Implantation: Mutual Activity of Sex Steroid Hormones and the Immune System Guarantee the Maternal–Embryo Interaction

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Abstract

Implantation is strictly dependent on the mutual interaction between a receptive endometrium and the blastocyst. Hence, synchronization between blastocyst development and the acquisition of endometrial receptivity is a prerequisite for the success of this process. This review depicts the cellular and molecular events that coordinate these complex activities. Specifically, the involvement of the sex steroid hormones, estrogen and progesterone, as well as components of the immune system, such as cytokines and specific blood cells, is elaborated.

Keywords

► endometrium
► immune cells
► estrogen
► progesterone
► cytokines

Development of a Receptive Endometrium

The preparation of the endometrium is hormone dependent. The pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone stimulate the ovary to secrete the sex steroids E2 and P4. Proliferation of the endometrial cells is induced by E2, which is mainly secreted during the first half of the menstrual cycle, referred to as the proliferative phase. The predominant sex steroid in the second half of the cycle, known as the secretory phase, is P4, which induces the differentiation of the endometrial cells. This phase of the cycle is characterized by the formation of glands that secrete large amounts of cytokines and growth factors, vascularization, infiltration of the endometrium by a variety of immune cells from the blood, edema of the tissue caused by a localized increase in vascular permeability, and decidualization of the stromal cells.1 It is important to note that in humans, decidualization (differentiation of the stromal cells) begins spontaneously during the secretory phase, unlike rodents, in which decidualization requires embryo implantation or another local mechanical stimulation.

Regulation of Endometrial Receptivity by E2 and P4

The ovarian steroid hormones, E2 and P4, are key regulators of endometrial function. Binding of these hormones to their receptors in the uterus drives the proliferation and differentiation of the endometrial tissue, preparing it for the implanting blastocyst. Activation of downstream genes by E2 and P4 stimulates a crosstalk between the endometrial stroma and epithelium that is crucial for the development of a receptive...
uteros. Different studies, most of which employed mouse experimental models, showed that of the two E2 receptors, ERα and ERβ, the ERα is the predominant mediator of the activity of this hormone in the endometrium. Mice that are Erα–/– exhibited hypoplastic uteri that could not support implantation, whereas in Erβ–/– mice, implantation was normal. Specifically, epithelial cell directed deletion of ERα caused a decrease in the synthesis of epithelial lactoferrin, an E2-regulated secretory protein, and led to apoptosis (Fig. 1).

A predominant mediator of E2 action in rodents is leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 cytokine family. Endometrial expression of LIF in mice is indispensable for implantation. Upon its secretion, LIF binds to its specific receptor, LIFR, in the luminal epithelium, and through gp130, activates the janus kinase (JAK)-signal transducer and activator of transcription (STAT) JAK-STAT pathway that leads to acquisition of endometrial receptivity. Using a model of endometrial epithelium specific, conditional Stat3 knockout, Pawar et al demonstrated that LIF-induced STAT3 activation triggers reorganization of the uterine epithelium by modulating different specific components of the junctional complex, such as E-cadherin, α and β-catenin, and claudins. Moreover, epithelial STAT3 regulates proliferation and differentiation of stromal cells during decidualization.

This paracrine effect of the epithelium on the stroma is mediated by the epidermal growth factor (EGF)-like growth factors, Egf, Hbegf, and Areg, that are produced downstream to the LIF-STAT3 signaling. Although LIF is a vital player in the mouse endometrium, its role in the human is still inconclusive. This cytokine reaches its peak level in human epithelium in the mid-secretory phase, the WOI, which is the receptive phase of the cycle. Some clinical studies indicate that low levels of LIF as well as its receptor are associated with unexplained infertility and with endometriosis, whereas other studies could not demonstrate such correlation. Furthermore, it was reported that LIF administration to in vitro fertilization (IVF) patients did not improve pregnancy outcome.

The predominant hormone during the receptive phase of the human endometrial cycle is P4, secreted by the corpus luteum that stimulates endometrial cell differentiation following E2 priming. In fact, P4 regulation is crucial for the transition of the endometrium from its nonreceptive to its receptive phase. This hormone modulates the expression of implantation-associated genes in the endometrium and is a key regulator of the transformation of the stromal cells to decidual cells, known as decidualization. Like E2, the activity of P4 is exerted through its binding to two receptors, PRA and PRB, that function in a tissue-specific manner.
P₄-induced reproductive functions, necessary for female fertility, are mediated by PRA, while PRB is required to elicit P₄-induced proliferation and differentiation of the mammary gland.¹⁶,¹⁷ The signaling via PR depends on its interaction with molecular chaperones, such as immunophilins. Immunophilin FKBP52 (also called FKBP4) is one of the main regulators of the uterine P₄ function. Its elimination in mice leads to implantation failure due to impaired endometrioid responsiveness to P₄.¹⁸ In human, lower expression of FKBP52 is associated with endometriosis.¹⁹ It was suggested that FKBP52 is critical for decidualization.²⁰ Decidualization, induced by P₄, is mediated by the homeobox transcription factors in the Hox gene family. Lack of murine endometrial Hoxa-10 impairs the responsiveness of stromal cells to P₄ that lead to an aberrant decidualization.²¹ This reduced responsiveness to P₄ is attributed to the direct effect of Hoxa-10 on FKBP52 expression.²⁰ The Hox gene family is also involved in embryogenesis.²²,²³ Mutation of Hoxa-10 in female mice caused implantation failure due to abnormal embryo development as well as impaired decidualization.²⁴

Other molecules that regulate/mediate P₄ activity are the steroid receptor coactivator 2 (SRC2), Indian hedgehog (IHH/Ilh) and bone morphogenetic protein (Bmp). Deletion of murine SRC2 leads to implantation failure due to impaired P₄-induced decidualization.²⁵ The IHH/Ilh is an example of the P₄-induced crosstalk between the epithelium and the stroma, during acquisition of uterine receptivity. It is expressed in the epithelium in response to P₄ and acts as a paracrine signal upon binding to its specific receptors on the stromal cells, inducing their proliferation. Deletion of Ihh gene, negatively affects uterine receptivity, leading to implantation failure.² In the human, expression of IHH increases during the secretory phase of the cycle in response to the P₄ receptor modulator CDB-2914.²⁶ It was further shown that chicken ovalbumin upstream promoter-transcription factor (Cox-2), a downstream target of IHH, is responsible for keeping balance between ER and PR activities.²²,²⁷ Unlike IHH, the P₄-induced transcription factor, Hand2 is expressed in the stroma and suppresses the E₂-induced fibroblast growth factor (FGF) production. Deficiency in uterine Hand2 results in implantation failure that is associated with high estrogenic activity and epithelial cell proliferation, suggesting that Hand2 mediates the antiproliferative activity of P₄ and by that allows epithelial differentiation, facilitating the acquisition of uterine receptivity.²⁸ Conventional ablation of the Bmp-2 leads to a complete infertility, due to impaired decidualization. Microarray analysis in vivo and in vitro identified FKBP6s and WNT ligands as Bmp-2 downstream targets.²⁹,³⁰ Taken together, these studies suggest that the balance between uterine E₂ and P₄ activities is crucial for normal receptivity and that this balance is regulated by stromal-epithelial communication.

**Other Genes that Are Involved in the Development of a Receptive Endometrium**

One of the genes that is up-regulated during the WOI is proprotein convertase 6 (PC6), localized mainly at the site of implantation. In vivo ablation of the PC6 in mouse endometrium completely inhibited implantation.³¹ This protein is crucial for decidualization in mice and humans.³¹,³² Knockdown experiments in human endometrial epithelial cells demonstrated its critical role in blastocyst adhesion.³³ It has been suggested that normal implantation takes place in an inflammatory environment provided by the receptive endometrium.³⁴,³⁵ In this context, prostaglandins (PGs) were demonstrated to be crucial for successful embryo implantation. Uterine depletion of cytosolic phospholipase A₂, cyclooxygenase-2 (COX-2), and lysosphosphatic acid receptor LPA₄, which have a central role in PG synthesis, leads to implantation failure in the mouse.³⁶–³⁸ In human, defective PG synthesis correlates with repeated implantation failure in patients undergoing IVF treatment.³⁹ It was suggested that the levels of PGE₂ and PGF₂α in the uterine fluid, 24 hours before embryo transfer, could predict pregnancy outcome. In vitro experiments demonstrated that embryo adhesion to the epithelium is significantly reduced in epithelial cells pretreated with inhibitors of PG synthesis.³⁹

**Involvement of the Immune System in Acquisition of Endometrial Receptivity**

In contrast to its classical role in protecting the organism from invading foreign antigens, the endometrial immune system supports invasion and maintenance of the fetal semi-allograft.⁴⁰ This paradoxical immune activity is a result of specific E₂ and P₄-regulated cytokines and chemokines which serve to recruit specific leukocyte populations into the endometrium and regulate their differentiation. Leukocytes in human endometrium comprise different subpopulations, including uterine natural killer (uNK) cells, macrophages (Mac), and dendritic cells (DCs). The uNK cells, the abundance of which increases at the WOI and early pregnancy, compose approximately 70% of the decidual leukocyte population, and their role in stromal decidualization has been suggested.⁴¹ Circulating NK cells in the peripheral blood are cytotoxic, however, upon their infiltration into the endometrium, they undergo differentiation into uNK cells, losing their killing activity.⁴² The cytokine IL-15, secreted by DCs and endometrial cells, as well as transforming growth factor beta (TGF-β)1, secreted by Mac, are essential for this process.⁴³,⁴⁴–⁴⁶ Mac and DCs are the major antigen presenting cells in the endometrium. They are present in the endometrium throughout the menstrual cycle and demonstrate increased abundance during the WOI and early pregnancy.⁴⁷–⁴⁸

Accumulating evidence suggests that, in addition to their classical role in mediating immune response, DCs and Mac have a pivotal role in implantation. Transient deletion of DCs resulted in a faulty decidualization characterized by reduced proliferation, differentiation, and delayed angiogenesis.⁴⁹,⁵⁰ Supporting their role in implantation, therapy by DCs administration significantly decreases the rate of spontaneous resorption of embryos in the mouse uterus.⁵¹ As initially reported by us, and later by others, endometrial biopsy substantially increases implantation and clinical pregnancy rates in IVF patients with repeated implantation failure.⁵²–⁵⁸ This increase in pregnancy rate was associated with elevation of DCs and Mac abundance.⁵⁹ Endometrial Mac and DCs

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**Mutual Activity of Sex Steroid Hormones and the Immune System**

Gnainsky et al. 339

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**Seminars in Reproductive Medicine** Vol. 32 No. 5/2014
secrete both proinflammatory and anti-inflammatory cytokines, by which they may affect Th1/Th2 cytokine balance as well as tissue remodeling and growth,\textsuperscript{66–62} thus inducing endometrial regeneration. Based on in vitro experiments, we suggested that endometrial biopsy-induced tumor necrosis factor (TNF)\textsubscript{α} mediates the injury-induced secretion of macrophage inflammatory protein (MIP)-1B, growth-regulated protein (GRO)\textsubscript{α}, IL-15 by the endometrial tissue.\textsuperscript{59} Other studies showed that TNF\textsubscript{α} induces secretion of IL-11 and LIF, cytokines that are essential for decidualization,\textsuperscript{63,64} further supporting the contribution of inflammation to the development of receptive endometrium.

It is important to note that during the menstrual cycle, the steroid hormones, E\textsubscript{2} and P\textsubscript{4}, modulate the production of different proinflammatory cytokines such as IP-10 and MIP-1B.\textsuperscript{65,66} Interestingly, E\textsubscript{2} synthesis is reciprocally regulated by the proinflammatory cytokine IL-6. This cytokine acts synergistically with TNF\textsubscript{α} to increase aromatase, 17-β-hydroxysteroid dehydrogenase and estrone sulfatase activity, enhancing local E\textsubscript{2} biosynthesis.\textsuperscript{67} In this context, local injury of the endometrium during the proliferative phase substantially increases endometrial ER expression in the following secretory phase.\textsuperscript{68} Previous experiments in mice revealed that endometrial niche cells expressing ERα directly respond to E\textsubscript{2} by transmission of proliferative signals to neighboring endometrial stem/progenitor cells.\textsuperscript{69,70} Taken together, it seems that in IVF patients undergoing endometrial biopsy, stem cells that are present in the endometrium may respond to the injury by proliferation in an E\textsubscript{2}-dependent manner.\textsuperscript{72} Therefore, in IVF patients with recurrent implantation failures, when steroid hormones are apparently insufficient in provoking endometrial receptivity, mechanical intervention by endometrial biopsy may elicit the inflammatory response required for successful implantation.

**Implantation**

Embryo implantation is divided into three sequential stages: apposition, adhesion, and invasion.

**Apposition**

Apposition is a dynamic process, during which the free-floating blastocyst and the receptive endometrium initially interact. Having tethering and rolling over, "scanning" the uterine surface, the embryo spots the specific site of implantation and attaches the endometrium. A similarity between the rolling blastocyst in the uterus and the leukocyte migration on the endothelial wall has been suggested.\textsuperscript{73} In both processes, interaction is established by binding of the L-selectin molecule expressed on the leukocytes as well as on the trophoblast cells, to its oligosaccharide ligands, localized on the endothelial wall and on the luminal epithelium, respectively.\textsuperscript{74} Surprisingly, L-selectin-deficient mice are fertile.\textsuperscript{75} However, in human, elevated level of the L-selectin ligand, MECA-79, is associated with improved implantation, while decreased expression of sulfotransferase, GlicNAc6ST-2, which is involved in the generation of functional L-selectin ligands in the endometrial cells, was associated with infertil-
Adhesion

Embryo–endometrium interaction is stabilized by adhesion molecules such as troponin, cadherins, and integrins (ITGs).\(^{104}\) ITGs that are expressed by both, the endometrium and the blastocyst, bind to several ECM Arg-Gly-Asp (RGD) containing ligands such as fibronectin, vitronectin, thrombospondin, and osteopontin (OPN). This binding serves as a bridge between the luminal uterine epithelium and the blastocyst.\(^{104}\) It was suggested that OPN, the expression of which increases in the endometrium during its receptive phase, plays a crucial role in establishing the embryo–endometrium interaction.\(^{59,105}\) Interestingly, although OPN KO mice are fertile, functional blocking of endometrial OPN with specific antibodies, significantly reduced the number of implantation sites.\(^{106}\) Moreover, it has been shown that OPN increases blastocyst adhesiveness by binding to its surface receptors, CD44 and/or ITGs. This binding initiates focal adhesion kinase and PI3K/AKT signaling pathways that induce the formation of functional ITG adhesion complexes.\(^{107}\) ITG αvβ3 is a well-characterized OPN receptor in human endometrium, the production of which is elevated at the WOI. In vivo studies in mice showed that ITGβ3 – deficient mice are infertile, and that functional blocking of endometrial ITGαvβ3, by intrauterine injection of specific antibodies, significantly reduces the number of implantation sites.\(^{106,108}\) Similar to these results, blastocysts failed to attach to epithelial cells pretreated with ITGβ3 siRNA in vitro.\(^{109}\) Nevertheless, the correlation between endometrial ITGβ3 levels and infertility is still inconclusive.\(^{13,110–116}\) The expression of this ITG is regulated by P4, mediated by EGF, HB–EGF, and Hoxα-10.\(^{84,117–119}\) Simón et al.\(^{120}\) demonstrated that human endometrial epithelial cells induce the secretion of the cytokines IL-1α and IL-1β by the blastocyst, and these in turn, increase ITG expression by the epithelial cells. A bidirectional crosstalk between the blastocyst and the endometrium is also suggested by a recent demonstration that factors released to the medium by human blastocysts that successfully implanted following embryo transfer, alter messenger RNA levels of several genes in the epithelial cells, in vitro, facilitating their adhesive properties.\(^{121}\)

Invasion

Invasion is the final step of implantation, during which the trophoblast fractures the epithelial lining of the uterus and penetrates into the endometrial stroma. Decidualized stromal cell and uNK cells secrete factors that stimulate trophoblast invasion by altering expression of key regulators such as, ITGs, MMPs, and their tissue inhibitors of metalloproteases (TIMPs).\(^{81,122–124}\) They also induce vascular growth and remodeling by secretion of large amount of angiogenic factors, MMP-2 and MMP-9.\(^{92,125}\) Moreover, uNK cells secrete factors that trigger endometrial stromal cells to produce cytokines/chemokines, such as IL-15, which in turn supports uNK differentiation, while others, IL-8, CCL8, and CXCL1, act synergistically with uNK-secreted chemokines to induce trophoblast migration.\(^{126}\)

Regulation of endometrial remodeling and clearance of apoptotic trophoblast cells during trophoblast invasion is attributed to Mac.\(^{127,128}\) The invading trophoblast secretes chemokines, such as CXCL16 and CXCL12 that recruit and activate immune cells to the decidua.\(^{129–131}\) Monocytes that are recruited and “educated” by the trophoblast cells, positively affect trophoblast development and function, by both secretion of cytokines such as IL-6, IL-8, MCP-1, and GROα and stimulating trophoblast to secrete cytokines that in an autocrine manner facilitate their own growth, survival, and invasion.\(^{130}\)

Proteases pave the way for the invading trophoblast by digesting the extracellular matrix. The human MMP gene family includes 23 members,\(^{132}\) of which MMP-2 and -9 were mostly studied. Almost all MMPs are produced by the trophoblast, decidual stromal cells, and uNK cells,\(^{133}\) and their expression is tightly regulated by hormones, cytokines, and growth factors as well as by the TIMPs. It was demonstrated that EGF and cytokines, such as IL-6, IL-1β, TNFα, and IL-8, stimulate trophoblast invasion by increasing levels of trophoblast MMP-2 and -9.\(^{134–138}\) Activation of STAT3 by LIF was shown to regulate trophoblast invasiveness by TIMP-1 down-regulation.\(^{124}\) However, TGF-α1 inhibits the proteolytic activity of cytotrophoblast cells by up-regulating TIMP-1 and 2,\(^{138,139}\) Another negative regulator of MMP activity in the trophoblast cells is P4 that inhibits the expression of MMP-2, MMP-3, MMP-7, and MMP-9 and increases the expression of TIMP-3, thus restricting trophoblast invasion.\(^{15}\) In addition to MMPs, the invasive trophoblast expresses various members of a disintegrin and MMPs (ADAMs), such as ADAM8, -12, -19, and -28.\(^{140}\) In contrast to the secreted forms of MMPs, most of the ADAM family members are membrane proteins. It was suggested that ADAM family members regulate trophoblast invasion by proteolytic shedding of the membrane anchoring ectodomains, thus activating variety of chemokines, cytokines, growth factors, receptors, and their ligands.\(^{141}\)

Epilogue

Although protocols for infertility treatments are being constantly improved, implantation remains the rate-limiting step for their success. In IVF patients, who generate high-quality embryos but fail to conceive, it is apparently inadequate uterine receptivity that is responsible for implantation failure. Many efforts have been invested in identifying endometrial biomarkers that can predict implantation competence. The studies discussed in this review suggest different implantation-associated genes/proteins as promising candidates. Using the “omics” approach (transcriptomics, proteomics, and secretomics) in endometrial biopsies/uterine aspiration, groups of genes/proteins that characterize a receptive endometrium were proposed.\(^{142}\) A new assay defined as endometrial receptivity array (see article “Endometrium and Implantation Clinical Management of Endometrial Receptivity” by Blesa and Ruiz-Alonso in this issue) seems to provide accurate and consistent parameters for the evaluation of uterine receptivity.\(^{143–146}\) Evaluating uterine receptivity using the appropriate biomarkers could provide a potential tool for assessing the success of infertility.
treatments. Furthermore, high probability of implantation argues in favor of the transfer of a single embryo in IVF treatment, avoiding the subsequent severe complications of multiple pregnancies.

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Seminars in Reproductive Medicine Vol. 32 No. 5/2014
Mutual Activity of Sex Steroid Hormones and the Immune System


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