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Uterine Receptivity to Human Embryonic Implantation: Histology, Biomarkers, and Transcriptomics

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Abstract

Embryonic implantation is a dynamic process of paracrine interactions between the maternal compartment and the conceptus and involves a receptive endometrium and a developmentally competent blastocyst. Herein, we review histology, clinical approaches, and the promise of transcriptomics in elucidating mechanisms underlying implantation and development of biomarkers of uterine receptivity - with an eye to diagnose and treat implantation-based disorders of miscarriage, fetal growth restriction, pre-eclampsia, and infertility.

Keywords

human implantation; endometrium; microarray; blastocyst

1. Challenges and approaches in studying the process of human implantation

Clinical implications

Successful embryo implantation is a crucial event in natural and assisted human reproduction. Blastocyst implantation is a dynamic process, involving embryo apposition, attachment to the maternal endometrial epithelium, and invasion into the endometrial stroma [1]. With *in vitro* fertilization (IVF), implantation failure can occur due to several factors [2], including embryonic defects such as chromosomal abnormalities, which are the most common cause of implantation failure [3,4]. Another widely acknowledged barrier to successful blastocyst implantation and, therefore, successful treatment of infertile women with implantation failure, is an inappropriately developed endometrium. It is well established that embryos cannot implant in a poorly matured endometrium [5], and this may be responsible for low implantation rates with transfer of “good quality” embryos. The success of embryonic implantation further relies upon cross-talk between the embryo and a receptive endometrium [5]. Optimization of IVF results are important for many reasons, and achieving optimal implantation is even more important in the setting of single embryo transfer, a common practice aimed to avoid multiple pregnancies.

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The implantation window and challenges of studying it

The endometrium is receptive to blastocyst implantation during a spatially and temporally restricted “window”, when the luminal epithelium is favorable for blastocyst implantation even in the absence of an implanting blastocyst [6,7]. This period, at the mid-secretory phase, is limited to approximately 48 hours and is characterized by up-regulation of several endometrial growth factors, cytokines and adhesion molecules [8-10]. For ethical and practical reasons, it is not possible or extremely difficult to study the human implantation process *in vivo*. The implantation process varies among species, and results from animal studies cannot always be extrapolated to humans. Therefore, investigation about the human implantation process is limited to evaluation of human endometrium in non-pregnant cycles or responses of endometrial cellular components to placental and/or embryonic secreted products.

1.1 Approaches to Studying Human Implantation

Endometrial Tissue

A common way to study human implantation is to study human endometrium *in vitro*, usually obtained by biopsies or from uteri after hysterectomies. A disadvantage of a biopsy in the implantation window is a possible disturbance of an ongoing implantation. There are limited, and hence controversial, reports about the effect of the procedure on pregnancy rates. Some authors [11] claim that the endometrial biopsy at the time of embryo transfer does not exclude viable pregnancy. Others suggest that analysis of protein patterns in endometrial secretions may offer a more safe and non-invasive method of assessing endometrial receptivity during treatment cycles [12].

Blood and Uterine Fluid Biomarkers

The least invasive method of endometrial fertility assessment would be blood sampling and analysis of a biomarker or a panel of biomarkers predictive of optimal endometrial receptivity. Leukaemia inhibitor factor (LIF) is an example of such a biomarker of the “window of implantation” [13,14]. While LIF measurements in serum do not reflect fertility status [15], its low concentrations in uterine flushings are predictive of unsuccessful implantation [15]. Similar data were reported for glycodelin, the main secretory product of luteal phase endometrial epithelium and one of the markers of a receptive endometrium. Its concentration in serum and uterine fluid increase in ovulatory cycles [16,17] and decrease in uterine fluid of infertile women, with no difference in serum levels in those patients [18,19]. Indeed, most proposed endometrial receptivity markers are not specific to the uterus, and thus it would be difficult to determine whether their serum concentrations play a role in predicting successful implantation. An alternative to peripheral blood analysis could be investigation of uterine flushings; however, this technique is not standardized, and the concentrations of components therein may vary, depending on variations in the procedure [10].

Cervical Mucus

Examination of cervical mucus affords another non-invasive method to study the cytokines and growth factors produced by a receptive endometrium (and transport to the cervical mucus). To the best of our knowledge, there are only two studies that aimed to correlate endometrial expression of cytokines implicated in endometrial receptivity with their levels in cervico-vaginal secretions. The earlier study focused on LIF and demonstrated its presence in cervical mucus [20]. In that analysis, levels of LIF in cervical mucus were highly correlated with its production by endometrial tissue in the periovulatory period. However, in another study LIF was not detectable in cervical secretions throughout the menstrual cycle except for menses [21]. Macrophage-colony stimulating factor (M-CSF), epidermal growth factor (EGF), interleukin-1 beta (IL-1beta), transforming growth factor beta -1 (TGF-beta1) and TGF-beta

2 were detected in cervico-vaginal secretions [21]. However, no correlations between cytokine levels in cervico-vaginal secretions and serum, and between the level of cytokine gene expression in secretory endometrium and the concentration of cytokines in serum collected on the day of endometrial biopsy were found. Only EGF exhibited a significant positive correlation between the level of gene expression in secretory endometrium and its concentration in cervico-vaginal secretions collected on the day of endometrial biopsy, suggesting that EGF may have a predictive value as a minimally invasive marker of uterine receptivity [21].

Ultrasound

Another approach has been to use ultrasound evaluation as a predictive method for a receptive endometrium. Jarvela et al [22] reported that in women undergoing IVF, when using three-dimensional Doppler ultrasound, a triple-line pattern after FSH stimulation and a decrease in endometrial volume was associated with conception. However, Sterzik et al. [23] concluded that ultrasonography is an inadequate method to predict endometrial receptivity in IVF cycles, since neither the endometrial thickness nor the echo pattern correlate with the histologic findings. Earlier, Hambartsoumian [24] evaluated a possible relationship between endometrial LIF secretion (endometrial biopsy at cycle day 10) and endometrial growth during IVF cycles. It was demonstrated that LIF production was negatively correlated with endometrial thickness and echo pattern, specifically, and suggested that over-expression of LIF might lead to an inhibitory effect on the endometrium. Taken together, prospective studies on biomarkers as predictors of conception are needed to develop non-invasive methods in assessment of endometrial receptivity.

2. Endometrial dating and the window of implantation

A normal, ovulatory menstrual cycle (natural, spontaneous cycle) has approximately the same length in each cycle, but can vary in length between women. A standardized (ideal) menstrual cycle that lasts 28 days can be morphologically “dated” according to criteria of Noyes et al. [25]. The histological maturation of the endometrium develops in a distinct pattern that follows the progression of the menstrual cycle. Recently, on a large number of women, it was demonstrated that histological dating of the endometrium does not have the accuracy or the precision necessary for the diagnosis of luteal phase deficiency, or to guide clinical management of women with reproductive failure [26,27]. It was proposed that the dating of endometrium should be related to the serum LH surge, which corresponds to ovulation, rather than to the “ideal” 28-day cycle [28]. Endometrial histology is most consistent in biopsies from days LH -3/-2 to days LH +7/+8, when changes occur with a high degree of regularity regardless of the length of the preovulatory and postovulatory phases [28]. It is now a common practice to date endometrium according to days after the LH peak [29] [30]. Ultrastructural changes in human endometrium throughout the cycle also allow dating of the endometrium [31,32] and assist in determining the “implantation window”. The implantation window is a short interval during the mid-secretory phase, when the endometrium is most receptive to blastocyst implantation. It begins on days 20–24 of an ideal menstrual cycle or 6–10 days after the LH surge and is believed to last less than 48 hours [33–35]. Endometrium appears to be the only tissue in which period of embryo implantation is time restricted, i.e. embryo can implant only during a specific time period – “implantation window”, whereas extrauterine pregnancy with embryo implanting in various tissues in peritoneal cavity can occur at any time [36,37].

3. Molecular and structural markers of endometrial receptivity

A principal event of mammalian implantation is the development of a receptive endometrium and its subsequent differentiation into decidua. Many molecular markers such as integrins, IL-1, calcitonin, amphiregulin, EGF, HB (heparin binding)-EGF, colony stimulating factor-1,

LIF, mucins, leptin, selectin-L ligands, Hoxa genes, and COX (cyclooxygenase) have been proposed to identify this period of receptivity [9,38–42]. The beginning of the implantation window is also characterized by remarkable ultrastructural changes in endometrial epithelial cell morphology [31,32]. However, the implantation process is a complex and multifactorial event, with association and interplay of the different factors involved. Therefore, it is important to know how different markers of implantation correlate with each other. Several studies have demonstrated correlation between pinopode appearance and expression of integrin $\alpha_v\beta_3$ [43], osteopontin [44], LIF and LIF receptor [45], HB-EGF [46], glycodelin [47], progesterone receptors [48], antioxidant enzyme glutaredoxin [49] and cyclooxygenases 1 and 2 [50] in the same endometrial tissue samples. These observations are consistent with an important role of the cytokines, growth factors, and other molecules in the implantation process and reveal interesting spatio-temporal aspects of the expression of some of these.

4. Transcriptomic approaches to markers of uterine receptivity

With the development of microarray technology, global approaches have been pursued to identify novel pathways involved in implantation events [51]. Five studies were published, within a short time frame, reporting the transcriptome human endometrium during the window of implantation [52–56] (Table 1). Remarkably, all studies used the same microarray platform to perform their analysis. This makes a comparison between the studies possible; however, there are differences in the type of data analysis, as well as number of patient samples used, ages of subjects, cycle phases compared, and pooling or not pooling samples (Table I). Two studies [53,55] pooled RNA from different patients to run on microarrays. Four out of five studies [52,53,55,56] used pre-defined fold change cut off of 2.0 as evidence of change in gene regulation, while Riesewijk et al. [54] used a more stringent approach and a 3.0 fold change as a cutoff. The latter study is the only one that analyzed samples from the same woman in the early and the mid-secretory phase of the menstrual cycle. These differences in study design may be responsible for the differences in the list of regulated genes identified. The main difference between the studies comes from the analysis of functionally and structurally different stages of the menstrual cycle (proliferative and secretory), which is reflected by the large variability of the up- and down-regulated genes. Interestingly, however, as noted above, Riesewijk et al. [54] performed microarray analysis of endometrium from the same women at LH+2 and at LH+7, which minimizes inter-patient variability. However, caveats include reactive inflammatory changes and subsequent gene alterations as a result of the first biopsy [56], and different microenvironments and/or complement of cell types may be represented in the two biopsies from the same subject.

Figure 1 represents the distribution of significantly changed genes among different groups of genes in the studies by Kao et al. [52], Carson et al [53], Borthwick et al [55], Riesewijk et al. [54] and Mirkin et al. [56]. Genes with unknown biological function represented one of the biggest groups of regulated genes. Cell surface proteins, extracellular marker components, and growth factors/cytokines represent a large fraction of the up-regulated genes in mid-secretory phase in these studies, as well as genes encoding intracellular signaling and cell cycle proteins. Immune genes were also represented in four out of five studies, implicating an important role for the immune cells and processes in endometrium prior to and during embryonic implantation. Genes encoding DNA binding proteins, transcription factors and DNA modifying enzymes were highly represented among down-regulated genes, as well as genes with unknown function and ESTs (Figure 1). These approaches have enabled identification of some genes not previously known to be involved in the implantation process [56], and further studies are required to elucidate their potential roles.

We have analyzed the studies above in an effort to identify common genes present in all studies and which change in similar way during the receptive period in mid-secretory endometrium.

Similar analyses were performed earlier by Horcujadas et al. [57] and Mirkin et al. [56]. There are very few genes that were significantly and similarly regulated during the window of implantation in at least four out of the five reports. These surprising results could be explained by some differences in study design among all studies (in particular, comparison of mid-secretory phase endometrium to either proliferative or early secretory endometrium). Up-regulated transcripts in common between studies are: osteopontin (SPPI) – the only gene up-regulated according to all studies, decay accelerating factor for complement (CD55, Cromer blood group system), growth arrest and DNA damage-inducible protein (GADD45), apolipoprotein D, Dickkopf/DKK1, monoamine oxidase A (MAOA), interleukin 15 (IL15) and mitogen-activated protein kinase kinase kinase 5 (MAP3K5); the down-regulated gene in common in those studies was olfactomedin-related ER localized protein. A brief overview of those factors is presented.

The significant up-regulation of osteopontin in all five studies is remarkable, since it is acknowledged to be involved in the implantation process. This glycoprotein is a ligand for $\alpha v \beta 3$ integrin [58,59], it mediates cellular adhesion and migration during embryo implantation, is regulated by progesterone, and its maximal expression in endometrial epithelial cells has been observed in the window of implantation [60–62]. Moreover, the localization of osteopontin on the surface of endometrial pinopodes has been demonstrated [44].

Decay accelerating factor for complement (DAF) is a complement protective protein, highly expressed in the human endometrial epithelium in mid- to late secretory phase and regulated by HB-EGF and EGF [63–65]. It was highly up-regulated in our recent microarray study in normal mid-secretory endometrium with subsequent decrease in the late secretory phase [62] and was decreased in endometrium from women with luteal phase defect and patients with recurrent pregnancy loss associated with antiphospholipid syndrome [66,67]. It functions probably by protecting the endometrial integrity from complement components increased in secretory endometrium [64].

GADD45 is member of a group of stress inducible genes. Its up-regulation in mid-secretory endometrium reflects the escalation of endometrial protective mechanisms in anticipation of implantation and invasion of a blastocyst. High expression of GADD45 in high receptivity cell lines compared to the low receptivity lines was reported [29].

Apolipoprotein D is a multi-ligand, multi-functional transporter involved in lipid metabolism and cholesterol transport; it can bind cholesterol, progesterone, pregnenolone, bilirubin and arachidonic acid, but it is unclear which of these represent its physiological ligands [68]. Up-regulation of this gene in four out of five studies underscores the regulation of lipid metabolism during endometrial maturation.

DKK-1 is a potent inhibitor of the Wnt signaling pathway [69]. It was recently found to be specifically up-regulated by progesterone in *in vitro* decidualized endometrial stromal cells [70], was up-regulated in microarray study of mid-secretory endometrium [62] and down-regulated in eutopic endometrium from women with endometriosis [52].

Monoamine oxidase A (MAOA) is localized in the outer membrane of mitochondria and catalyses the oxidative deamination of a variety of monoamines using flavin adenine dinucleotide as cofactor [71]. Henriques et al [72] reported up-regulation of MAOA gene and protein in mid-secretory compared to early secretory endometrium, and decreased MAOA expression in donor oocyte recipients with implantation failure. Interleukin 15 is progesterone regulated gene in endometrial stromal cells and is important as chemoattractant and stimulator of natural killer (NK) cell replication [73,74]. Interestingly, in women with unexplained recurrent abortion, there are elevated levels of endometrial IL-15 compared with control

endometrium [75,76]. This chemokine was shown to play a central role in postovulatory recruitment of CD16(–) NK cells from peripheral blood into human endometrium [77].

MAP3K5 is a member of mitogen-activated protein kinase (MAPK) signaling cascades. Expression and activation of this pathway in human endometrium is increased in secretory phase, in particular in mid-secretory phase [78] and alteration in its phosphorylation may be involved in pathogenesis of endometriosis [79].

Olfactomedin-related ER localized protein, or olfactomedin 1, is a down-regulated gene in common in the five microarray studies. The exact function of the protein is not known. Its expression was increased in endometrium from women with unexplained recurrent spontaneous abortion, and it induced cell arrest in a human endometrial cell line [80].

Unexpectedly, several biomolecules with an established role in human implantation process by one-to-one approach (LIF, HOXA10, L-selectin, HB-EGF etc) did not pass the defined fold-change threshold in the microarray studies described above. The existing discrepancies among the array studies confound identification of definitive markers of uterine receptivity. Analysis of endometrial tissue obtained by standard operating procedures shared by all investigators and from large numbers of well-characterized subjects, with the same study design is anticipated to be extremely informative.

5. Model of early embryonic implantation

There are multiple interactions that must occur for successful embryonic implantation into a receptive endometrium, and the transcriptome of the endometrium in the implantation window provides the beginning to understanding this complex process (Figure 2). Notably, the endometrial transcriptome in the mid-secretory phase studied to date is a static snapshot of events occurring in this tissue as it awaits embryonic implantation. Of equal importance are the close spatio-temporal relationship between the conceptus and the maternal compartment and how their physical interactions affect/change their respective transcriptomes. A few studies have used *in vitro* models of human implantation that focused mainly on ultrastructural relationships between the attached blastocyst and the endometrial epithelium, e.g. in a three dimensional cell culture system [81–84]. Carver et al. [85] demonstrated hCG-secreting activity of a blastocyst implanted into endometrial stromal cells monolayer. Mercader et al [86] reported results of a 5 year-long study, where blastocysts and endometrial epithelial cells were co-cultured in IVF and oocyte donation programs. Such an approach dramatically increased implantation and pregnancy rates in IVF patients. Further research by the same group, studying the secretome of human blastocysts cultured in sequential (routinely used) media versus co-culture with endometrial epithelial cells, identified, in a preliminary report, IL-6 as a predictor of implanting versus non-implanting blastocysts [87]. Microarray studies of the whole genome of endometrial cells during/after physical contact with the human blastocyst *in vitro* would be valuable and informative about the molecular pathways driving the initiation of pregnancy, although this is a challenge experimentally. Finally, *in vitro* models of the invasive phase of implantation, such as co-cultures of endometrial and trophoblast cells and treatment of endometrial cells with trophoblast conditioned medium, are informative about the invasive phase of implantation (and are reviewed elsewhere [88]).

7. Summary and conclusions

A transcriptomic approach has been very helpful to understand biological processes, biochemical pathways, and signaling pathways that are operating within the implantation window in human endometrium as it awaits embryonic implantation, as well as those in the blastocyst as it is ready to initiate nidation. The biggest challenge is to elucidate the processes during the cross-talk between the implanting embryo and the maternal endometrium during

the early phases of implantation, during placentation, and in the microenvironments of the endometrium that are participating in these events or affected by endocrine interactions during pregnancy establishment. When these are identified and understood, targeted therapies may be introduced to enhance the implantation process and potentially minimize implantation disorders including miscarriage, pre-eclampsia, and intrauterine fetal growth restriction.

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Figure 1.

Distribution of changes in gene expression among different functional classes. A – results from Kao et al., 2002; B – Riesewijk et al., 2003; C - Carson et al., 2002; D –Borthwick et al., 2003; E – Mirkin et al., 2005.

Figure 2. Model of events in early human implantation

LIF- leukemia inhibitory factor; N-Ac6-ST - N-acetylglucosamine-6-O-sulfotransferase; CPE receptor - Clostridium perfringens enterotoxin receptor; IDO - Indoleamine 2,3-dioxygenase; IL-15 – interleukin 15.

Table 1
Comparison of five studies on human implantation window using the microarray technology approach.

Reference	N of samples biopsy1/ biopsy2	Age (median)	Endometrial biopsy 1	Endometrial biopsy 2	Array	N of genes on the array	Defined fold change	N of genes up-regulated	N of genes down-regulated
Kao et al., 2002	4/7	28–39	Cycle day 8–10	LH + 8 - + 10	Affymetrix HG-U95A	12 686	2	156	377
Carson et al., 2002	3/3	not mentioned	LH+ 2 - + 4	LH+ 7 - + 9	Affymetrix HG- U95A	~ 12 000	2	323	370
Rieswijk et al., 2003	5/5	23–39	LH+ 2	LH+ 7	Affymetrix HG- U95A	~ 12 000	3	153	58
Borthwick et al., 2003	5/5	23–44 (35.7)	Cycle day 9–11	LH+ 6 - +8	Affymetrix HG- U95A-E	60 000	2	90	46
Mirkin et al., 2005	3/5	24–32	LH+ 3	LH+ 8	Affymetrix HG-U95A	12 686	2	49	58