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Uterine receptivity to implantation of blastocysts in mammals

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1. ABSTRACT

Reproduction in mammals is a highly complex biological process. The critical importance of reproduction to propagation of species required the natural evolution of various strategies that vary considerably across species. Regardless of species, a dialogue between the developing conceptus (embryo-fetus and associated placental membranes) and maternal uterus must be established during the peri-implantation period. The uterus must provide a microenvironment that supports growth and development of the conceptus and is receptive to implantation. During the same period, the conceptus must provide its pregnancy recognition signaling to sustain the functional life of corpora lutea for production of progesterone which is essential for implantation and placentation; critical events for successful pregnancy. However, it is within the peri-implantation period that most embryonic deaths occur due to deficiencies attributed to uterine functions or to the failure of the conceptus to develop appropriately, signal pregnancy recognition and/or undergo implantation and placentation. The challenge is to understand the complexity of key mechanisms that are characteristic of successful reproduction in humans and animals and to use that knowledge to enhance fertility and reproductive health or to establish acceptable methods for control of fertility.

2. OVERVIEW: PREGNANCY RECOGNITION SIGNALING FOR ESTABLISHMENT OF PREGNANCY AND IMPLANTATION

2.1. Primates

Pregnancy recognition signaling in primates extends corpus luteum (CL) function at least until the time of the luteal-placental shift when production of progesterone (P4) by the placenta is adequate to support pregnancy (1,2). Primate blastocysts begin implantation following attachment to uterine luminal epithelium (LE) on Days 7 to 9 post-ovulation in macaques and humans or Days 11 to 12 in marmoset monkeys. Syncytiotrophoblast cells of human conceptuses secrete chorionic gonadotropin (CG) from Days 8 to 10 for pregnancy recognition and implantation begins on Days 7 to 9 post-ovulation. CG produced by primate blastocysts signals maternal recognition of pregnancy through its luteotrophic actions

via luteinizing hormone CG receptor (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 (GnRH) from the uterus as GnRH receptors are detectable in placental tissue. Importantly, GnRH receptor agonists enhance and antagonists suppress CG secretion. In many primates, CG production decreases at the time of the luteal-placental shift in production of P4. Further, exogenous CG increases P4 production and extends CL lifespan in both women and monkeys.

2.2. Rodents

In rodents, mating induces release of prolactin (PRL) from the anterior pituitary and it is the initial luteotrophic signal for CL formation and production of P4 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 P4 (3). The gestation period for rats, mice and hamsters is 20 to 22 days, and functional CL are required for production of P4 through Day 17. Thus, maintenance of functional CL and the production of P4, requires two endocrine events in rodents: 1) mating-induced diurnal and nocturnal surges of PRL that increase LH receptors on luteal cells for formation of CL and suppress 20 alpha-hydroxysteroid dehydrogenase activity to prevent conversion of P4 to 20 alpha-hydroxy P4 that does not support pregnancy or uterine decidualization; and 2) production of lactogenic hormones by uterine decidua and placentae act via receptors for prolactin on CL to maintain production of P4 throughout gestation.

2.3. Ruminants

Interferon tau (IFNT), the pregnancy recognition signal in ruminants, suppresses transcription of estrogen receptor alpha (ESR1) and, therefore, estrogen-induced expression of the oxytocin receptor (OXTR) gene in uterine LE and superficial glandular epithelium (sGE) to abrogate development of the endometrial luteolytic mechanism involving oxytocin-induced luteolytic pulses of prostaglandin F2-alpha (PGF) (4,5). 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. The absence of PGR in uterine epithelia is required for expression of P4-induced and IFNTstimulated genes in ovine uterine LE/sGE (4,5).

2.3.1. Goats

Caprine IFNT is secreted between Days 16 and 21 of gestation to prevent pulsatile release of luteolytic PGF (6). Intra-uterine injections of ovine IFNT in nanny goats extends CL lifespan.

2.3.2. Cows

Bovine IFNT is secreted between Days 12 and 38 of pregnancy and activates mechanisms that prevent secretion of luteolytic pulses of PGF (7) by uterine epithelia. As for ewes, neither exogenous estradiol (E2) nor OXT stimulate uterine release of PGF in pregnant cows. This indicates that *ESR1* and *OXTR* mRNAs are either less abundant or not responsive to E2 and OXT in endometria of pregnant as compared to cyclic cows or in cows that received intra-uterine injections of ovine IFNT. In any event, intra-uterine injections of bovine IFNT abrogate uterine production of luteolytic pulses of PGF.

2.4. Pigs

Pig conceptuses secrete estrogens on Days 11 and 12 of pregnancy which activate mechanisms to redirect PGF secretion away from the uterine vasculature towards the uterine lumen (exocrine secretion). 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 antiluteolytic; (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 and/or metabolized to prevent it from acting on CL to cause luteolysis (8). In addition, PGE2 and lysophosphatidic acid (LPA) have been proposed to have 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 (9). In addition LPA: (i) increases in uterine luminal fluids of pigs; (ii) its receptor, EDG7, is expressed by pig conceptuses; and (iii) its expression is increased by estrogen in endometrial epithelia during early pregnancy (10). Indeed, LPA3 is critical for embryo migration and spacing (11), events critical to implantation and placentation in pigs.

2.5. Horse

The equine conceptus produces an unknown factor(s) that inhibits uterine release of luteolytic PGF (12). In cycling mares, concentrations of PGF in uterine venous plasma and uterine flushings increase between Days 14 and 16 when luteolysis occurs and concentrations of P4 in plasma decline. Receptors for PGF (PTGFR) are abundant on luteal cells between Day 14 of the estrous cycle and estrus, as well as Day 18 of pregnancy. The equine conceptus migrates between the two uterine horns until fixation on Day 18 of pregnancy to activate an antiluteolytic mechanism. This results in reduced amounts of PGF in uterine fluids and uterine venous plasma and PGFM in peripheral plasma due to abrogation of the mechanism for pulsatile release of PGF in pregnant mares. Further, the presence of the conceptus abrogates endometrial production of PGF in response to both cervical stimulation and exogenous OXT, indicating the absence of or a reduction in expression of endometrial OXTR in pregnant mares. Equine conceptuses produce increasing amounts of E2 between Days 8 and 20 of gestation; however, attempts to prolong CL lifespan in mares using exogenous E2 have yielded variable results. The equine conceptus also secretes proteins of 400, 65 and 50 kDa between Days 12 and 14 of pregnancy, as well as IFND (13), but the role(s) of the unidentified proteins and IFND in pregnancy recognition signaling are not known.

2.6. Cat

Cats have a bipartite uterus and the male deposits semen in the anterior vagina at ejaculation (14). Ovulation occurs 25 to 50 hours post-coitum (pc) and frequent matings reduce the time to ovulation. Fertilization takes place in the oviduct, up to 48 hours after ovulation and embryos enter the uterus at the blastocyst stage four to six days post-ovulation. Blastocysts hatch from the zona pellucida on Day 11 and implantation occurs on Days 12 to 13 of pregnancy. The cat has an endotheliochorial type placenta with zonary villous distribution. Following mating, concentrations of P4 in plasma increase to 15 to 90 ng/ml between Days 10 and 40 of pregnancy and Days 13 to 30 of pseudopregnancy. Pseudopregnancy typically lasts 40 days whereas the length of gestation is 63 to 65 days, but ranges from 56 to 71 days. By Day 30, circulating levels of P4 are higher in pregnant than pseudopregnant queens.

The placentae of cats does not produce sufficient P4 to maintain pregnancy as ovariectomy on Day 45 results in a rapid decline in circulating P4 and abortion within 6 to 9 days (15). Circulating concentrations of PRL increase in blood during the last trimester of gestation to peak values at parturition (5-10 ng/ml) and remain elevated during lactation in response to the stimulus of suckling. Prolactin is considered an important luteotrophin in late gestation. Relaxin is produced by the fetal-placental unit and concentrations in plasma increase to 5-10 ng/ml during the second half of gestation. Relaxin, acting in concert with P4, maintains a quiescent uterus and facilitates parturition by softening the connective tissues of the pelvis. Following parturition, queens experience anestrus during lactation and resume cycling 2 to 3 weeks after weaning kittens (16).

2.7. Dog

Oöcytes are fertilized 2 to 5 days after ovulation in the bitch and blastocysts enter the uterine lumen on Day 10 where they are free-floating until hatching and implantation on Day 16 (17). The dog has an endotheliochorial placenta with zonary villous distribution. The CL are the primary sources of P4 as both ovariectomy and hypophysectomy at any stage of pregnancy results in abortion. Since CL lifespan in pregnant and pseudopregnant bitches is similar, a pregnancy recognition signal does not seem to be required for CL maintenance during pregnancy (18). Concentrations of P4 in plasma are similar in pregnant and pseudopregnant bitchs prior to implantation, but values for P4 increase post-implantation in parallel with increases in circulating levels of RLX. Secretions of PRL and RLX increase in parallel independent of effects of E2 produced by CL. Prolactin is essential for CL maintenance and function, whereas LH is permissive to CL function. Luteolysis precedes parturition due to release of PGF beginning about 36 hours prepartum, but the roles of estrogens in parturition are not known.

2.8. Rabbit

The rabbit has a duplex uterus so the male deposits semen into the anterior vagina so that sperm can be transported into each uterine horn to fertilize ova which are ovulated from each ovary approximately 10 hours post-coitum (pc) (19). Fertilization occurs at the ampullary-isthmic junction of the oviduct 1 to 2 hours post-ovulation, embryos enter the uterus on Day 3 and blastocysts undergo implantation on Day 7. Rabbits have a hemochorial placenta with discoid villous distribution. The CL are the sole source of P4 required for establishment and maintenance of pregnancy. Following sterile mating, CL form and persist for 14 to 16 days, a period known as pseudopregnancy. For both pseudopregnant and pregnant does, circulating concentrations of P4 increase from Day 2 to Day 8 (12-20 ng/ml), but P4 profiles between pregnant and pseudopregnant does diverge as values decline rapidly to basal levels by Days 16 to 18 of pseudopregnancy. Pregnant does have elevated levels of P4 until 3 to 4 days prior to parturition between Days 28 and 36 pc (kindling). Circulating levels of P4 and E2 are not different between pregnant and pseudopregnant does until after implantation (19).

Maternal recognition of pregnancy in does occurs between Days 10 and 12 pc in response to estradiol (E2) and an unidentified placental luteotropin (20). Luteal cells contain LHCGR; however, LH does not stimulate P4 production *in vivo*. Rather, estrogen exerts its luteotrophic effect by uncoupling P4 production from cyclic AMP (cAMP). If E2 is withdrawn from does, exogenous CG stimulates luteal cAMP and both CG and cAMP stimulate P4 production. The luteotrophic effect of the placenta does not result from increased concentrations or affinity of luteal ESR1 for E2. Rabbit placentae secrete immunoreactive GnRH-like activity which appears to act locally on the uterus, but not directly on luteal cells (21). A 6 to 8 kDa placental luteotrophic factor enhances P4 production by cultured luteal cells either alone or in conjunction with E2 (22). Further, a 12 to 14 kDa rabbit placental luteotrophin that is acidic, trypsin- and heat-sensitive has been reported (23). This factor stimulated production of P4 by luteal explants in the presence of E2; however, 200 μg/ml of conceptus protein was necessary to achieve a modest increase in P4 production. Rabbit placental giant cells contain immunoreactive CG and cytotrophoblast cells contain immunoreactive CSH1/PRL. However, effects of CSH1 and PRL on luteal cells are not known (24). Circulating levels of E2 increase, P4 levels decrease and PRL levels increase about 2 days pre-partum with onset of nest-building behavior, but these hormonal changes mainly affect lactogenesis (25).

3. OVERVIEW: KEY EVENTS IN IMPLANTATION

Uterine receptivity to implantation varies among species, and involves coordinate changes in expression of genes associated with attachment of trophectoderm to uterine LE and sGE, as well as mid- to deep uterine glandular epithelium (GE), modification of phenotype of uterine stromal cells, silencing of PGR and ESR1 in uterine epithelia, suppression of genes for immune recognition of trophectoderm, 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 cells and stromal cells in response to progesterone, estrogens, glucocorticoids, prostaglandins and interferons influence uterine receptivity to implantation in mammals.

3.1. Temporal and Spatial Changes in Steroid Receptors in the Uterine Endometrium During the Peri-Implantation Period of Pregnancy

Uterine receptivity to implantation is established by actions of P4 and, in some species, E2 that regulate or are permissive to the actions of locally produced cytokines and growth factors including interferons, CG, prolactin and placental lactogen (1,3-5,26-28), homeobox transcription factors and cyclooxygenase-derived prostaglandins through autocrine and paracrine pathways (29-31). A fundamental paradox of early pregnancy 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. Thus, effects of P4 are mediated via PGR expressed in stromal and myometrial cells of the uterus, perhaps by stromal cell-derived growth factors known as "progestamedins" (32,33).

In the ewe, down-regulation of PGR in uterine epithelia is a prerequisite for the expression of genes for uterine secretions and for transport of other molecules into the uterin lumen that are collectively known as histotroph. These genes are induced by P4 in uterine GE; however, co-administration of E2 and P4 to ovariectomized ewes up-regulates expression of PGR in uterine epithelia and disrupts expression of genes for uterine secretions such as secreted phosphoprotein 1 (SPP1) mRNA and protein (34,35). 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 insulin-like growth factor binding protein 1 (IGFBP1) by uterine LE/sGE that stimulate migration and attachment of trophectoderm cells to uterine LE. Further, silencing expression of *PGR* in uterine epithelia allows P4 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 (36) or FGF7, HGF and retinoic acid in primates (26,37). These progestamedins exert paracrine effects on uterine epithelia and conceptus trophectoderm that express receptors for FGF7 and FGF10 (*FGFR2IIIb)* and HGF (MET; protooncogene *Met*). In sheep, many genes are progesterone-induced and IFN-stimulated and this appears to involve novel non-classical cell signaling pathways, independent of PGR and STAT1 (5). 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 (38). Interestingly, all 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 (39).

In the 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 (39) The limited number of estrogen-stimulated genes localized in endometrial of pigs include: *AKR1B1, B2M, CD24, EDG7, FGF7, IRF2, MX1, NMB, SLAs 1, 2,3, 6, 7, 8, SLC5A1, SPP1, STC1 and* (40). 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 IGFI (41). FGF7 is an established stromal cell derived paracrine mediator of hormone-regulated epithelial growth and differentiation (42). However, there is novel expression of FGF7 by uterine LE between Days 12 and 15 of the estrous cycle and pregnancy in pigs. FGF7 binds to and activates FGF2IIIb expressed by uterine epithelia and conceptus trophectoderm. Estrogen increases *FGF7* expression only after P4 suppresses expression of PGR by uterine epithelia. The FGF7, in turn, increases cell proliferation, phosphorylated FGFR2IIIb, the mitogen-activated protein kinase cascade and expression of urokinase-type plasminogen activator, a marker for trophectoderm cell differentiation (42). From about Day 20 of pregnancy, FGF7 is expressed by uterine GE in pigs in response to P4 and is presumed to continue to affect uterine epithelia and conceptus development (G.A. Johnson, R.C.Burghardt and F.W. Bazer, unpublished results). The increased secretion of estrogens between Days 15 and 30 of pregnancy increase expression of endometrial receptors for PRL, uterine secretory activity and uterine blood flow (5).

3.2. The Implantation Cascade

Although strategies for implantation differ significantly between species, blastocysts of all eutherian mammals share the events of shedding the zona pellucida followed by apposition and adhesion to the uterine LE which is characterized as the implantation/attachment/adhesion cascade (Figure 1). Available information indicates that initial conceptus attachment requires alterations in the expression of antiadhesive components, mainly mucins, contained in the glycocalyx of LE that sterically inhibit attachment in mammals (43). 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 (44). Unmasking adhesion molecules on the surface of uterine LE permits initial contacts with trophectoderm in a process that has been termed "rolling and tethering" that is similar to the extravasation of immune cells from the vasculature. Attachment to the uterine LE progressively develops into more stable adhesion through interactions between trophectoderm and maternal extracellular matrix

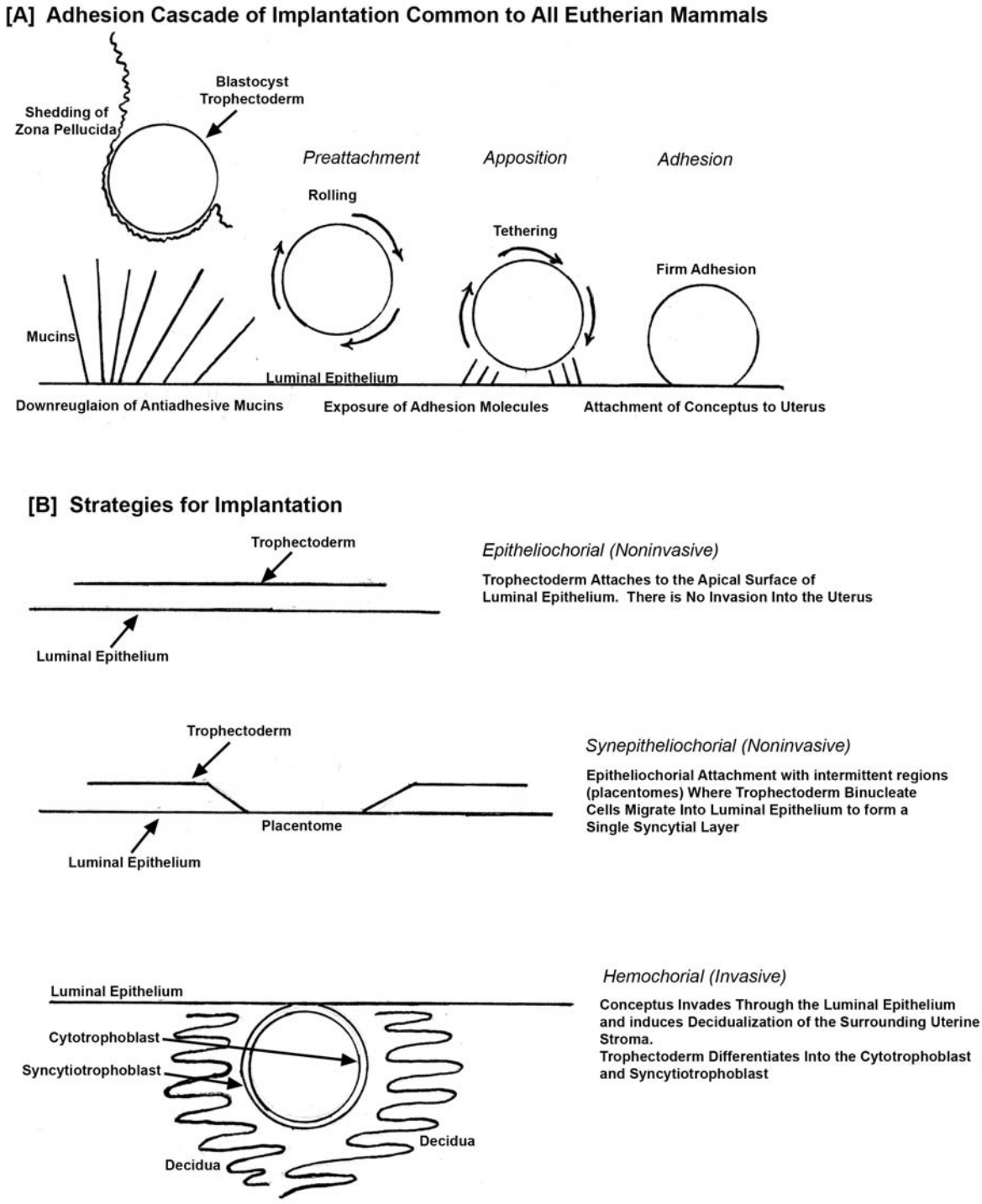

Figure 1. [A] Implantation in all eutherian mammals initiates through an adhesion cascade characterized by down-regulation of antiadhesive mucins on the apical surface of uterine luminal epithelium which allows for the attachment of conceptus trophectoderm. The rolling conceptus first tethers to the luminal epithelium through carbohydrate-lectin interactions which are subsequently reinforced through integrin-ECM binding. [B] Different mammalian species have varied strategies for placentation following initial attachment for implantation. Pig trophectoderm simply attaches to luminal epithelium resulting to intact epithelial sheets at the uterine-placental interface. Binucleate cells in sheep trophectoderm invade into the luminal epithelium at sites destined to form placentomes to form a single layer syncytium. Human blastocysts invade past the lateral surfaces of luminal epithelial cells and embed in the stroma, induce decidualization of the stromal cells and the torphectoderm differentiates into cytotrophoblast and syncytiotrophoblast.

(ECM), as well as stromal cells encountered beyond the uterine LE during invasive implantation (45).

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 (29). Low affinity interactions are followed by stable adhesion involving integrins expressed on trophectoderm and uterine LE and their ECM bridging ligands that also have roles in adhesion, migration, invasion, cytoskeletal organization and bidirectional signaling (45). In humans, expression of alpha v beta 3 and alpha 4 beta 1 integrins increase in uterine LE during the window of implantation (46). In pigs alpha v beta 6 on conceptus trophectoderm and alpha v beta 3 on LE bind the ECM molecule SPP1 (47). These and other integrins at both maternal and conceptus interfaces along with integrin-binding matrix proteins such as fibronectin, oncofetal fibronectin, vitronectin, laminin, the latency associated peptide linked to transforming growth factor-beta (TGFB), galectin-15 (LGALS15) and IGFBP1 are critical in species having non-invasive and invasive implantation (45,48,49).

3.3. Strategies for Implantation.

Implantation may be non-invasive (central) or invasive (interstitial or eccentric) depending on whether or not the conceptus 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 pre-implantation period (the pre-receptive 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 and transform morphologically from spherical to tubular and filamentous forms. Pre-attachment conceptus development is accompanied by growth and differentiation of the trophectoderm that secretes a pregnancy recognition signal.

During the initial stages of implantation conceptus/maternal interactions differ between domestic animals (non-invasive implantation) and rodents, carnivores and primates (invasive implantation) (4,5,39). Differences among species exist in the extent of interactions between trophectoderm (gives rise to chorion) and maternal uterus at the interface for development of placental structures. For example, intimate contact between chorion and an intact uterine LE is maintained in pigs throughout pregnancy (epitheliochorial placenta). Ruminant conceptuses form binucleate trophectoderm cells which migrate and fuse with uterine LE and each other to form plaques of multinucleated syncytia (synepitheliochorial placenta). The binucleate trophectoderm cells are the source of placental lactogen as well as other hormones such as P4 (50). 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 (51).

Carnivores, rodents, and primates exhibit invasive implantation as the blastocyst invades and implants deeply into the endometrial stoma 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 syncytiotrophoblast 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 sinusoids in hemochorial placentae of higher primates and rodents, whereas the hemoendothelial 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 syncytium.

3.4. Cytoskeletal Changes Mediate Blastocyst Expansion and Elongation of Conceptus Trophectoderm in Non-invasive Implantation and Trophoblast Outgrowth and 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 the trophectoderm. Pig conceptus trophectoderm cells in the elongation zone are columnar compared to cuboidal in areas peripheral to the elongation zone and this structural modification is associated with changes in length and orientation of microfilaments (52). That is, orientation of microfilaments in pig trophectoderm cells change from horizontal to parallel relative to the lateral cell borders suggesting that elongation is initially through migration or condensation of trophectoderm 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 trophectoderm cells and extension of filapodia from extra-embryonic endodermal cells allow movement and redistribution of cells toward the ends of tubular blastocysts. There are also changes in the actin cytoskeleton in trophectoderm during the transition from spherical to tubular and filamentous forms as follows:1) early cleavage stage embryos have filamentous actin concentrated at sites of contact between blastomeres; 2) compacting morulae accumulate actin at the margins of blastomeres; and 3) trophectoderm 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 (53,54). The actin cytoskeleton is essential for force generation during conceptus elongation as constricted regions along the length of filamentous conceptuses contain polarized trophectoderm 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 (47). Recently, SPP1 was shown to bind directly to the integrin heterodimers αvβ6 on trophectoderm and αvβ3 on uterine LE to induce assembly of focal adhesions that promote migration and attachment of trophectoderm to uterine LE that is considered critical to conceptus elongation and implantation (47).

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: 1) 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; 2) 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 3) microtubules, larger hollow polymers of tubulin that extend across the cytoplasm to the cell periphery (55,56). 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' (55) 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 (57). Cells are therefore able to respond to both internally generated or externally applied forces and can sense the rigidity and anisotropy of the ECM (58). Pulling on ECM tugs on integrins and associated focal adhesion proteins to deform mechanosensory molecules that elicit biochemical signals which likely changes intracellular metabolism and gene expression in elongating conceptus trophectoderm. 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 (59). 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 (55). 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 trophectoderm required for implantation (60,61). These amino acids regulate motility and outgrowth of trophectoderm cells 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 (62,63). Implantation of the human embryo and migration of human extravillous trophectoderm requires multiple Rho GTPase family members in both trophectoderm cells and endometrial stromal cells into which they invade (64,65). Rho GTPases including RhoA, Rac1 and Ccd42 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 (66).

The mTOR signaling pathway has been linked to elongation of conceptus trophectoderm in sheep. For ovine conceptus development during implantation and placentation, integrin activation by SPP1 binding and arginine appear to stimulate remodeling of trophectoderm cells for elongation and adherence to uterine LE/sGE 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) (67). For ovine trophectoderm cells, SPP1 binds alpha v beta 3 and alpha 4 beta 1integrins to induce focal adhesion assembly, a prerequisite for adhesion and migration of trophectoderm cells through activation of: 1) RPS6K via crosstalk between FRAP1/mTOR and mitogen-activated protein kinase (MAPK) pathways; 2) mTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate cell migration; and 3) 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 trophectoderm cells essential for expansion and elongation of conceptuses and attachment to uterine LE for implantation (67).

3.5. Decidualization.

Penetration of the uterine LE barrier by invasive trophectoderm cells triggers a series a stromal responses collectively termed decidualization (68). During decidualization, hyperplasia and hypertrophy transforms small spindle-like endometrial stromal cells into enlarged polygonal epithelial-like cells with extensive cell-cell contacts (69,70). As they differentiate, these cells express additional or different arrays of cytoskeletal proteins (71) and exhibit marked accumulation of filamentous proteins which include microtubules, microfilaments, intermediate filaments and the microtubular lattice (72). Two cytoskeletal proteins characteristic of decidual cells are α-smooth muscle actin (73) and desmin (74,75). These

cytoskeletal proteins appear to be physically involved with changes in growth, shape and protein secretion by stromal cells during the decidualization process (76). Functionally, decidualized stromal cells secrete prolactin (77,78) and IGFBP1 (79,80) which likely function in complex gene networks that restrain trophoblast invasion (81,82), as well as many other endocrine and paracrine factors (69,83,84). Decidual cells also accumulate ECM proteins including SPP1, laminin and fibronectin. The SPP1 expressed by decidual natural killer cells in mice (85) and uterine decidual stromal cells in humans (86) may be involved in angiogenesis within the decidua. The result is formation of a morphologically and functionally distinct tissue that produces hormones, promotes nutrition of conceptuses, prevents fetal allograft rejection and regulates placentation by limiting trophoblast invasion through generation of a local cytokine environment which promotes trophoblast attachment over invasion (87,88). 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.

While decidualization is characteristic of primates and rodents with invasive implantation, it is not thought to be a property of species with central and noninvasive implantation. Interestingly, the uterine stroma of both pigs and sheep undergo a fibroblast-to-myofibroblast differentiation that is similar to that in species in which uterine decidualization occurs. In sheep, expression of α-smooth muscle actin detected on Day 35 of pregnancy is accompanied increased expression of desmin and SPP1 and α-smooth muscle actin is detected on Day 45 of pregnancy in pigs (89). Incorporation of the smooth muscle isoform into stress fibers confers high contractile activity that is transmitted to the extracellular matrix (90). This neodifferentiation of myofibroblasts is a response to a change in the composition, organization and mechanical properties of the extracellular matrix (91) and to cytokines locally released by inflammatory and resident immune cells (92). In particular, TGFB1 is considered the pro-fibrotic cytokine that causes ECM production and organization, increases tissue inhibitor of metalloproteinases (TIMP) synthesis, decreases protease synthesis and induces the transition of fibroblasts into contractile myofibroblasts resulting in a mechanically strained environment that is the hallmark of tissue remodeling and wound healing (93). The timing of myofibroblast differentiation in domestic animals indicates that they may play a role in mechanotransduction and adaptation to mechanical forces imposed by the growing conceptus.

3.6. Molecular Markers of the Window of Implantation

Global gene profiling comparing 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 (94-97). 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 E2 receptor (EP2), interleukin-15 (IL15) and TGFB type II receptor for which expression increased. Notably, expression of SPP1 by uterine GE increased 12-fold during the receptive phase in women (94) and up to 60-fold during pregnancy in rats (95), suggesting its 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 (98). 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 1, alpha v beta 5, alpha v beta 6 and alpha 8 beta 1, as well as alternative binding sequences for interactions with alpha 4 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 (99).

4. UTERINE RECEPTIVITY TO IMPLANTATION OF BLASTOCYSTS IN PRIMATES, PIGS, RODENTS AND SHEEP

4.1. Primates

Pregnancy recognition signaling in primates extends CL function at least until the time of the luteal-placental shift when production of P4 by the placenta is adequate to support pregnancy (1,2). Primate blastocysts begin implantation following attachment to uterine LE on Days 7 to 9 post-ovulation in macaques and humans or Days 11 to 12 in marmoset monkeys. In humans, expression of alpha v beta 3 and alpha 4 beta 1 integrins increase in uterine LE during the window of implantation (100). These and other integrins at both maternal and conceptus interfaces along with integrin-binding matrix proteins such as fibronectin, oncofetal fibronectin, vitronectin, SPP1, laminin, IGFBP1 and the latency associated peptide linked to one or more isoforms of TGFB are critical for both non-invasive and invasive implantation (101,102). These and other ECM molecules are likely bridging ligands for stable adhesion between apically expressed maternal and fetal integrins.

Global gene profiling comparing endometrial tissues from late proliferative and secretory phases of the menstrual cycle indicated that 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 IGFBP-1 and -2, PGE_2 receptors, IL15 and TGFB Type II receptor (94,103). Notably, SPP1 expression by uterine GE increased 12-fold

during the receptive phase in women and up to 60-fold during pregnancy in rats suggesting a direct role in embryo-uterine interactions (100).

Compaction of morulae and formation of blastocysts are similar for baboon, macaque and human, including differentiation of blastocysts prior to implantation. The earliest indications of implantation in primates include attachment to and penetration of uterine LE by trophoblast followed by its invasion into adjacent uterine glands; however, most of the trophoblast remains superficial to the basal lamina of uterine LE. Then, ectoplasmic processes of syncytial trophoblast penetrate the basal lamina, invade into subjacent superficial maternal blood vessels and form junctional complexes with endothelial cells. Consequently, maternal blood vessels are dilated and the stratum compactum stroma is edematous at implantation sites and some uterine glands become surrounded by trophoblast cells that do not penetrate the basal lamina. Vascular lacunae then form and fill with maternal blood in areas with syncytial clefts formed by a single layer of syncytiotrophoblast over cytotrophoblast with numerous microvilli protruding into the cleft in baboons, macaque and humans. During early stages of lacunar formation, cytotrophoblast cells are present within superficial maternal capillaries and enter maternal arterioles as early as Day 12 and are common by Day 14 of gestation. The blood-filled lacunae enlarge and lift the developing placental disk above the endometrial surface and stromal edema increases at the implantation site. In addition, endovascular cytotrophoblast cells migrate into venules and uterine glands, but not into the endometrial stroma. During the lacunar stage, epithelial plaques form around the necks of uterine glands so that the developing placenta is subsequently superficial to the endometrial surface. Then, decidualization of the endometrial stroma occurs in response to implantation and this is followed by formation of the placenta.

Invasive implantation induces uterine decidualization which involves hyperplasia and hypertrophy of stromal cells and their secretion of prolactin, ECM proteins, SPP1, laminin and fibronectin, invasion by numerous immune cells and formation of cell-cell contacts. Decidualized stromal cells produce many endocrine and paracrine factors that control trophoblast invasion by generating a local cytokine environment that promotes trophoblast attachment. Varying degrees of decidualization occur in all species with extensive stromal transformation in species with invasive implantation (rodents and primates), moderate transformation in species with synepitheliochorial placentae (ruminants) and minor changes in species with epitheliochorial placentae (pig, horse).

In the human placenta, there are many amino acid transporters (104,105), but little is known about their expression in the uterus. In contrast, various glucose transporters have been identified in preimplantation embryos that provide a mechanism for glucose uptake and utilization. The Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 1 (SLC2A1) may be important for transporting glucose into the uterus and/or into the conceptus (106), because of its ubiquitous nature and high abundance in peri-implantation blastocysts (107), while SLC2A3 may be involved in uptake of glucose by cells of the conceptus (106) and expresion of SLC2A4 in syncytiotrophoblast may be regulated by concentrations of insulin and glucose in maternal blood (108).

There is abundant information on uterine receptivity to implantation in baboons, but the focus here is on humans (1). Syncytiotrophoblast cells of human conceptuses secrete CG from Days 8 to 10 for pregnancy recognition and implantation begins on Days 7 to 9 post-ovulation. During pregnancy, PGR expression is limited to endometrial decidual cells to insure a P4-responsive endometrium permissive to establishment and maintenance of pregnancy. The window of uterine receptivity to implantation in women is between Days 6 and 10 post-ovulation and invasion of the conceptus into the endometrium begins on about Day 11. The CG acts via LHCGR expressed by uterine epithelia and stromal cells to induce decidualization of stromal cells which: 1) secrete prolactin and IGFBP1; 2) increase edema; 3) express alpha smooth muscle actin and prostaglandin-endoperoxide synthase 2 (PTGS2) genes; 4) increase angiogenesis and blood flow; and 5) express leukemia inhibitory factor (LIF). Interleukin 1 beta (IL1B) from trophoblast also increases expression of IGFBP1 and decidualization of endometrial stromal cells.

During the window of implantation in humans, P4 stimulates morphological development of uterine glands and secretory activity by GE and a decrease in ESR1 marks the onset of uterine receptivity. Thereafter, ESR1 and PGR are restricted to the basalis zone of the endometrium prior to implantation and PGR remain abundant in uterine stromal cells. The window of implantation in humans is characterized by expression of integrin heterodimers alpha 1 beta 1, alpha 4 beta 1 and alpha v beta 1 which can bind fibronectin, vitronectin, thrombospondin, von Willebrand factor, bone sialoprotein 1, L-selectin and SPP1. L-selectin binding to $\alpha_4\beta_1$ is associated with establishment of connections between invading conceptus trophectoderm and maternal vasculature that extends to placentation while $\alpha_v\beta_3$ and SPP1 localize to pinopods of endometrial LE as a marker of implantation. Glycodelin may be required for implantation, whereas LIF and calcitonin are considered essential for implantation.

Secretions of uterine GE increase in response to P4 and conta*in* *utero*globin, histone A2, spermidine/spermine acetyltransferase 2, secretory leukocyte protease inhibitor and metallothionine. Stromal cells of humans and macacque also secrete proprotein convertase 6 at the implantation site. However, P4 also suppresses expression of proteins including TGFB, MMP11, proenkephalins, cysteine/glycine rich protein 2, collagen type $VII_{\alpha 1}$, and frizzle related protein 4, while FGF7 has anti-apoptotic effects on uterine GE.

Type I and Type II IFNs produced by human placenta and decidual cells (109) may: 1) regulate

proliferation of trophoblast or other cells in the uterus; 2) exert immunosuppressive effects by suppressing mitogen-induced proliferation of T- and B-cells; 3) protect the conceptus from viral infections; 4) regulate cellular differentiation and expression of cell surface antigens; 5) stimulate expression of ε-globin, a component of embryonic hemoglobin; and 6) suppress expression of proto-oncogenes such as EGFR, c-erB2 and c-fms to affect trophoblast growth and differentiation. As noted earlier, ISGs are among the most upregulated genes in human endometrial stromal cells treated with human trophoblast conditioned medium (103). Kumar *et al.* (110) found guanylate binding protein 1 (GBP1) to be induced by both IFNA and IFNG and it is considered a marker of uterine receptivity to implantation although its function is not known. The Mx proteins, also GTPases, are induced by Type I IFNs and may protect against viral infection. Li *et al.* (111) reported expression of p27 (cyclin-dependent kinase inhibitor 1b; CDKN1B), which has high homology to interferon regulated gene 1 (IRG1), to increase in Ishikawa cells in response to IFNA and that estradiol and IFNA exert synergistic effects to increased the abundance of p27 that preceded cell proliferation. The p27 gene is also expressed during the window of implantation in humans and is considered essential for control of normal endometrial proliferation (112). The shift in endometrial production from PGF to PGE is associated with implantation. In humans, IFNA suppresses progesterone-regulated production of basal PGF, but not PGE2 (113). Of particular interest is a report that IFNA stimulates transcription of the *CGB* gene without effects on cell proliferation (114); however, these results are from studies using a bladder tumor cell line and have not been confirmed using trophectoderm cells. The cytokines regulating development of human conceptuses may also have undesirable effects. For example, the combined effects of tumor necrosis factor-α, IFNG and IL1B may lead to pregnancy failure due to loss of blood supply and conceptus death (115).

4.2 Pigs

In pigs, the 1-cell fertilized ovum or zygote undergoes cleavage to form a 2-cell embryo by 26 hours after fertilization (116). Embryos remain in the oviduct before entering the uterus at the 4- to 8-cell stage between 48 and to 56 hours post-fertilization. Blastocyst formation is a key stage in early embryonic development when cells segregate into the embryonic disc, trophectoderm, extra-embryonic endoderm and blastocoel necessary for continued development and differentiation to a conceptus (embryo and associated extra-embryonic membranes). Before blastocysts develop into a conceptus, they "hatch" from the zona pellucida and increase in size to Day 10 of pregnancy (2-6 mm) before undergoing a morphological transition to large spheres of 10 to 15 mm diameter and then tubular (15 mm by 50mm) and filamentous (l mm by 100-200 mm) forms, primarily by cellular remodeling on Day 11. During the transition from tubular to filamentous forms, pig conceptuses elongate at 30 to 45 mm/hour, primarily by cellular remodeling of trophectoderm. However, hyperplasia is responsible for subsequent growth and elongation of the conceptus to 800 to 1000 mm length by Day 15 of pregnancy. The period of rapid elongation of pig conceptuses is accompanied by production of estrogens (117,118), as well as IFNG and IFND (119) by trophectoderm.

In pigs, oxytocin released in a pulsatile manner by the uterus binds oxytocin receptors to stimulate pulsatile release of PGF from uterine epithelia (120-122). PGF is the uterine luteolysin in pigs as CL regression occurs in response to pulsatile release of PGF into the uterine venous drainage beginning on Days 15 and 16 of the estrous cycle (123) and hysterectomy extends CL lifespan to about 120 days (121). However, the exact roles of prostaglandins in the pig uterus remain to be clarified. Inhibitors of PG synthesis fail to protect the CL from luteolysis (124), amounts of PGF and PGE2 in the uterine lumen are greater in pregnant than cyclic pigs (8), uterine PGF is processed into an inactive metabolite through a utero-ovarian countercurrent vascular pathway within the broad ligament (125), and PGE2 synthase:PGF synthase ratios are higher in CL from pregnant than cyclic pigs, but not between CL ipsilateral or contralateral to the pregnant uterine horn. Therefore, it has been suggested that molecules from the conceptus are transported within the mesometrium to the ovaries to enhance CL maintenance (126).

Pregnancy recognition is the result of conceptus secretion of estrogens secreted by pig conceptuses on Days 11 and 12 of pregnancy that redirects PGF secretion from the uterine vasculature into the uterine lumen (8). Interestingly, estrogen secretion by the conceptus is coordinate with its secretion of IL1B, which may, in turn, modulate uterine responses to this cytokine (127). In addition to pregnancy recognition, conceptus estrogens modulate uterine gene expression required for implantation (128). The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on Days 9 and 10 results in degeneration of all pig conceptuses by Day 15 (128,129). It should be noted that PGE2, as well as lysophosphatidic acid have proposed roles in pregnancy signaling events. Expression of PGE2 synthase by trophoblast and endometrium decreases production of PGF in favor of PGE2 that is proposed to support CL maintenance (9). In addition lysophosphatidic acid in uterine luminal fluid and its receptors in uterine epithelia increase in response to estrogen during early pregnancy (10). Indeed, lysophosphatidic acid is critical for migration and spacing of embryos in mice (11) and these events are critical to implantation and placentation in pigs.

Pig conceptus trophectoderm is unique in secreting both Type I and Type II interferons (IFNs) during the peri-implantation period. Cultured conceptuses from Day 11 of pregnancy secrete proteins that cross reacted with antiserum against IFNA (130), but peak production of antiviral activity in uterine flushings or conceptus culture media is on Days 14 and 15 of pregnancy (131). The major species (75% of antiviral activity in pig conceptus secretory proteins) is the type II IFNG and the other (25%) is the novel type I IFND (119). Abundant *IFNG* mRNA is detectable in porcine trophectoderm between Days 13 and

20 of pregnancy, whereas *IFND* mRNA is detectable in Day 14 conceptuses only by RT-PCR analysis (40). On Day 15 of pregnancy, immunoreactive IFNG and IFND proteins co-localize to peri-nuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus, as well as cytoplasmic vesicles within clusters of trophectoderm cells along the uterine LE (119). This expression is characterized by *de novo* appearance of zona occludens one (ZO1), a marker of epithelial tight junctions, on their basal aspect, suggesting changes in endometrial polarity (119). In contrast to sheep conceptuses, in which IFNT is the signal for maternal recognition of pregnancy (4,5), the IFNs produced by pig conceptuses do not appear to be antiluteolytic. Daily intrauterine infusion of conceptus secretory proteins from Days 12 to 15 of the estrous cycle had no effect on inter-estrous interval or temporal changes in concentrations of P4 in plasma, but did increase concentrations of PGE2 in uterine venous plasma (132,133). Although pig conceptus IFNs do not appear to influence pregnancy recognition, their paracrine effects are suggested by localization of IFN receptors on endometrial epithelial cells (134), increased secretion of PGE2 (132), expression of several known IFN-responsive genes in the endometrium and modulation of gene expression uterine stromal cells and GE gene expression by the IFNs in conceptus secretory protein preparations (40).

Interactions of estrogen and IFNs regulate cell-specific expression of multiple genes in the pig endometrium to elicit the complex interplay between endometrium and conceptus for pregnancy recognition and implantation. Examples of genes regulated by conceptus estrogens and IFNs are *SPP1* and *STAT1,* respectively. The timing of estrogen secretion by the conceptus correlates with the induction of *SPP1* expression in uterine LE, whereas stromal induction of *STAT1* correlates with IFNG and IFND secretion by the conceptus. Indeed, administration of exogenous estradiol to ovariectomized pigs induces *SPP1* mRNA in endometrial LE (135), while intrauterine infusion of conceptus secretory proteins, which contain IFND and IFNG, into cyclic pigs treated with exogenous estrogen increases *STAT1* (136) which is similar to expression patterns for these genes in the uterine endometrium during the peri-implantation period of pigs. Upregulation of *SPP1* within uterine LE and *STAT1* within stroma and GE in close proximity to the implanting conceptus implies paracrine regulation of genes by conceptus estrogens and IFNs. The effects of conceptus estrogens on the endometrium are restricted to regions near the conceptus due to the metabolic activity of trophectoderm. During pregnancy, the pig endometrium rapidly converts estradiol to estrone and then converts it to the biologically inactive estrone sulfate which is present in high concentrations within the uterine lumen and blood of pregnant pigs (137). The trophectoderm has sulfatase enzyme activity converts estrone sulfate to biologically active estrone to act locally in, for example, increasing expression of gene such as *SPP1* in uterine LE. In contrast, it is somewhat surprising that initial increases in stromal *STAT1* are restricted to sites of intimate association between the conceptus and uterus, given that *IFNG* synthesis and secretion by pig conceptuses appears to be similar in magnitude to *IFNT* production by sheep conceptuses (27). Indeed, *STAT1* increases universally in the stroma and GE of pregnant ewes without regard to conceptus location within the lumen, presumably due to the very high levels of secretion of IFNT by conceptuses (136,138). One explanation for the spatial pattern of *STAT1* expression in the pig uterus is that IFND and IFNG act synergistically to upregulate ISGs as is the case for other interactions between Type I and Type II IFNs (139). It is plausible that high levels of IFNG act on uterine stromal and GE cells to increase intracellular stores of interferon-stimulated gene factor 3 (ISGF3) so that the much lower levels of IFND can maximally upregulate *STAT1* in close proximity to the implanting pig conceptus. To date, only a limited number of estrogen- and IFN-stimulated genes have been localized in the pig endometrium. These include the estrogen-regulated genes *AKR1B1, B2M, CD24, LPAR3EDG7, FGF7, IRF2, MX1, NMB, SLAs 1, 2, 3, 6, 7, 8, SLC5A1, SPP1,* and *STC1*, and the IFN-regulated genes *B2M, IRF1, ISG15, MX1, SLAs 1, 2, 3, 6, 7, 8, STAT1* and *STAT2* (see 40) and cathepsins and cystatins (140).

Implantation in pigs is influenced by estrogens secreted by conceptuses, and progesterone secreted by the CL. The best described estrogen-regulated adhesion molecule for implantation in pigs is SPP1 which is a secreted ECM protein that is up-regulated during the initial stages of pregnancy in uteri of pigs (135,141), as well as sheep, goats, humans, rabbits and mice (98,141). *SPP1* contains an Arg-Gly-Asp (RGD) sequence that mediates binding to cell surface integrin receptors, including alpha v beta 3 (143), alpha 5 beta 1(144), alpha v beta 1 (145), alpha v beta 5 (146), alpha v beta 6(147) and alpha 8 beta 1 (148). Alternative binding-sequence interactions between *SPP1* and integrins such as alpha 4 beta 1 (147), alpha 9 beta 1 (150) and alpha 4 beta 7 (151) can also occur. *SPP1* mRNA is initially detected in discrete regions of uterineLE juxtaposed to the conceptus just prior to the beginning of implantation on Day 13. This expression is induced by conceptus estrogens that are secreted beginning on Days 11 and 12 to signal pregnancy recognition. SPP1 expression expands to the entire LE by Day 20 when firm adhesion of conceptus trophectoderm to uterine LE occurs; however, *SPP1* mRNA is not present in the pig conceptuses (135,141). In contrast to mRNA, SPP1 protein is abundant along the apical surface of uterine LE, on trophectoderm, and along the entire conceptus-uterine interface during pregnancy (135,140). Burghardt and co-workers (45) have shown that multiple integrin subunits are expressed at this interface that potentially form heterodimer receptors that bind SPP1, including alpha v beta 3, alpha v beta 1, alpha v beta 5, alpha 4 beta 1 and alpha 5 beta 1 . Recently the porcine trophectoderm cell line (pTr2) and primary porcine uterine epithelial (pUE) cells were used in a series of experiments to examine interactions between membrane integrin receptors and exogenous SPP1 (151). The results indicated that: 1) pTr2 cells and pUE integrins bind directly to SPP1; 2) SPP1 binds alpha v and beta 6integrin subunits only; and 3) pUE cells bound SPP1 via alpha v and beta 3 integrin subunits (47). Therefore, it appears that SPP1 directly binds the alpha v beta 6 integrin heterodimer

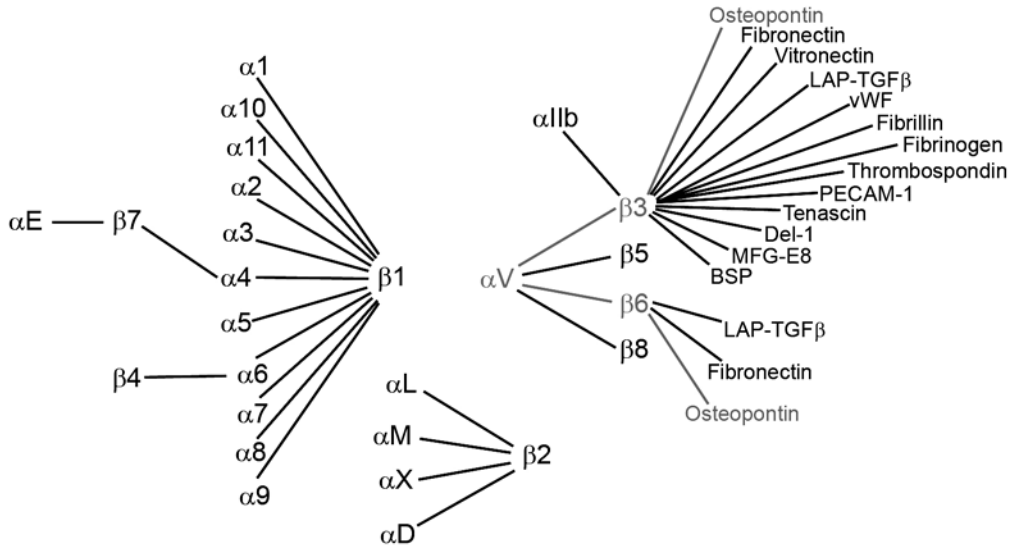

Figure 2. Integrin receptors are transmembrane glycoproteins composed of noncovalently linked alpha and beta subunits that integrate signals from the outside to the inside of cells and visa versa. A total of 18 alpha subunits combine with 8 beta subunits to form 24 known discrete heterodimer receptors. Integrin receptors bind a large array of ECM molecules. During the implantation cascade and placentation, integrins stabilize attachment of conceptus trophectoderm to uterine luminal epithelium. Recently, αvβ3 on luminal epithelium and αvβ6 on trophectoderm were demonstrated to bind the ECM molecule osteopontin during implantation in pigs (47).

receptor on conceptus trophectoderm cells and alpha v beta 3 on uterine LE cells (47). Interestingly the conceptus alpha v beta 6integrin binds discretely to only three ECM proteins, each of which is expressed prominently at the conceptus-endometrial interface of pigs (Figure 2) namely SPP1, fibronectin and the latency associated accociated peptide of TGFB (135,153,154).

4.3. Rodents

In mice, leucine or arginine is required for expanded blastocysts to exhibit motility and outgrowth of trophectoderm required for implantation (60,61). Development of the preimplantation rodent embryo is marked by principal transitions from fertilization, cell division, establishment of cell polarity, compaction to form a morula and then a blasocyst that can undergo implantantation after it hatches from the zona pellucida. The mature blastocyst has an outer epithelial trophectoderm, a primitive extra-embryonic endoderm and the inner cell mass. Implantation begins when the trophectoderm makes the first physical and physiological connections with the uterine LE. The state of activity of the blastocyst determines the window of implantation that involves increased expression of genes that regulate cell-cycle, cell-signaling and energy-metabolism, as well as expression of key genes activated by blastocysts that include heparin-binding EGF-like growth factor and its receptors ErbB1 and ErbB4 and many genes expressed by epithelial and/or stromal cells of the uterus.

The sequential events in implantation involve: 1) apposition as trophectoderm becomes closely opposed to uterine LE; 2) attachment of trophectoderm to uterine LE sufficient to resist dislodging of the blastocyst occurs on the evening of Day 4 of pregnancy and increases stromal vascular permeability at the site of blastocyst attachment; 3) penetration or invasion of the blastocyst through the uterine LE and basal lamina into the stroma to stimulate the decidualization reaction and establishment of a vascular relationship between the conceptus and maternal uterus; and 4) decidualization involving loss of uterine LE and extensive differentiation of uterine stromal cells in response to signaling molecules that include cytokines, homeobox transcription factors, cell-cycle molecules, extracellular-matrix remodelling factors and lipid mediators, uterine angiogenesis and establishment of the uterine–embryonic axis. Angiogenesis is essential for implantation and placentation, and is mediated by vascular endothelial growth factor, angiopoietins and prostaglandins that coordinate VEGF signaling.

Catecholoestrogens produced in the uterus from metabolism of estrogens from the ovary activate blastocysts as do other signaling molecules that include endocannabinoid anandamide which is crucial to implantation in mice. Other locally produced signaling molecules, including cytokines, growth factors, homeobox transcription factors, lipid mediators and morphogens, together with ovarian hormones, serve as autocrine, paracrine and juxtacrine factors to specify uterine receptivity to implantation in mice. Available evidence indicates that the following genes are required for uterine receptivity to implantation in mice*:* Basigin, ESR1, FK506-binding protein-4, GP130/signal transducer and activator of transcription, homeobox-3, lysophosphatidic acid receptor-3, leukaemia inhibitory factor, PGR, phospholipase A2, peroxisome proliferator-activated receptor, and PTGS2 (see 155). In that same review, genes essential for

decidualization in mice include: FK506-binding protein-4, Homeobox A10, Homeobox A11, IL1 receptor-1, PGR, and PTGS2 (155).

Type I IFNs are present in high concentrations in placenta, but not maternal or fetal tissues in mice and viral infections as early as Day 7 of pregnancy induce IFNA, IFNB and IFNG (156). Reese *et al.* (156) reported increased expression of IFNB and several ISGs in uterine implantation sites of mice following treatment with estradiol to terminate delayed implantation. Li *et al.* (111) found expression of IFNA and IRG1 in uteri of pregnant rats to increase between Days 1 and 4 and then decrease following implantation. Interferon regulatory factor 1 mRNA was most abundant in uteri of rats treated with both estradiol and IFNA. In support of findings of Li *et al.* (111), Austin *et al.* (158) and Bany and Cross (159) reported that mouse trophoblast giant cells express IFNA that induces expression of ISG15 during the peri-implantation period.

Microarray analyses revealed significantly altered expression of genes at implantation sites in mice, including up-regulation of interferon-activated gene 202 and down-regulation of genes for histocompatibility 2, T region locus 23, IRF6 and MHC Class I and Class II (157). IFNG is expressed by uterine LE and GE, trophoblast cells and degenerating metrial gland cells of mice (160); however, most IFNG in decidua of mice at mid-gestation may be from uterine NK cells (161). Major changes in uterine spiral arteries between Days 9 and 10 of gestation in mice do not occur in IFNG null or Type II IFNR null mice or in alymphoid mice (160-162). Thus, endothelial cells, vascular smooth muscle cells and stromal cells of the uterus may be targets of action of IFNG. Further, IFNG is targeted to heparan sulfate which is essential for implantation in mice (163) and this may protect IFNG from inactivation and increase its stability (164) to allow protracted effects of IFNG on vascular development in decidual tissue. Type I and Type II interferons modify gene expression at implantation sites in rodents to either affect the conceptus directly or to exert indirect effects through actions on blood vessels and decidual cells required for successful implantation and pregnancy (165).

4.4. Ruminants

Uterine receptivity and implantation of blastocysts for ruminants includes: 1) hatching from zona pellucida; 2) precontact with uterine LE and orientation of blastocyst; 3) apposition between trophectoderm and uterine LE; 4) adhesion of trophectoderm to uterine LE and 5) limited endometrial invasion (165). Pregnancy recognition signaling and initial stages of implantation in sheep on Days 12 and 13 coincide with loss of PGR from uterine epithelia, but not stromal or myometrial cells (34). Loss of PGR by endometrial epithelia coincides with loss of expression of genes, such as *MUC1*, preceding the attachment phase of implantation. Further, uterine receptivity and implantation are prevented if uterine LE/sGE express PGR, which supports the concept that regulation of LE/sGE and GE functions must be directed by progestamedins produced by PGR-positive stromal cells (4,34).

IFNT induces or increases expression of several ISGs in endometria that are hypothesized to be important for conceptus implantation (see 99). Since expression of ISGs increases in a stage-specific manner within endometria of diverse species, including domestic animals, laboratory rodents, primates, and humans during early pregnancy, they may be universally important in establishment of uterine receptivity to conceptus implantation. A number of transcriptional profiling experiments conducted with human cells, ovine endometrium, and bovine endometrium have elucidated genes regulated by IFNT during pregnancy (167-170).

Bovine endometrial, ovine endometrial and human 2fTGH fibroblast cells have been used to determine that IFNT activates the classical JAK-STAT-IRF (janus kinase-signal transducer and activator of transcription-interferon regulatory factor) signaling pathway used by other Type I IFNs (170). Numerous ISGs are induced or stimulated in the endometrium during conceptus elongation in both cattle and sheep (4,99). Many classical ISGs, such as *ISG15* (ISG15 ubiquitin-like modifier), are expressed in LE of the ovine uterus on days 10 or 11 of the estrous cycle and pregnancy, but are undetectable in LE by Days 12 to 13 (172,173). In response to IFNT from elongating conceptuses, *ISG15* is induced in the stratum compactum stroma and GE by Days 13 to 14, and expression extends to the stratum spongiosum stroma, deep glands, and myometrium as well as resident immune cells of the ovine uterus by Days 15 to 16 of pregnancy (172,173). As IFNT production by the conceptus declines, expression of ISGs also declines, but some remain abundant in endometrial stroma and GE on days 18 to 20 of pregnancy. Similar temporal and spatial alterations in *ISG15* expression occur in the bovine uterus during early pregnancy (174).

Curiously, *in vivo* studies revealed that many classical ISGs (*B2M*, *GBP2, IFI27, IFIT1, ISG15, IRF9, MIC, OAS*, *RSAD2*, *STAT1,* and *STAT2*) are not induced or upregulated by IFNT in endometrial LE of the ovine uterus (172,175-179). This finding was initially surprising, because all endometrial cell types express *IFNAR1* (interferon (alpha, beta and omega) receptor 1) and *IFNAR2* subunits of the common Type I IFN receptor (180). However, it was discovered that IRF2, a potent transcriptional repressor of ISGs, is expressed specifically in uterine LE to represses transcriptional activity of IFN stimulated response element (ISRE)-containing promoters (176,181). Thus, IRF2 in LE appears to restrict IFNT induction of many ISGs to stroma and GE of the ovine uterus. In fact, all components of the ISGF3 transcription factor complex (*STAT1, STAT2, IRF9*) and other classical ISGs (*B2M, GBP2, IFI27, IFIT1, ISG15, MIC, OAS*) contain one or more ISREs in their promoters. Further, suppressors of cytokine signaling (SOCS1-3) are also upregulated in endometria by pregnancy and IFNT (182). Depending on their cell-specific expression in the uterus, SOCS1-3 may be involved in negative regulation of the JAK-STAT pathway activated by IFNT (183). The silencing of *MIC* and *B2M* genes in endometrial LE during pregnancy may be a critical mechanism preventing immune rejection of the conceptus semi-allograft (177). Of

particular note, several reports indicate induction or increases in ISGs in peripheral blood lymphocytes and the CL during pregnancy or in ewes receiving intrauterine injections of IFNT (184-186). Thus, IFNT or IFNT-stimulated immune cells may traffic out of the uterus to exert systemic effects that alter maternal physiology.

Given that the critical signaling components of the JAK-STAT signaling system (STAT1, STAT2, IRF9) are not expressed in uterine LE, IFNT must utilize a non-classical STAT1-independent cell signaling pathway to regulate expression of genes in ovine uterine LE. Transcriptional profiling of human U3A (STAT1 null) cells and ovine endometrium treated with IFNT were used to discover novel ISGs in the uterine LE/sGE during pregnancy including *WNT7A* (wingless-type MMTV integration site family, member 7A), *LGALS15*, *CTSL*, and *CST3* (168, 178, 187, 188). Previous studies determined that *GRP, PTGS2, HSD11B1, IGFBP1, SLC2A1, SLC5A11*, and *SLC7A2* are expressed in ovine uterine LE/sGE (49,189,190). Interestingly, these LE/sGE-specific genes are progesterone-induced, via P4-induced downregulation of the PGR or stimulation of progestamedins from uterine stromal cells, and further stimulated by IFNT (4,5,99). Collectively, those genes are likely essential regulators of conceptus elongation by effects on trophectoderm cell proliferation, migration, and attachment. The effects of the genes may be direct in the case of secreted factors (LGALS15, IGFBP1, WNT7A) or indirect due to the production or transport of biologically active substances including proteases, protease inhibitors, prostaglandins, cortisol, and amino acids. Thus, CG and IFNT have similar functions as they both are pregnancy recognition signals that maintain production of P4 by CL and regulate genes involved in uterine receptivity to implantation.

4.4.1 Servomechanisms regulating uterine gland morphogenesis and secretory function

During early pregnancy, ovine and bovine uteri are exposed sequentially to estrogen, progesterone, IFNT, and CSH1 which is proposed to initiate and maintain endometrial gland morphogenesis and differentiated secretory functions (4,5,32,192). Placentae of many species, including rodents, humans, nonhuman primates, and ruminants, secrete hormones structurally related to pituitary prolactin and growth hormone that are termed CSH1 (alias placental lactogen) (3). Ovine CSH1 is produced by trophoblast giant BNC from Days 15 to 16 of pregnancy which is coordinate with onset of expression of *UTMP (uterine milk proteins or SPINT1)*, *SPP1*, *GRP* (gastrin-releasing peptide), and *STC1* (193-196) which are excellent markers for GE differentiation and secretory function during pregnancy in sheep. A homodimer of the PRLR (prolactin receptor), as well as a heterodimer of PRLR and GHR (growth hormone receptor), transduce signals by ovine CSH1/placental lactogen (197). In the ovine uterus, *PRLR* gene expression is unique to GE (195, 198). Temporal changes in circulating levels of CSH1 are correlated with endometrial gland hyperplasia and hypertrophy and increased production of SPP1 and UTMP during pregnancy. Sequential exposure of the pregnant ovine endometrium to progesterone, IFNT, and CSH1 appears to be required to activate and maintain endometrial remodeling, secretory function of GE, and perhaps uterine growth during gestation. Chronic treatment of ovariectomized ewes with progesterone induces *SPP1*, *UTMP* and *STC1* expression by GE (196,199,200,201). However, intrauterine infusions of CSH1 further increases *SPP1*, *STC1*, and *UTMP* gene expression in the ovine uterus, but only when ewes receive P4 and intrauterine infusions of IFNT (200,202). The effects of IFNT may be attributed, in part, to increasing *PRLR* in the endometrial glands (203). Available evidence indicates that placental hormones play key roles in stimulating endometrial gland morphogenesis and differentiated functions during pregnancy that are required for conceptus development in ruminants. Indeed, similar servomechanisms are proposed for the human trophoblast and endometrium during early pregnancy (204).

5. SUMMARY AND PERSPECTIVE

The common aspects of uterine receptivity and implantation of blastocysts include: 1) hatching from zona pellucida; 2) precontact with uterine LE and orientation of blastocyst; and 3) apposition between trophectoderm and uterine LE; and 4) adhesion of trophectoderm to uterine LE. Thereafter, implantation can be accomplished with no invasion of the uterine LE, limited endometrial invasion or complete invasion of the blastocyst into the uterine stroma and induction of deciduas. These differences among species in the extent of interactions between trophectoderm (gives rise to chorion) and maternal uterus are at the interface for development of placental structures. Intimate contact between chorion and an intact uterine LE is maintained in pigs throughout pregnancy (epitheliochorial placenta) whereas ruminant conceptuses form binucleate trophectoderm cells which migrate and fuse with uterine LE and each other to form plaques of multinucleated syncytia and specialized placentomes (synepitheliochorial placenta critical for exchange of nutrients and gases across the placenta. Carnivores, rodents, and primates exhibit invasive implantation characterized 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 sinusoids in hemochorial placentae of higher primates and rodents, whereas the hemoendothelial placenta of carnivores retains the endothelial layer. The outcome is to increase placental efficiencies for placental exchange of nutrients and gases, as well as maternal heart work as uterine blood flow decreases from 600 to 800 ml/kg/min to 200 to 250 ml/kg/min to 50 ml/kg/min in epitheliochorial, synepitheliochorial and hemochorial and hemenodothelial placenta, respectively.

This review addressed the various mechanisms responsible for conceptus-endometrial interactions that provide for blastocyst/conceptus growth and development, implantation and establishment of pregnancy in humans, rodents, companion animals and agriculturally important species of livestock that also serve as animal models for biomedical research. However, many of the key mechanisms underlying these events are yet to be

discovered. We must gain a better understanding of nutrients and other molecules in the uterine lumen that support growth and development of the conceptus so that we can formulate better culture media for embryos during assisted reproduction. There is a gap in knowledge about the requirement for loss of expression of PGR by endometrial epithelia as a prerequesite for implantation, expression of genes for secretory proteins, and selective transport of molecules into the uterine lumen to support conceptus growth and development. The identification of progestamedins or estramedins unique to uterine epithelial cell functions, implantation and establishment of pregnancy in mammals must be understood along with how progestamedins and /or estramedins act individually or in concert with cell signaling pathways activated by secretions from the conceptus such as IFNs, lactogenic hormones, and prostaglandins.

Comparative reproductive biology is essential to advance understanding key aspects of the biology of reproduction. For example, the ewe is a proven model for research to understand the roles of IFNs during the peri-implantation period because trophectoderm or immune cells at sites of implantation of most, if not all mammals, express Type I and/or Type II IFNs. Indeed, IFN-stimulated genes are among the most highly upregulated genes in human decidualized stromal cells treated with trophoblast conditioned medium and in the uteri of domestic and laboratory animals. Understanding effects of ovine IFNT and porcine IFNG and IFND on gene expression in the uterus will advance our understanding of novel mechanisms whereby P4 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 likely 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.

There is a clear need to improve the frequency of successful outcomes for women who choose to use assisted reproductive technologies to overcome fertility problems. This can best be accomplished by capitalizing on advances that arise from basic research designed to understand aspects of uterine biology and conceptus development that may then be used to develop strategies for fertility control that are acceptable to people from various religious, cultural and ethnic backgrounds. There is also the issue of fertility control in abandoned companion animals, i.e., dogs and cats that must be addressed as well as the overpopulation situation for wildlife species, particularly deer and feral pigs.

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