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PROGESTERONE RECEPTOR-REGULATED GENE NETWORKS IN IMPLANTATION

Indrani C. Bagchi, Yong-Pil Cheon, Quanxi Li, and Milan K. Bagchi

Dept. of Veterinary Biosciences and Molecular & Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61822

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1. ABSTRACT

The steroid hormone progesterone (P) plays a pivotal role in the establishment and maintenance of pregnancy. The well-known function of P during early pregnancy is to regulate (i) uterine receptivity for blastocyst attachment, (ii) progressive phases of embryo-uterine interactions, and (iii) differentiation of the endometrial stroma that maintains an environment conducive for the growth and development of the implanting embryo. The cellular actions of P are mediated through intracellular progesterone receptors (PRs), which are well-studied gene regulators. It is postulated that hormone-occupied PRs trigger the expression of specific gene networks in different cell types within the uterus and the products of these genes mediate the hormonal effects during early pregnancy. In the present article, we provide a brief description of the molecules that have emerged as candidate markers of progesterone action in rodents and humans during implantation.

2. INTRODUCTION

Progesterone (P) plays a crucial role during early pregnancy by coordinating a complex series of interactions between the implanting blastocyst and the receptive uterus (1-5). In mice, implantation is initiated four days after fertilization when the blastocyst reaches the uterus (1,6). Studies by Psychoyos and coworkers demonstrated that in rodents, the attachment of the blastocyst to the endometrium, which initiates implantation and establishes pregnancy, could occur only for a brief period of time known as the receptive phase (1). This phase is transient and tightly regulated by P and E. Both of these hormones also regulate the transformation of a receptive endometrium to the subsequent refractory status during which embryo attachment can no longer occur (1,7-9).

While the combined action of E and P is essential for acquisition of uterine receptivity, P is the critical hormone for decidualization (1,10). Decidualization involves differentiation of the fibroblast cells of endometrial stroma into morphologically distinct cells, termed decidual cells, which show unique secretory and biosynthetic properties (10,11). This differentiation, induced by P following a brief priming by E, is a prerequisite for successful implantation. Decidua is a transient tissue, which first develops at the time of blastocyst attachment on day 4.5. During the next three days, the decidual cells proliferate and differentiate extensively, and then they start to undergo apoptosis. By the end of the invasive period (day 10.5), the decidua is totally regressed. A variety of functions have been attributed to the decidua, such as providing nutrition to the embryo, being a source of hormones and cytokines, and serving an immunoregulatory role during pregnancy (12-14). However, the most widely accepted role of decidua is its regulation of trophoblast

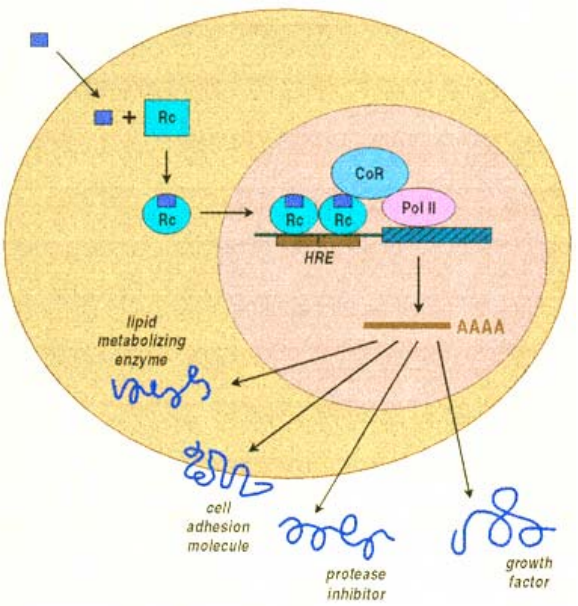

Figure 1. The signal transduction pathway of progesterone receptor. The hormone-bound PR (Rc) homodimer interacts with the hormone response element (HRE) at a target gene to regulate initiation of transcription by RNA polymerase II (pol II) machinery. CoR denotes cellular coregulators (e. g. coactivator) that act in concert with PR to regulate transcription. The RNA transcript is translated into a protein that carries out a particular cellular function.

invasion (12-14). If uncontrolled, trophoblast invasion of the endometrium could be destructive. It is, therefore, strictly controlled by maternal factors secreted by the decidualizing stroma (15). The P-induced signaling molecules that participate in the formation and promote the function of the decidual tissue remain poorly understood.

3. PROGESTERONE RECEPTORS INDUCE SPECIFIC GENE EXPRESSION IN PERIIMPLANTATION UTERUS

Although much of the molecular and cellular mechanisms by which P controls the reproductive tract function during early pregnancy are unclear, there is little doubt that the majority of the physiological effects of this hormone are mediated by interaction with intracellular progesterone receptors (PRs) (16). The strongest evidence in favor of an essential role of PR in P-mediated responses in various reproductive tissues came from a mouse model carrying a null mutation of the PR gene (17). The female PR knockout (PRKO) mice, which are infertile, display multiple reproductive abnormalities. They fail to ovulate because of a defect in follicular rupture. Furthermore, the uteri of these mutant mice are hyperplastic and fail to respond to an artificial decidual stimulus, indicating that in the absence of PR, the endometrial tissue of the homozygote uterus is refractory to the implantation signals of the preimplantation embryo.

PR, a member of the nuclear receptor superfamily, is a transcription factor (16,18,19). It exists as two protein isoforms, termed PR-A and PR-B (16). Both isoforms are expressed from a single gene in rodents and humans as a result of transcription from two alternative promoters and translation initiation at two different codons (20,21). The selective physiological roles of the two isoforms of PR are predicted to differ based on different structural and functional properties of the individual proteins observed using in vitro assay systems (22,23). Consistent with this paradigm, more recent studies have shown that mice lacking PR-A isoform are infertile and exhibit severely impaired uterine phenotype analogous to PR null animals (24). In contrast, mice in which PR-B was ablated are fertile and appear to possess normal uterine phenotype (25). Most interestingly, the availability of the PR isoform-specific KO mice showed, for the first time, that PR-A and PR-B regulate different sets of target genes in a reproductive tissue (24,25).

In a P-responsive cell, the hormone binds to its intracellular receptor and triggers its gene regulatory function (Figure 1). The hormone receptor complex interacts with specific cellular target genes to modulate their expression (16). It is likely that P triggers the expression of a network of genes in the endometrium during early stages of pregnancy and these eventually lead to the synthesis of new proteins, which prepare the uterus for establishment and maintenance of gestation.

4. POTENTIAL ROLES OF NEWLY DISCOVERED P-INDUCIBLE GENES IN IMPLANTATION

Although PR plays a crucial regulatory role in the implantation process, the molecular pathways that mediate the uterine effects of this receptor remained mostly unexplored. Previous studies in rodents have identified only a handful of P-regulated genes in the uterus. These include the genes encoding the growth factor amphiregulin, the homeobox proteins Hoxa-10 and Hoxa-11, peptide hormones calcitonin and proenkephalin, and the enzyme histidine decarboxylase (5, 26-30). The recent advent of microarray-based analysis, however, provided, for the first time, a comprehensive profile of PR-regulated gene networks with potential roles during implantation (31). These studies have identified a variety of novel PR regulated molecules, such as transcription factors, growth factors and their cell surface receptors, cell remodeling and adhesion molecules, and protease inhibitors, which may play important roles in the uterus during implantation (31). The current challenge is to link these molecules and their pathways to previously well characterized morphological, physiological, and biochemical events that are associated with the process of implantation. We provide below a brief discussion of the plausible roles that some of these molecules might play in mediating P effects in the pregnant uterus.

4.1. Transcription and growth factors

It is known that during the preimplantation period, a rise in the level of P suppresses E-regulated proliferation of epithelial cells (4). P simultaneously promotes differentiation of epithelial cells and induces proliferation of stromal cells. With the onset of

implantation, the stromal cells undergo P-induced differentiation to decidual cells. This sequential triggering of P-controlled proliferation and differentiation of different uterine cell-types is essential for acquisition of receptivity (32). It is, therefore, not surprising that osteoblast-specific factor 2 (Osf2), a transcription factor involved in cell differentiation and growth factors such as amphiregulin are under the influence of P in the uterus at the time of implantation (26, 31). Both Osf2 and amphiregulin are expressed at a low level on day 3 of pregnancy, the expression peaks on day 4, the day of implantation, and declines the following day (26, 31). *In situ* hybridization revealed that Osf2 and amphiregulin expression in the mouse uterus is restricted to the luminal epithelial cells of the uterus (26, 31). The functional role of these molecules in implantation, however, has not been established yet. While the Osf2 null mice die due to a complete lack of bone formation, the amphiregulin knockout mice are healthy and fertile and show no apparent defect in implantation (33, 34). The lack of phenotype in amphiregulin null mice is attributed to the fact that other family members of EGF that are expressed in the uterus during early pregnancy could potentially compensate for the loss of amphiregulin.

4.2. Homeobox genes and Morphogens

P regulates the expression of Hox family of genes including Hoxa-10 and Hoxa-11 in the uteri of pregnant mice (27). Homeobox or Hox genes are developmentally regulated transcription factors that control morphogenesis of the reproductive tract (35, 36). While morphogenesis of most organs is completed by adulthood, the uterus undergoes a remarkable program of cellular proliferation and differentiation during early pregnancy. It is likely that Hox genes expressed in the adult uterus may be involved in these processes during implantation. In response to rising P level, both Hoxa-10 and Hoxa-11 are expressed in the stromal cells starting from day 2 of pregnancy (37, 38). The expression continues during transformation of stromal cells into the decidual cells following implantation. The maternal expression of Hoxa-10 is required for female fertility (37, 39, 40). Homozygous Hoxa-10 mutant mice are either sterile or give birth to small litters. Although the proximal uterus of Hoxa-10 mutant mice shows partial homeosis into an oviduct like structure, this is not the major cause of infertility in these mice. In Hoxa-10 null mice, decidualization is severely compromised, indicating a maternal requirement for Hoxa-10 in the periimplantation uterus (40). The Hoxa-11 mutant mice also exhibit female infertility due to uterine defects such as stromal and glandular cell development (38, 41).

Recent studies have shown that the expression of another morphogen, Indian hedgehog (Ihh) in the pregnant uterus is under P regulation (42, 43). Ihh is a member of the developmentally regulated morphogens, the Hedgehog gene family, which regulates both cell proliferation and differentiation (44). Unlike Hox genes, however, Ihh is expressed in the luminal and glandular epithelial cells starting on day 2 of pregnancy (42, 43). The expression peaks on day 3, declines on day 4 and disappears by day 5 of gestation. A targeted null mutation of Ihh has been made (45). However, the Ihh null mutants exhibit short-limbed dwarfism and perinatal lethality, which is presumably caused by respiratory failure. The role of Ihh in the uterus during implantation therefore, remains to be determined.

4.3. Peptide Hormones

Previous study identified two peptide hormones, proenkephalin and calcitonin, as P-regulated endocrine signals in the uterus (28, 29). The expression of the precursor of enkephalin was reported to increase dramatically in the mouse endometrium at the onset of implantation and continued during gestation (31, 46). Interestingly, enkephalin appears to be involved in regulating peristalsis of the intestines and in inhibiting contractions of the vas deferens (47, 48). A similar role for enkephalins in controlling muscle contractility during implantation is conceivable. This scenario is particularly attractive since P is known for its role in inhibition of uterine contractility and maintenance of tranquil environment during gestation.

Unlike enkephalin, which is expressed throughout gestation, the peptide hormone calcitonin, is transiently expressed in the rat uterine epithelium overlapping the window of implantation (49). The expression of calcitonin mRNA or protein increases by day 2 of gestation and reaches a peak on day 4, the day before implantation. On day 5, the day implantation occurs, the expression of the gene starts to decline and by day 6, when implantation is completed, the calcitonin level falls to below detection limits (49). The timing and location of calcitonin synthesis in the epithelium raise the possibility that calcitonin is secreted by the glands into the uterine lumen and its principal function may be to regulate blastocyst implantation in an autocrine or paracrine manner. Studies have indeed shown that the administration of antisense oligodeoxynucleotides (ODNs), targeted specifically against calcitonin mRNAs, into the lumen of the preimplantation phase uterus results in a dramatic reduction in the number of implanted embryos (50). Similar treatment with the corresponding sense ODNs exhibits no effect on implantation, suggesting that the block in embryonic implantation upon administration of the antisense ODNs into the uterus is a direct phenotypic consequence of the suppression of calcitonin gene expression in the implantation phase of gestation (50). Recently, the mechanism by which calcitonin regulates implantation was investigated (51). Interestingly, these studies suggest that calcitonin regulates implantation by inhibiting the expression of calcium-dependent cell adhesion molecule E-cadherin from epithelial cell-cell contact sites, leading to the relaxation of adherent junctions at the time of trophoblast invasion.

4.4. Extracellular matrix and Cell Adhesion Molecules

P-regulated molecules may also control cell shape, motility and adhesion during implantation. During early stages of implantation, the embryonic trophectoderm cells become closely apposed to the luminal epithelium. In mammals, especially the rodent, a generalized stromal edema occurs before the beginning of apposition (52). This event leads to the closure of the uterine lumen, which

results in interdigitation of microvilli of the trophectoderm and luminal epithelia (53). Adhesion molecules expressed at the surface of the luminal epithelial cells could potentially facilitate the implantation process. It is noteworthy that P markedly induced the expression of Immune responsive gene 1 (Irg1), a putative proteoglycan harboring motifs for glycosaminoglycan attachment site (31, 54). Although the function of Irg1 is unknown, studies indicated that Irg1 mRNAs appear in the luminal epithelial cells precisely on day 4 of pregnancy, coinciding with the adhesive phase of the uterus (54).

4.5. Enzymes

Previous studies have shown that, during the preimplantation phase in mice, the oxygen consumption by the uterine tissue increases and glucose incorporation reaches a peak at the time of implantation (55). Consistent with this paradigm, uterine expression of various metabolic enzymes, such as pyruvate carboxylase, PEPCK, peptidylarginine deiminase, and carbonic anhydrase, are altered in response to RU486 at the time of implantation (31). Interestingly, leukocyte- and epidermal-12/15 lipoxygenases (12/15-LOX), which are involved in oxidative metabolism of arachidonic and linoleic acid are induced by P at the time of implantation (31). These enzymes are known to generate metabolites, such as hydroxy-eicosatetraenoic acids (HETES) and hydroxy-octadecadienoic acids (HODES), which serve as cell differentiation signals (56). Recent studies have shown that the uterine levels of the 12/15-LOX-derived metabolites, 12-HETE, 15-HETE, and 13-HODE, increased dramatically on the day of implantation and this increase was blocked by antiprogestin RU (57). Administration of a 12/15-LOX-specific inhibitor to pregnant mice led to a drastic decline in the production of these metabolites and a concomitant inhibition of steroid hormone-regulated uterine receptivity during implantation (57). Collectively, these results indicate that P-induced 12/15-LOX enzymes in the uterus play a critical role during implantation.

The enzyme histidine decarboxylase (HDC), which, is involved in the biosynthesis of histamine, is also induced in response to P in the epithelial cells of the uterus during early pregnancy (30). An epithelial accumulation of HDC occurred predominantly on day 4 of pregnancy, which then declined by day 5 of gestation. Participation of histamine in embryo implantation has long been considered important. Consistent with this notion, recent studies have shown that uterine-derived histamine interacts with embryonic H2 receptors in a paracrine fashion to initiate the process of implantation (58).

4.6. Protease inhibitors

Recent studies have shown that P regulates the expression of two protease inhibitors in the pregnant uterus: p12 (a serine protease inhibitor) and cytotoxic T lymphocyte activator 2β (a cysteine protease inhibitor) (31). p12 exhibits extensive sequence homology at the amino acid level with members of the Kazal family of secretory serine protease inhibitors, and appears to be the mouse homologue of human pancreatic secretory trypsin inhibitor (PSTI) (59). Cytotoxic T lymphocyte activator 2β is secreted from T lymphocytes and mast cells (60).

Both p12 and CTLA-2β exhibited unique decidual stage-specific expression during early pregnancy (31). The mRNA corresponding to p12 and CTLA-2β mRNAs are induced in the mouse endometrium on day 4.5 of pregnancy, attains a peak during days 6-8, and then declines to undetectable levels after day 10 with the completion of the decidual phase (31). A role of protease inhibitors in regulating embryo-uterine interactions is consistent with the observation that the final stage of implantation involves invasion of the epithelium by the trophectoderm. Trophoblast invasion requires endometrial extracellular matrix proteolysis, as well as cellular migration through the maternal deciduas (61). These processes are precisely regulated and require a balanced interplay between the factors that promote and restrain trophoblast invasion. Existing evidence indicates that factors that promote invasion are primarily trophoblast-derived (61, 62). Consistent with this notion, mouse trophoblast has been shown to synthesize and secrete serine proteases (plasminogen activator), metalloproteases (gelatinase and stromelysin), and the cysteine proteases (cathepsin L) (63-67). The maternal decidua is thought to participate in the control of invasion by secreting the inhibitors of these proteases. It is, therefore, possible that p12 and CTLA-2β secreted during the decidual phase is involved in the regulation of trophoblast invasion.

5. DYNAMICS OF EXPRESSION OF PR AND ITS TARGET GENES IN THE PREGNANT UTERUS: CLUES TO FUNCTION

PR is expressed in different uterine cell types during early pregnancy. The pattern of total PR (PR-A plus PR-B) expression in the pregnant mouse uterus has been analyzed by immunohistochemistry (31). PR is undetectable in the endometrium on day 1 of pregnancy. A modest level of PR protein is expressed in the epithelium and stroma on day 2 of pregnancy. The PR accumulation increases dramatically on day 3 and is detected in both epithelium and stroma. On the day 4 of pregnancy, the level of PR in the luminal epithelial cells decreases slightly, but a marked increase in the level of stromal PR is observed. While a high level of PR continues to express in the stromal cells during the post-implantation days 5-7, the level of PR in the luminal epithelial cells declines sharply. These results demonstrate that PR is expressed in a cell- and stage-specific manner in the uterus during early pregnancy.

Analysis of spatio-temporal expression of PR-regulated genes revealed distinct patterns of gene expression (Figure 2). Most interesting among these is the expression of a set of genes precisely overlapping the implantation phase (days 3-5) (Figure 2, left panel) and another set overlapping the decidual phase (days 5-10) (Figure 2, right panel) of pregnancy. It is conceivable that the array of genes induced by PR in the epithelium within the preimplantation window of receptivity (days 3-5) may include critical regulators of embryo-uterine interactions during implantation. These include genes that encode Osf2, amphiregulin, Ihh, HDC, lipoxygenase, and Irg1. The PR-regulated genes that are expressed during days 5-10 in the

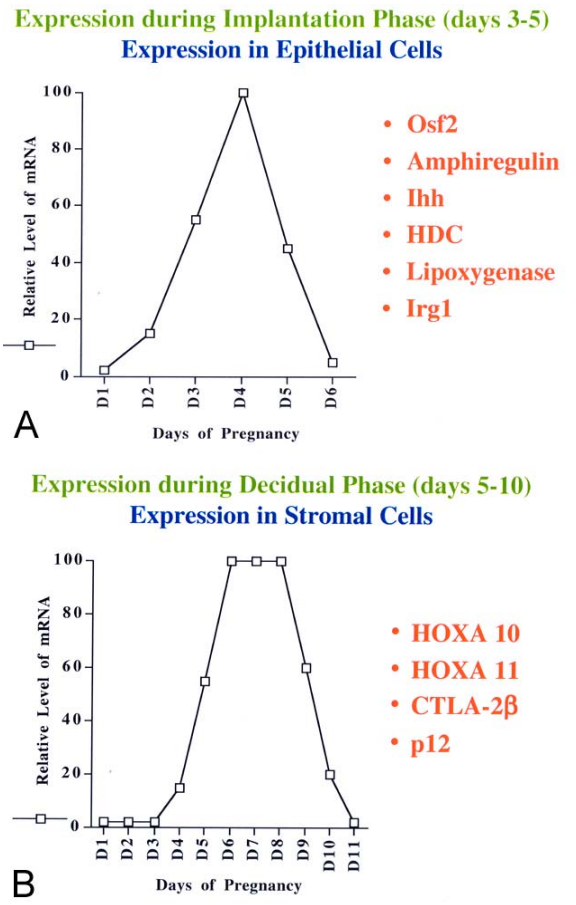

Figure 2. Temporal Expression of P-regulated Genes in Pregnant Mouse Uterus. A: Representative profiles of mRNA expression during the implantation phase. B: Representative profiles of mRNA expression during the decidual phase.

stromal compartment might be involved in various functional aspects of the decidualization process. Prominent members of this group are Hoxa 10 and 11, p12 and CTLA-2β. The abundant expression of PR in the stromal cells may directly control the expression of certain of these genes. It is also likely that PR-regulated gene products synthesized in the stromal cells may act in a paracrine manner to regulate gene expression in other cell-types within the uterus. A potential example of this is the P-dependent expression of p12 in the glandular cells during decidualization. Although PR expression in the glands declines in the post-implantation period, the P-dependent expression of p12 in these cells peaks during days 6-8 (31). We postulate that paracrine effectors produced by PR in the stromal cells drive p12 induction in the glands. Further analysis of uterine expression and function of specific PR target genes will help us to understand how different signaling pathways acting in distinct cell-types may interact or merge to regulate complex physiological events such as implantation and decidualization.

6. MOLECULAR MARKERS OF PROGESTERONE ACTION IN HUMAN ENDOMETRIUM

In order to understand the mechanisms that govern P-mediated responses in human pregnancy, the mouse and the rat have been used extensively as convenient model systems. Although evolutionarily very different, the two species have a similar hemochorial or an "invasive" type of placenta and also have many similarities in terms of hormone-responsive gene expression in the uterus.

Unlike rodents, however, the existence of a limited period of uterine receptivity or an "implantation window" remains to be established in human. The current understanding is that the fertilized ova arrives in the uterine cavity around day 17 (day 14 is taken as day of ovulation of a 28-day cycle), and remains there as a free-floating embryo until about day 19; implantation then occurs between day 19 to 22 (68-72). The implantation process in human is also under strict hormonal control (73, 74). A marked rise in the level of P along with a moderate increase in the level of E occurs during the post ovulatory or luteal phase of the cycle. However, in contrast to rodents, no direct argument in favor of an obligatory role of this E rise in human implantation is available so far. P on the other hand, is absolutely critical for the onset and maintenance of pregnancy in humans as evidenced by the detrimental effect of corpus luteum ablation in early pregnancy (75) and by the abortive action of the antiprogestin RU486 (76). Consistent with this notion, inadequacy of the luteal phase, which correlates with low P level during the menstrual cycle, has been identified as one of the factors for human infertility (77, 78).

The human endometrium undergoes cyclical cellular and molecular development in response to steroid hormones (73, 74). An intense proliferation of both epithelial and stromal cells occurs in response to rising E level during the first half of the cycle or proliferative phase. At mid-cycle, ovulation takes place and ovarian P production is initiated. During the second half of the cycle or secretory phase, P action dominates in the endometrium. It suppresses proliferation and induces endometrial differentiation. Extensive epithelial differentiation, characterized by glandular secretion, is observed 5-7 days following ovulation (days 19-21). This is followed by stromal cell differentiation, which is evident 9 or 10 days after ovulation (day 23-24). During this process, the stromal cells differentiate to a distinct morphological appearance accompanied by a unique biosynthetic and secretory phenotype. In the event of pregnancy, decidual changes become more extensive and are essential for a successful implantation. The molecules that have emerged as targets of PR action in the human endometrium during the window of implantation are described below.

6.1. Homeobox genes

The expression of both HOXA-10 and HOXA-11 has been investigated in the human endometrium during the menstrual cycle (79, 80). The signals corresponding to HOXA-10 and HOXA-11 mRNAs were enhanced in the glandular epithelium and stromal cells of the P-dominated

secretory phase of the menstrual cycle (79, 80). The expression of these homeobox genes in cultured endometrial cells were stimulated by progesterone or estrogen suggesting that sex steroids regulate HOX gene expression in the human endomterial cells (79, 80).

6.2. PeptideHormones

The synthesis of calcitonin mRNA and protein has been monitored in the human endometrium at different days of the menstrual cycle (81). Studies showed that calcitonin expression in human endometrium is temporally restricted to the epithelium of mid-secretory phase of the cycle, which closely overlaps with the putative window of implantation (81). It was also observed that, as in rodents, P regulates calcitonin expression in human endometrium (81). Calcitonin, therefore, emerged as a P-regulated potential marker of the receptive endometrium in the human.

6.3. Secreted Proteins

Previous studies have shown that P regulates the expression of two secreted proteins, glycodelin and IGFBP-1, in the human endometrium (82-85). Glycodelin is synthesized during implantation and early gestation from the glandular cells of human endometrium. *In vitro* studies using human endometrial adenocarcinoma cells have shown that ligand-activated PR increases the promoter activity of glycodelin through two Sp1 sites (86). The IGFBP-1 gene product, on the other hand, is synthesized and secreted from the decidualized endometrial stromal cells. Analysis of the promoter activity of IGFBP-1 in endometrial stromal cells revealed that ligand-bound PR acts through distinct progesterone response elements to activate this promoter (87, 88). Recently, a global gene expression profiling has been performed in human endmetrium during the window of implantation (89). Interestingly, both glycodelin and IGFBP-1 have appeared in the analysis, further confirming the expression of these molecules in response to rising P level during the mid secretory phase of the menstrual cycle overlapping the window of implantation.

7. PERSPECTIVES

Considering the central role played by P in controlling the physiology of early pregnancy, the identification of its downstream targets and their subsequent functional characterization is crucial for understanding this hormonal regulation. The advent of the DNA microarray methodology has facilitated the identification of novel P-inducible gene pathways that drive the events underlying uterine receptivity, control of embryo invasion, and stromal cell decidualization. The recent development of PR-A and PR-B null mice will allow a comprehensive analysis of the PR isoform-specific regulation of these genes. Furthermore, gene knockout mouse models can be developed to perform functional analysis of selected genes. Collectively, these approaches will help us to identify molecules that are critical mediators of P regulation of embryo-uterine interactions during blastocyst implantation. Certain of these P-regulated genes may also emerge as markers of the fertile human endometrium. The identification of such markers may assist in the diagnosis of female infertility due to failure of implantation and facilitate management of clinical therapy for affected women.

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Send correspodence to: Dr Indrani C. Bagchi, Dept. of Veterinary Biosciences and Molecular & Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61822, Tel: 217-333-7986, Fax: 217-244-1652 E-mail: ibagchi@uiuc.edu