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GENE EXPRESSION IN THE RHESUS MONKEY ENDOMETRIUM: DIFFERENTIAL DISPLAY AND LASER CAPTURE MICRODISSECTION

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1. ABSTRACT

The primate endometrium is a complex heterogeneous tissue that requires proper maturation to achieve a hospitable environment for implantation. Endometrial differentiation and maturation is primarily controlled through the action of progesterone during the secretory phase. Many of the genes and gene networks that are involved in this process are likely to be induced or inhibited in a temporal, spatial, and cell-type specific context within the endometrium. We have used several approaches to address these latter issues in the rhesus monkey endometrium. The use of differential display with hormonally distinct endometrial cDNA populations prepared from artificially controlled menstrual cycles has allowed us to identify different P-dependent mRNA regulatory patterns during simulated secretory phases. In addition, we have coupled differential display with laser capture microdissection to further study region and cell-type specific changes in the primate endometrium.

2. INTRODUCTION

In the human and menstruating non-human primates, the primary signal that governs endometrial function is the changing pattern of the female sex steroids, estradiol (E) and progesterone (P) (1,2). The endometrial processes of growth, differentiation, shedding, and reconstruction are expected to be the result of concomitant changes in gene expression regulated by these steroid hormones (3-7). P is the primary hormonal influence that regulates growth and differentiation of the endometrium during the secretory phase. One of the major objectives of our laboratory is the identification of P-regulated genes in the rhesus endometrium and their potential function in endometrial maturation that would permit implantation.

The evolving model of steroid hormone action in normal tissues requires the binding of hormone to its cognate receptor, which increases the affinity of the hormone-receptor complex to hormone response elements (HRE's). HRE's are discrete nucleotide sequences that appear as short or imperfect palindromic sequences near the promoters of steroid regulated genes. In addition to the up-regulation of specific genes, P has also been shown to specifically inhibit the transcription of target genes via interaction with its cognate HRE in upstream regulatory sequences (8-10). A recent study has mapped a negative modulation domain to a 120 residue stretch within the amino terminal portion of the PR (11). Recent studies have also shown that many transcriptional actions of P (as well as other members of the steroid receptor superfamily) require interactions with corepressors and coactivators (12-14). As might be expected, female PR knockout mice are infertile (15).

Studies aimed at the identification of implantation specific factors in rodent and rabbit models have implicated a number

of factors e.g. mucins and proteoglycans (16,17), members of the EGF family (amphiregulin (18), epiregulin and betacellulin (19)), calcitonin(20), histidine decarboxylase(21), and leukemia inhibitory factor (LIF) (22) (See (23,24) for reviews). Although these studies are important, it is unclear how they may relate to the human. For example, there is evidence that the anti-adhesive MUC1 is reduced during the receptive phase in the rat (25) and thereby potentially providing a more adhesive luminal epithelium for implantation. In contrast, this mucin appears to be elevated during the secretory phase in women (26). There is, however, recent evidence to suggest that a local reduction of MUC1 on the luminal epithelial surface is associated with implantation sites in the rabbit (17) and such an event could play a role in other species (27,28). In contrast to women, implantation in both mice and rats requires a surge of E just prior to the receptive window for implantation to occur (29). Importantly, these studies suggest that a single factor is unlikely to be solely responsible for the endometrial preparation required for receptivity. It is more likely that a number of temporally controlled levels of gene expression ultimately lead to an appropriate uterine environment for implantation.

3. STRUCTURAL FEATURES OF THE PRIMATE ENDOMETRIUM

3.1. Zonation

The two major morphological components of the primate endometrium are the upper functionalis region and the lower basalis region adjacent to the myometrium. The functionalis is a transient layer most of which is sloughed during menses. Using histological criteria Bartelmez et al (3) identified two different zones within each of these endometrial regions: the functionalis is composed of zone I, the luminal epithelia and densely packed stroma, and zone II, the upper third segment of the glands; the basalis is composed of zone III, the middle third of the glands, and zone IV, the deepest portion of the glands adjacent to the myometrium. A similar zonation of the endometrium is also apparent in the human (4). (See (30) for a schematic representation of the above)

3.2. Differential Cell-Type Composition

The number of different cell types that it harbors further defines the complexity of the endometrium. These cell types include; luminal and glandular epithelia, stromal fibroblasts, vascular smooth muscle cells, endothelial cells and cells of the lymphocytic system. The effect of a hormone on a target tissue has often been described in the past as an integral of the response to all cells and cell-types within the tissue. It has, however, become increasingly clear that different cells or cell-types within a target tissue can respond dissimilarly to the same hormone. For example, in our laboratory (31-33) and others (34-39) it has been shown by immunohistochemical techniques that progesterone inhibition of nuclear estrogen receptor is most pronounced in zones I,II and III while strong positive staining of glandular epithelia in zone IV is retained. Stromal cells in zones I, II, and III are also more rapidly affected by progesterone. In addition, it has been shown that there are zonal-dependent differences in proliferation during both E- or P-dominance during both natural menstrual cycles and artificial menstrual cycles in the rhesus monkey (40-43). These studies and others strongly support the concept that the primate endometrium contains distinctive microenvironments that can respond differentially to the same hormonal stimulation.

4. ANIMAL MODEL - RHESUS MONKEY

4.1. Artificial Menstrual Cycles

The development and use of artificial menstrual cycles in the rhesus monkey was first described by Hodgen (44). These studies showed that the rising and falling levels of E and P in a natural menstrual cycle could be simulated by the timed insertion and removal of silastic implants of E or P in ovariectomized animals. These seminal studies also showed that this simulation was sufficient to allow the endometrium to support implantation and eventual delivery (IVF and surrogate transfer).

The use of artificial menstrual cycles for studies on endometrial function allows the procurement of more reproducible and precisely timed endometrial samples than could be obtained reliably from natural cycles. In addition, the use of implants to simulate steroid profiles also allows us to simulate defects in steroid patterns particularly during the secretory phase i.e. luteal phase defects. Luteal phase defects in women are purported to be the most common endocrinopathy in infertility and recurrent abortion wherein low secretory P levels are not sufficiently elevated to achieve appropriate endometrial maturation (2,45-48). Both short and inadequate luteal phases similar to those found in women have been described in the rhesus monkey (49). These latter studies also provide support for the usefulness of the rhesus monkey as a model for luteal phase defects in women (46). We have created our inadequate secretory phase cycles to mimic the P levels found in the above studies in the rhesus monkey. Our previously published studies (32,42,50-52) describe in detail the protocols for creation of adequate and inadequate secretory cycles. These studies showed that the hormone levels produced by these protocols are coincident with those observed in the natural menstrual cycle (50) as well as inadequate secretory phases (46).

5. DIFFERENTIAL DISPLAY ANALYSIS

Differential display RT-PCR (differential display) is an approach for the identification of mRNAs that show differences in expression level between two or more experimental groups or that are unique to a cell type, tissue or developmental stage (53,54). The method allows random cDNAs to be amplified by a pair of short arbitrary (10-mer) PCR primers (55,56). The sequence of the primers dictates which panel of cDNA fragments of the total will be amplified: only those gene fragments containing sequences complementary to the primers will be amplified. Use of different primer sets will result in different patterns of gene fragments that are amplified, and we have utilized this method to broaden the scope of genes to be analyzed. Because the fragments are small (approximately 400 bp) they can be quickly sequenced and compared by homology to GenBank database entries.

5.1. Proliferative versus Secretory Endometrium

Using this approach we first analyzed cDNA populations prepared from estrogen-dominant endometria (EcDNA) or progesterone-dominant endometria (PcDNA)(57,58). Our first criteria for selection of these fragments required that the fragment be absent or in very low abundance in EcDNA and strongly expressed in PcDNA. Using different sets of arbitrary primers we identified 8 fragments (P1-P8) that clearly met our criteria (56). P1 is a 103 bp fragment and its DNA sequence matched an as yet uncharacterized human PAC clone from chromosome 6. Fragments P2, P3 and P4 yielded no significant sequence homologies to GenBank entries. P5 is 91 bp and showed limited, gapped DNA homology to an uncharacterized human BAC clone from chromosome 7. P6 is 261 bp of which 71 bp exhibited limited homology to an uncharacterized human expression sequence tag (EST) from fetal brain. P7 and P8 showed no homology to current database entries. Since fragments P1-P8 have yielded no significant gene matches to date, we searched for potential encoded protein domains using the Prosite (Swiss Institute for Bioinformatics, Geneva, Switzerland) database. No significant homologies, however, were found.

Our next criteria for selection was to identify and select fragments that were highly expressed in EcDNA and absent or in very low abundance in PcDNA i.e. E-dependent fragments that are inhibited by P. We isolated, cloned and sequenced four of these fragments (E1-E4) (56). Fragments E2 and E9 contained no open reading frames (ORFs) and showed no significant homology to GenBank entries. These fragments represent undefined non-coding regions. E3 was significantly homologous (probability =e-36) to an as yet uncharacterized human STS clone. Fragment E4 was also homologous (e-17) to an uncharacterized human genomic DNA near the Downs Syndrome region on chromosome 21.

5.2. Adequate versus Inadequate Secretory Phase Endometrium

We have previously prepared and characterized a cDNA population that was prepared from endometria harvested from an inadequate secretory phase (IcDNA) (52). Coincident with the up-regulation of the P-dependent fragments noted above there was a corresponding under-expression of these fragments in IcDNA and therefore the full expression of these fragments requires adequate levels of secretory P. In addition, our differential display analysis showed several distinct bands that were overexpressed in IcDNA compared to either PcDNA or EcDNA (I1-I4). Fragment I1 contained an open reading frame (ORF) that matched (100%) human serine/threonine protein phosphatase 2A. Fragment I2 contained an ORF that showed significant homology (83%) to human oxobutanoate dehydrogenase. The DNA sequence of fragment I3 was homologous to an uncharacterized human EST clone, and fragment I4 contained an ORF, which was 88% identical to the primate line-1 reverse transcriptase homolog.

For the three fragments that corresponded to previously characterized mRNAs (I1, I2 and I4), we designed specific primers for semi-quantitative PCR in order to confirm their regulation by P. I1 and I2 were shown to be up-regulated (5 and 3-fold respectively) in IcDNA compared to E- and PcDNA, as expected. However I4 appears to be a false positive from DDRT-PCR since its expression is greatest in PcDNA (i.e. up-regulated by P in the normal secretory phase). This observation shows the necessity for confirming the regulation of differential display fragments and avoiding false positives. The above data suggest another level of P-dependent gene regulation may be operative. That is, an inadequate level of P, similar to the level of P during the early part of an adequate secretory phase, can induce P-dependent gene expression but an adequate level of P appears to exert an autologous down-regulation of these early secretory phase response genes.

6. LASER CAPTURE MICRODISSECTION (LCM) OF THE ENDOMETRIUM

6.1. Description and Methods

Traditionally, cell-type specific regulation of genes and gene products has been accomplished using in situ hybridization and immunohistochemistry respectively. While these techniques remain important tools in our experimental arsenal, a new technology, LCM initiated by the NIH, has substantially expanded our ability to examine cell-type specific responses (59,60). Microdissection using this technique allows the retrieval of specific cells from microscopic regions of tissue sections. Tissues such as the primate endometrium are complex heterogeneous structures (see above) whose components are difficult at best or impossible to study in isolation. For example, separation of endometrial stroma from epithelia has been shown to dramatically alter epithelial response to hormonal signals (30). In addition, in a diseased tissue such as endometrial cancer, cancerous cells could constitute less than 5% of the total tissue and can make analysis of whole tissue uninformative. Microdissection can overcome limitations of traditional means of analysis and allow the application of powerful molecular methods of analysis on specific cell-types within specific morphological units in the tissue of interest (61,62).

Four different cDNA populations were prepared from endometrial tissue harvested by LCM from adequate secretory cycles (days 21-23). These populations were: glandular epithelia and stroma from the functionalis (FG and FS respectively) and glandular epithelia or stroma from the basalis (BG and BS respectively) (63). One of the first objectives of our studies was to assess the quality and potential usefulness of endometrial tissue harvested in this manner for subsequent gene expression studies. There are numerous steps in the preparation of suitable genetic material from laser microdissected tissue any one of which could compromise the quality of a sample (59). Because of the time and effort that is required for an analysis of gene expression using this approach it is useful to have some guide to the relative quality of a sample. Tissue limitations for our laboratory and others may not allow traditional means of analysis e.g. RNA integrity or relative size of the mRNA population by agarose gel electrophoresis.

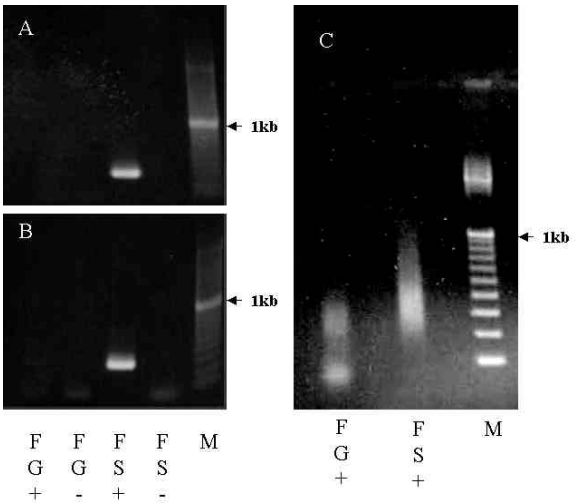

Figure 1. Detection of 18S ribosomal RNA expression before and after amplification of cDNA. (A) results after 30 cycles of PCR, (B) results after 40 cycles of PCR. (C) corresponding cDNA smears after amplification of adaptor-ligated cDNA. "+" signs indicate cDNA that has been amplified after adaptor ligation while "-" signs indicate double stranded cDNA before amplification. FG = cDNA from functionalis glandular epithelia; FS = cDNA from functionalis stromal cells. A 100 bp ladder is shown with the most intense band corresponding to 1Kb indicated by an arrow.

In an effort to overcome this drawback we have used an adapter-specific primer amplification approach to allow us to visualize a cDNA smear (57). This approach coupled with the detection of an appropriate housekeeping gene(s) can serve as a useful guide to estimate sample quality for those investigators faced with limited tissue/cells. This approach also provides considerable material (cDNA) from a single round of amplification (approximately 75-fold) that will subsequently allow a number of comparative studies on gene expression to be performed.

With the use of primers for a housekeeping gene it would be expected that its presence at the correct fragment size would provide evidence for the suitability of the genetic material for further analysis. While this may in part be true, the absence of an appropriate PCR product could mean either that the preparation is poor or that there is insufficient material for detection (false negative). Our data for 18S ribosomal RNA expression prior to and after amplification show that a false negative can be detected. For example, a detectable band of the correct size is only apparent following amplification for either 30 or 40 cycles (FS+ vs FS-) (Figure 1A,B).

The use of this amplification strategy cannot only allow detection of a false negative result but also can provide some additional information with regard to the quality of the genetic material through cDNA smears. For example the cDNA populations prior to or after amplification for glands from the functionalis did not show an 18S ribosomal RNA band (324bp) despite amplification (FG+, Figure 1B). These data would suggest that the quality of this material rather than the quantity is most likely at fault. The very low molecular weight smear of this cDNA population correlates with this result (Figure 1C). cDNA smears following amplification of these laser microdissected samples can also be useful in the design of an appropriate expected fragment size for a given housekeeping gene or other gene of interest.

6.2. LCM and Differential Display

In order to couple LCM with differential display we prepared cell-type specific cDNA populations (glandular epithelia or stroma) from both the functionalis and basalis of adequate mid-secretory endometria as described above. Following differential display analysis, we selected six fragments that showed a putative cell-type specific and/or a region-specific expression (63).

Although DDRT-PCR is a potentially powerful and important approach, a drawback as noted above can be the appearance of false positives (64). After cloning and sequencing, specific primers for each of the fragments were designed and used to verify their expression patterns. Although three fragments were shown to be false positives with regard to their regional or cell-type specificity, they remain potentially important because of their expression during an adequate secretory phase.

Two of these false positives showed strong homologies with a known human gene. The putative S1 (stromal specific) fragment was shown to have a 95% homology with human protein phosphatase 3 (formerly 2B). In eukaryotes four major types of protein serine/threonine phosphatases (PP1, PP2A, PP2B and PP2C) have been identified and they have shown to play a number of important roles in cellular processes including cell cycle regulation, growth factor signal transduction pathways, glycolysis and other metabolic processes (65,66). As noted above, we have previously shown that another protein phosphatase with very broad substrate specificity, PP2A, is highly up-regulated in the rhesus endometrium during the mid-secretory phase (56). The substrate specificity of protein phosphatase 3 (S1) is, however, much more restricted than PP2A, and comprises proteins that regulate other protein kinases and phosphatases (65). The putative BS-1 fragment showed a 93% identity with the human HNMT gene (67). The N-methylation by this enzyme results in the inactivation of histamine (68). Histamine has been proposed to play an important role in implantation in rodents (29) and the expression of this gene during the primate secretory phase could suggest one mechanism whereby the activity of histamine is modulated. The third false positive, FG-1, showed no significant homology with current GenBank entries.

The three fragments identified in laser microdissected samples showing the expected regulatory pattern were F1 (highly expressed in the glands and stroma of the functionalis), BG-1 (highly expressed in the glands of the basalis), and FS-1 (highly expressed in the stroma of the functionalis). Although BG-1 and FS-1 are currently

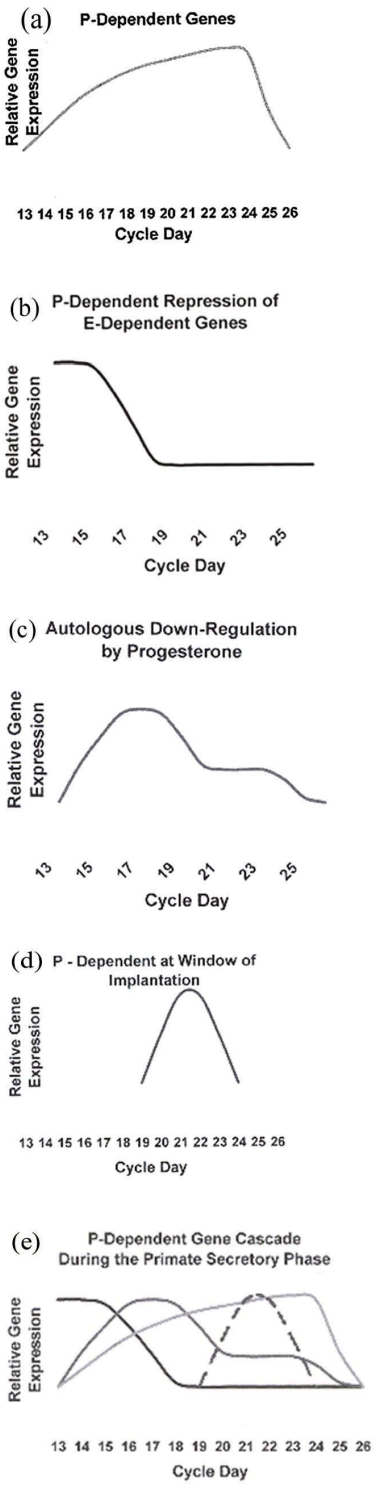

Figure 2. Schematic of relative gene expression patterns during the primate secretory phase. (a) P induction of genes during the course of the secretory phase; (b) P repression of E-dependent genes; (c) autologous down-regulation of P dependent genes; (d) P induction of gene expression during the putative window of implantation; (e) a composite cascade of gene expression patterns a-d.

uncharacterized gene fragments, F1 showed a 94% homology to a known gene, namely, the human leukotriene B4 receptor (69,70).

Leukotriene B_4 (LTB_4) is one of the most potent chemoattractant mediators, acting mainly on neutrophils but also on related granulocytes, macrophages and endothelial cells. LTB_4 activates inflammatory cells by binding to its cell surface receptors BLTR1 and BLTR2 and has been implicated in a number of inflammatory diseases (69). Interestingly, levels of leukotrienes were elevated in the endometrium of women with primary dysmenorrhrea and endometriosis (71). Levels of LTB_4 have also been shown to increase in the rat uterus during the peri-implantation phase implicating a role for this cytokine in the uterus receptivity for implantation (72). Importantly, the expression of this gene was localized to the endometrial functionalis, the target for blastocyst invasion/implantation. To our knowledge this is the first time expression of a BLTR2 receptor ortholog (F1) has been shown in the endometrium. Further studies will be required to identify the role of this receptor in proper maturation of the endometrium

7. SUMMARY AND OVERVIEW

The coordinated, steroid-induced activation and repression of many genes during the transition from E to P-dominance is expected to be important for proper endometrial maturation. Based on our work and results from other laboratories there are at least 5 different levels of P-dependent gene expression. During a normal secretory phase, the early phase of P regulation likely involves both induction of P-dependent genes (Figure 2a) and inhibition of E-dependent genes (Figure 2b) that are sensitive to the initial rise in serum P level. The rising titers of serum P lead subsequently to further inhibition of E-dependent genes and continued induction/maintenance of P-dependent genes. Superimposed on these two regulatory actions of P is an autologous down-regulation of some but not all P-dependent genes (Figure 2c) (56). In addition, several laboratories have shown that some genes are only activated just prior to and during potential implantation in the human and other animal models (17,18,73-77). Recent results from our laboratory have also demonstrated a similar temporal regulatory pattern for several genes in the rhesus endometrium (78). This scenario of a sharply restricted window of P-dependent gene regulation (activation) is also included in our working model (Figure 2d). Subsequently, P-dependent gene regulation necessary for proper maturation declines coincident with falling titers of P in the late secretory phase resulting in a loss in receptivity of the endometrium. Taken together, these patterns of induction, repression, or loss of gene expression describe some of the temporal changes in P action that occur during the primate secretory phase.

The above putative levels of P-dependent regulation must also be considered in the context of regional and cell-specific expression within the endometrium. As described above, the use of laser capture microdissection affords an important and powerful tool to

characterize and analyze these aspects of endometrial function. Indeed, our data using LCM further suggest that regional and cell-type differences in gene expression are a property of the primate endometrium and are likely to play important roles in the coordination of tissue gene expression patterns during an adequate secretory phase.

Our working model, described above, summarizes our hypothesis that waves of P-dependent gene regulation, a cascade, are central to proper maturation of the primate endometrium (Figure 2e). All levels of regulation are considered important because of their potential to be linked. That is, preceding gene expression patterns may control and direct subsequent expression that allows full endometrial maturation. It is anticipated that refinements or additions to this working model will result from future studies in our laboratory and others.

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