loss of pregnancy (Dunlap et al., 2006a). These results, together with the fact that HYAL2 mRNA, which functions as a cellular receptor for both JSRV and enJSRVs Env, is detected in the trophoblast giant BNC and multinucleated syncytia of the conceptus, suggest that expression of enJSRVs Env and HYAL2 is important for growth and differentiation of conceptus Tr in sheep (Fig. 1).

The abundant expression of ERVs in human and mouse placenta, in particular the presence of intact env genes in the syncytiotrophoblast, which have been preserved over millions of years, together with the observation that they elicit fusion of cells in vitro, suggests that independent ERVs were positively selected for a convergent biological role in placental morphogenesis during evolution (Harris, 1998; Knerr et al., 2004; Rawn and Cross, 2008).

In the endometrium, enJSRV expression fluctuates during the estrous cycle and early pregnancy, but increases in abundance between Days 1 and 13 as concentrations of progesterone in plasma increase (Palmarini et al., 2001). Moreover, long terminal repeats of some enJSRV loci have intact open reading frames for all their genes, it is plausible that enJSRV-derived viral particles are shed into the uterine lumen. Indeed, viral particles have been observed in uterine epithelia and ovine conceptus Tr, but it is not known whether these particles have any biological function (Kalter et al., 1975).

Thus, evidence from studies of primates, sheep and rodents suggests that ERVs influence mammalian evolution through effects on placental morphogenesis as retroviral env genes were co-opted to influence placental development. Given the structural diversity among the placentae of different orders of mammals, one might speculate that specific roles played by these viral genes may differ, perhaps depending on cellular expression patterns of ERV env and their specific receptors. Indeed, proviral inheritance may provide a better predictor of the diversities among syncytial morphologies than taxonomy (Stoye, 2009).

**Vascular regulation and angiogenesis**

The mammalian placenta undergoes rapid formation of new blood vessels from existing ones (angiogenesis) (Reynolds et al., 2006) to increase the supply of nutrients and oxygen from mother to fetus. Successful pregnancy requires complex remodeling of the endometrium to orchestrate implantation and ensuing angiogenesis that provides hematotrophic support for the developing conceptus (Charnock-Jones et al., 2004; Kaufmann et al., 2004; Red-Horse et al., 2004). Placenta facilitates these events and is defined as the expansion and juxtaposition of the microcirculatory systems of the uterus and placenta to optimize exchange of nutrients, gasses and metabolic wastes (Breier, 2000). Dysregulated endometrial angiogenesis underlies infertility in (i) endometriosis (Sirisatidis et al., 2006); (ii) fetal undernutrition that may lead to adult onset of diseases including cardiovascular disease and type 2 diabetes (Barker, 2004); and (iii) rudimentary endo-vascular invasion that contributes to pre-eclampsia (Norwitz, 2006). Thus, an understanding of signals that promote uterine and placental angiogenesis in pregnancy is critical.

In ewes, progesterone induces hypoxia inducible factors (HIF1A and HIF2A) and IFNt further stimulates expression of HIF2A in uterine LE/sGE (Song et al., 2008). HIF functions to control expression of over 200 genes, including EPO, CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), VEGF, solute carrier family 2 (facilitated glucose transporter) member 1 (SLC2A1; also termed GLUT1), and IGF2. Mice deficient in Hif1a, Hif2a, or aryl hydrocarbon receptor nuclear translocator (Amt) die at mid-gestation from vascular defects primarily involving embryonic and extraembryonic vasculature (Semenza, 2000). In contrast, mice deficient in Hif2b or Hif3b do not exhibit vascular abnormalities (Cowden and Simon, 2002). These results suggest that Vegf is primarily regulated by HIF1A, HIF2A and ARNT during embryonic development.

The placenta and the uterus produce classical angiogenic factors that include VEGF, basic fibroblast growth factor (FGF2), angiopoietins
(ANG), and their receptors that affect vascular development in the reproductive tract of sheep (Borowicz et al., 2007). Both VEGF and FGF2 are major angiogenic growth factors of the placenta and uterus that account for most of the heparin-binding angiogenic activity. VEGF stimulates vascular permeability, vascular endothelial cell migration and protease production to enhance angiogenesis. Mice lacking VEGF receptors (VEGFR-1 or flt-1 or VEGFR-2 or flk-1) experience defects in fetal-placental vasculogenesis and angiogenesis resulting in embryonic death by mid-gestation. Basic FGF also stimulates proliferation of both uterine and fetal placental arterial endothelial cells. Both VEGF and FGF2 likely regulate uterine and placental blood flow by stimulating production of NO by endothelial cells and NO, in turn, stimulates expression of VEGF and FGF2. ANG regulates vascular growth and development by activating Tie2-mediated cell signaling. Ang1 null mice exhibit cardiovascular defects and die by mid-gestation. ANG1 does not stimulate endothelial cell proliferation, but affects microvascular organization, stabilization and endothelial cell survival required for vascular remodeling.

Several other key signaling pathways that mediate angiogenesis are in response to molecules that include prostanoids (Jabbour et al., 2006), angiotensin (Ino et al., 2006; Sugawara and Ito, 2006), as well as integrins and metalloproteinases (Bayless and Davis, 2003).

Sphingosine-1 phosphate, another potent inducer of angiogenesis (Hla, 2004; Langlois et al., 2004) is emerging as a novel target in anticancer therapies (Milstien and Spiegel, 2006; Sabbadini, 2006; Visentin et al., 2006). S1P signals through activation of one or more of its five known high-affinity G-protein-coupled receptors, S1P1–S1P5 (Spiegel and Milstien, 2003a). S1P1 and S1P3 are expressed in endothelial cells and mediate angiogenic responses in multiple systems (LaMontagne et al., 2006), although the S1P2 receptor functions in an angiostatic capacity (Inoki et al., 2006). Sphingosine kinase 1 (SPHK1) phosphorylates sphingosine to biologically active S1P whereas S1P phosphohydrolases (SPP1 and SPP2) and sphingosine lyase (SPL) dephosphorylate S1P back to sphingosine. Importantly, the S1P pathway is highly evolutionarily conserved (Spiegel and Milstien, 2003b). Results from studies of transgenic mice revealed that expression of S1P receptors and modulating enzymes are important for angiogenesis, endometrial development and placentation (Kono et al., 2004). S1P1, S1P2, S1P3 and SPHK1 are up-regulated as pregnancy progresses and up-regulation of S1P synthesis is
coordinate with uterine decidualization between Days 5.5 and 7.5 of pregnancy in mice (Kaneko-Tarui et al., 2007; Mizugishi et al., 2007). Interestingly, S1P and its metabolites and modifying enzymes exhibit cross-talk with various signaling pathways. Ceramide, a sphingosine metabolite, induces PTGS2 (Bailou et al., 1992) which is the rate-limiting enzyme for prostanoid biosynthesis (Simmons et al., 2004). Further, S1P induced PTGS2 in human amniotic cells (Kim et al., 2003), predecidualized uterine stromal cells (Skaznik-Wiik et al., 2006), smooth muscle cells (Hsieh et al., 2006) and human chondrocytes (Masuko et al., 2007). Serrano-Sanchez et al. (2008) reported a correlation between SPHK activity and PTGS2 induction in rat myometrium. Inside-out signaling of S1P can be induced by receptor tyrosine kinase activation by various growth factors, where growth factor ligation of receptor via VEGF, TGFβ and IGF leads to SPHK activation and translocation to the membrane, which induces local production of S1P to stimulate G-protein coupled receptors (Lehman and Spiegel, 2008). Interestingly, S1P prevents HIF degradation independent of oxygen (Michaud et al., 2009) and, under hypoxic conditions, SPHK1 can modulate HIF levels (Ader et al., 2009). Also, S1P stimu-
lates eNOS translocation to the membrane and production of NO (Rikitake et al., 2002). We have shown that S1P synergizes with angiogenic growth factors (Bayless and Davis, 2003; Su et al., 2008) and wall shear stress to increase Akt phosphorylation and sprouting responses by endothelial cells (Kang et al., 2008). Akt is activated downstream of S1P (Lee et al., 2001) and phosphorylates eNOS on Ser1179 (Fulton et al., 2002) which leads to NO production, angiogenesis and vasodilation (Morbidelli et al., 2003). Thus, the S1P signaling pathway is intricately linked to other key signaling pathways, including growth factors, prostanoids, NO and HIFs, which render the S1P pathway perfectly suited to regulate angiogenic responses.

Following successful elongation of the conceptus, trophoblast outgrowth and implantation, the placenta of ruminants organizes into placentomal and interplacentomal regions. Development of placentomes, although morphologically distinct from humans, is driven by local alterations in integrins and ECM proteins common to the maternal-fetal interface of both primates and ruminants (Johnson et al., 2003a; Carson et al., 2006; Fouk et al., 2007). During placentome development, highly branched villous tree-like placental folds, termed cotyledons form by Day 20 of gestation (Cross et al., 2003). Cotyledonary chorionic villi lined by syncytiotrophoblast then protrude into crypts in the maternal endometrial caruncular tissue (a glandular area of the endometrium consisting of stroma covered by a single layer of LE) resulting in extensive interdigitation of endometrial and placental tissues by Day 40 of gestation to form a placenta that is somewhat analogous to the decidua in humans. Failure of placentomes to develop results in fetal loss because they allow for close proximity of maternal and fetal blood vessel interdigitation for exchange oxygen and nutrients (Wu et al., 2004; Reynolds et al., 2005). Approximately 90% of the blood from the uterine artery and umbilical vein is directed into the vasculature of maternal caruncules and fetal cotyledons, respectively (Caton et al., 1983). The doubling of capillaries per unit tissue in the caruncle from Days 50 to 70 coincides with an initial peak of NO production, whereas VEGF levels are declining (Kwon et al., 2004; Vonnahme et al., 2005). This suggests that another pro-angiogenic factor, such as S1P, is responsible for NO production and angiogenesis between Days 50 and 70. Treatment of ewes with a specific S1P receptor antagonist, FTY720, inhibits development of caruncles in placentomes (K. Bayless and G.A. Johnson, unpublished results).

Polyamines and NO are also essential to placental growth and angiogenesis as rats fed Arg-free diets experience reduced NO synthesis, increased fetal resorptions, IUGR, increased perinatal mortality and decreased numbers of live pups at birth (Wu et al., 2005, 2008, 2009). Also, oral administration of Arg (3 g daily for 4 weeks) to women with pre-eclampsia increased NO synthesis which was associated with reduced blood pressure, prolonged pregnancy, improved fetal well-being, enhanced fetal growth and uterine quiescence to prevent preterm labor (Wu et al., 2009). NO is a key regulator of angiogenesis during pregnancy that is derived from eNOS and/or iNOS expressed by placenta of rodents, humans, pigs and sheep. Placental synthesis of NO (the major vasodilator), like that of polyamines (other products of Arg catabolism), is essential for placental angiogenesis and growth) and increases markedly between Days 30 and 60 of gestation when placental growth and placentomal development are most rapid (Kwon et al., 2003). Inhibition of NO or ornithine decarboxylase (a key enzyme in polyamine synthesis) activity during early pregnancy markedly reduces placental size that leads to IUGR in rats. Increases in NO synthesis in sheep placentomes from Day 100 of gestation are coordinate with significant increases in placental-fetal blood flow and rapid fetal growth. Intriguingly, in ovine placentae and endometria, both NADPH and BH4 levels increase markedly between Days 40 and 60 of gestation along with increases in concentrations of citrulline (the precursor of arginine) and arginine in allantoic fluid. Between Days 80 and 100 of gestation, BH4 concentrations also increase in placentomes and endometria, as do concentrations of arginine in allantoic fluid. Arginine is a potential activator of the pentose cycle activity and a stimulator of endothelial GTP-CH expression critical for regulating the synthesis of NADPH and BH4 and NO production in placenta and endometrium which prevent or ameliorate IUGR and development of hypertension and pre-eclampsia (Wu et al., 2009).

In summary, this review addresses novel mechanisms responsible for conceptus-endometrial interactions during pregnancy. However, there are many events for which mechanisms are yet to be discovered. There is a gap in our knowledge about the requirement for loss of expression of PGR by endometrial epithelia as a prerequisite for implantation, expression of genes for secretory proteins, and selective transport of molecules into the uterine lumen to support conceptus growth and development for successful establishment of pregnancy. Thus, there is a clear need to identify the progestamedin or estramedin unique to each species and to understand mechanisms whereby they exert paracrine effects on uterine epithelia individually and in concert with cell signaling pathways activated by secretions from the conceptus such as IFNs, lactogenic hormones, and prostaglandins. Comparative reproductive biology is necessary to advance understanding of these mechanisms. For example, the ewe is a proven model for research to understand the roles of IFNs during the peri-implantation period because Tr or immune cells as the sites of implantation of most, if not all mammals, are now known to express Type I and/or Type II IFNs. Thus, IFN-stimulated genes are among the mostly highly up-regulated genes in human decidualized stromal cells treated with trophoblast conditioned medium and in uteri of domestic and laboratory animals. Understanding effects of ovine IFNT on gene expression in the uterus will advance our understanding of novel
mechanisms whereby progesterone and IFNs directly or indirectly act on cells of the reproductive system to induce ISGs critical to establishment and maintenance of pregnancy in mammals. Similarly, understanding the roles of novel endogenous retroviruses in reproductive tissues will advance our understanding of their roles in implantation, placentation and the endocrinology of pregnancy. This knowledge is essential for translational research into strategies to enhance reproductive efficiencies and reproductive health in humans and animals.

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