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Gestational Age-Dependent Expression of IL-10 and Its Receptor in Human Placental Tissues and Isolated Cytotrophoblasts

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Control of antifetal immune responses is thought to be regulated locally by the placenta. Because the physiologic programming of the placenta across gestation is likely to influence the local immunity, we hypothesize that a potent anti-inflammatory cytokine such as IL-10 may be produced in a gestational age-dependent manner. In the present study, we examined the expression of IL-10 and its receptor in placental explants or freshly isolated cytotrophoblasts from different gestational ages and compared it with the expression profiles of other cytokines. First and second trimester placental tissues from normal pregnancies predominantly expressed IL-10, whereas the levels of IL-2, IL-4, and IFN-γ were mostly below detection throughout pregnancy. The expression of IL-10, but not its receptor, diminished significantly in term placental tissues collected “before” the onset of labor and did not change appreciably “after” labor. On the other hand, TNF-α and IL-1β were significantly up-regulated in response to labor-associated conditions. IL-10 expression was transcriptionally attenuated at term as observed in cytotrophoblasts. In contrast to the placental cytokine milieu, autologous PBMCs, when activated with PHA, secreted significant amounts of IL-2, IL-4, IL-10, and IFN-γ, albeit with a statistically significantly enhanced IL-10 production in first trimester compared with age-matched nonpregnant women. These data suggest that IL-10 is expressed in the placenta in a gestational age-dependent manner and that its down-regulation at term may be an important mechanism underlying the subtle changes associated with parturition. The Journal of Immunology, 2000, 164: 5721–5728.

Placental regulation is required to program an intrauterine milieu that promotes fetal growth and development (1–5). Indeed, recent observations support previous predictions that suppression of maternal immunity provides an immune-privileged microenvironment for the fetus (6–11). Cytokines, nonclassical MHC molecule HLA-G, Fas, TNF-related ligands, and possibly catabolizing enzymes for T cell growth-promoting nutrients expressed at the decidua-placental interface have been implicated in modulating maternal immune competence (12–23). Importantly, a predominance of cytokines such as IL-4, IL-10, or IL-13, collectively called Th2 cytokines with anti-inflammatory characteristics, has been suggested to be compatible with a successful pregnancy (24, 25).

Several reports have now confirmed, at least in murine pregnancy, that unbalanced presence of Th1 cytokines IL-2, IFN-γ, and TNF-α during pregnancy results in fetal ablation. Pathogenic infections such as Leishmania major or abortion-prone mating (CBA × DBA/2) in mice associated with the predominant production of Th1 cytokines, including IFN-γ, IL-2, and TNF-α, culminate in fetal loss, which can be reversed by administration of the Th2 cytokine IL-10 during pregnancy (26–29). These studies suggest that systemic or placental presence of Th2 cytokines, particularly IL-10, would be supportive of normal pregnancy. In humans, placental and decidual tissues from normal pregnancies have been shown to express an array of pro- and anti-inflammatory cytokines (30–40). This implies that a potent anti-inflammatory cytokine(s) is produced locally to control fetal-ablating immune responses.

The debate on the role of Th2 cytokines in human pregnancy is likely to focus on IL-10. Although human IL-10 does not fit the classical Th2 cytokine profile because it can be produced by both Th1 and Th2 cells as well as non-T cells, this 18-kDa polypeptide exhibits predominantly inhibitory effects on inflammatory reactions (41). One major role of IL-10 is the down-regulation of chemokine and cytokine production by Th1 cells and macrophages (42, 43). IL-10 also interferes with Ag presentation and directly or indirectly inhibits CD8+ T cell or NK cell responses (44–48). Furthermore, IL-10 may also act as the mediator of several other intrauterine regulators. For example, progesterone, catecholamines, and prostaglandins have been shown to induce production of IL-10 (38, 49, 50). Recently IL-10 has also been shown to be an autocrine inhibitor of matrix metalloproteinase-9 production in human cytotrophoblasts (51). Because matrix metalloproteinase-9 is also induced in villous trophoblasts in response to labor (52), it is tempting to hypothesize that IL-10 expression may be subjected to physiological attenuation in these cells as a result of parturition-associated changes.

In the present study, we have studied IL-10 expression in human placental tissues and isolated cytotrophoblasts from different gestational ages, as well as in autologous PBMCs. IL-10 expression was compared with the expression of its receptor, IL-4 and its receptor, IL-2, and IFN-γ. Placental production of this cytokine was also evaluated “before” and “after” the onset of labor and...
compared with that of TNF-α and IL-1β. Taken together, our results support the hypothesis that IL-10 production by the human placenta is attenuated at term and that a balance between IL-10 and the onset of inflammatory responses may regulate the events conducive to parturition.

Materials and Methods

Tissue collection and processing

This study was approved by Women and Infants’ Hospital’s Institutional Review Board, and placental tissue samples were collected after informed consent had been obtained. Placental samples were obtained from pregnant women who met the following inclusion criteria: 1) age between 18 and 40 years, 2) singleton pregnancy, 3) normal pregnancy at the time of sample collection, 4) healthy women with no preexisting clinical conditions such as diabetes, hypertension, or autoimmune disease, 5) no previous history of spontaneous abortion, ectopic pregnancy or still birth, and 6) reliable gestational age by early ultrasound.

First trimester (7- to 11-wk gestation; n = 7) and second trimester placental samples (13- to 17-wk gestation; n = 7) were collected after elective pregnancy termination. End of pregnancy (term) placental samples before the onset of labor (39- to 41-wk gestation; n = 7) were collected at the time of elective cesarean section with no rupture of fetal membranes. Term placental samples after the onset of labor (39- to 41-wk gestation; n = 7) were collected from normal spontaneous vaginal deliveries associated with rupture of fetal membranes occurring less than 8 h before the time of delivery. Placental samples were used only if there was no clinical evidence of infection.

A small portion of the placenta was used for assessment of morphological anomalies and infection. Any sample showing histological evidence of infection was discarded. Before use, placental tissues were separated from fetal membranes and decidua. Fresh tissues were processed immediately.

Antibodies

Cytokine-specific ELISA kits were purchased from Endogen (Cambridge, MA). mAbs used in immunohistochemical detection of IL-4 and its receptor α-chain, IL-10 and its receptor, IFN-γ, IL-2, and isotype-matched control IgG were purchased from R&D Systems (Minneapolis, MN).

ELISA

After collection, placental tissue samples were washed several times with RPMI 1640 to remove blood. A fixed weight (5 g) of each sample was sheared into pieces using scissors and scalpel, washed again, and incubated (0.2 g of wet tissue/ml) in RPMI 1640 supplemented with 2.5% FBS and antibiotics at 37°C in 5% CO2. Culture supernatant was collected at incubation time periods indicated, centrifuged, and stored at −80°C until the time of cytokine assay. Cytokine concentrations were measured in duplicate using commercial ELISA kits according to the manufacturers’ instructions and were subjected to calculations according to standard protein values expressed in pg/ml. The final results were statistically analyzed using nonparametric analysis as described below. The intra- and interassay coefficients of variation were <10%.

Cytotrophoblast isolation

Cytotrophoblasts were isolated according to a modified method of Kliman et al. (53). The conditions were standardized for term and second trimester placental tissues. Placental tissues were digested with decreasing concentrations of trypsin-DNase 1 (trypsin, 1 mg/ml; and DNase, 1.5 mg/ml) at least four times at 37°C for 20 min each. The cells from the first digestion were excluded. The cell mass collected in the following steps was treated with a lysis buffer (0.15 M NH4 Cl, 1 mM KHCO3, and 0.1 mM EDTA (pH 7.3)) for 5 min at room temperature with constant shaking to lyse the RBCs, which if not removed disturb separation on Percoll gradients. Cytotrophoblasts isolated in this manner were stained for cytokeratins or CD45 (a marker for immunocytes) to ascertain their purity (>95%). Placental tissues from second trimester were processed in a similar manner, except that trypsin-DNase 1 digestion was limited to the first two rounds and cells from the first round digestion were not excluded.

RT-PCR

Total RNA was isolated by the single-step guanidium isothiocyanate-CsCl gradient method. Total RNA (5 μg) was used in the RT-PCR reaction to assess expression of IL-10 in cytrophoblasts from different sets of tissues. Freshly prepared cytrophoblasts were treated according to specific requirements for each experiment, and RNA was isolated. For RT-PCR, the IL-10 primer set, which produces 360 bp product, was as follows: sense, 5′-CACCCAGTCTGAACAGTGCC-3′; and antisense, 5′-ACCTGTCTC CACGGCTCTGTGCT-3′. For an internal control, we used a GAPDH primer set: sense, 5′-ATTCTTACCACGGCAAAATCTCAATGGG-3′; and antisense, 5′-AGGGCGGAGATGATGAC-3′, which amplifies a 220-bp fragment. First, 5 μl of total RNA was subjected to cDNA synthesis using murine leukemia virus reverse transcriptase. To rule out chromosomal DNA contamination of total RNA, cDNA synthesis was also conducted in the absence of reverse transcriptase. cDNA synthesis and amplification were conducted in GenAmp 9600 PCR system (Perkin-Elmer, Norwalk, CT). The conditions for PCR were based on the germinal center content of the primers. In the case of IL-10, we have used the following conditions: 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, for a total of 30 cycles. For the semiquantification of amplified products, the GAPDH product was simultaneously amplified. PCR products were analyzed by electrophoresis through 2% agarose gels and stained with ethidium bromide.

Statistical analysis

Statistical significance of experimental differences was assessed using nonparametric (Mann-Whitney U test) analysis. The differences were considered to be statistically significant when the p value was <0.05.

Results

Production of IL-10 by human placental explants from different gestational ages

Because a determination has to be made for the ability of placental explants from different gestational ages to secrete cytokines in vitro, we first sought to assess the time course of maximum cytokine production using IL-4 and IL-10 as a model. Two different placental samples from each gestational age were independently minced into small pieces (explants) and cultured, and supernatants were analyzed for IL-4 or IL-10 production using ELISA as described in Materials and Methods. IL-10 production was detected...
as early as 20 min of culture, suggesting an in vivo activation of IL-10 expression. In contrast, we failed to detect IL-4 at any time point. Maximum IL-10 production was observed at around 18 h in all gestational age placental tissues. Therefore, the 18-h culture period was used for analysis of cytokine production by placental tissues in all subsequent experiments.

Next, the production of IL-2, IL-4, IL-10, and IFN-γ (Fig. 1) was analyzed in seven placental tissues from first trimester, second trimester, or term pregnancies. IL-4 and IL-2 (data not shown) were below the detection level in these assays. On the other hand, although there was significant interindividual variability among subjects, IL-10 was produced at significant levels in first and second trimester placental explants (mean values, 400 pg/ml and 576 pg/ml, respectively), but its production significantly declined at term (mean value, 42 pg/ml). IFN-γ was produced only at the end of pregnancy but at very low levels. Significantly, all placental explants secreted IL-10 without in vitro treatment with activators. Thus, these data show that the human placenta preferentially produces the anti-inflammatory cytokine IL-10 in a gestational age-dependent manner.

Immunohistochemical detection of IL-10 in placental tissues

To further confirm the expression profile of IL-10 in placental tissues immediately after their procurement, formalin-fixed tissues from different gestational ages were subjected to immunohistochemical analysis using an IL-10-specific mAb as described in Materials and Methods, and the results are shown for a representative placental tissue section. Examination of first trimester tissue revealed intense IL-10-positive staining in the villi, localized mainly to the trophoblast layer, which is comprised of both cytotrophoblast and syncytiotrophoblast cells (Fig. 2A). Similarly, cytotrophoblasts from 16-wk second trimester gestation (E and G) and 39-wk term gestation (F) were processed for immunohistochemistry using IL-10 mAb or control IgG as described in Materials and Methods. Magnification, ×40 in A–D and ×100 in E–G.

From the data presented in Figs. 1 and 2, A–D, it is not clear whether the reduced staining in the trophoblast layer is related to the decreased number of cytотrophoblasts, which normally occurs at the end of pregnancy, or if it is due to down-regulation of IL-10 production at the stem cell (cytotrophoblast) level. To address

FIGURE 1. Cytokine secretion by cultured human placental explants. Minced placental tissues (5 g) from different gestational ages were cultured for 18 h, and supernatant was collected as described in Materials and Methods. Using specific ELISA kits, concentrations of different cytokines were measured. The data are only shown for IL-10, IL-4, and IFN-γ. Note a statistically significant difference between term samples and those from first and second trimesters.

FIGURE 2. Immunodetection of IL-10 in placental tissue blocks from first trimester, second trimester, and term (before onset of labor) as well as in isolated cytotrophoblasts. A 5-μm placental section was fixed and stained with an IL-10- or control IgG. Sections are representative of a minimum of seven placental tissues examined for each gestational age. IL-10-specific immunostaining was mainly localized to the trophoblast layer in first (A) and second (B) trimester tissues. Third trimester tissue block (C) showed little or no IL-10-specific staining in the trophoblast layer. A dense villous core in term placental tissue (C) appears to present a homogeneous, nonspecific staining pattern with IL-10 mAb. Placental tissues from all gestational ages (only first trimester shown here for reference) showed no staining with control isotype-matched IgG (D). Similarly, cytotrophoblasts from 16-wk second trimester gestation (E and G) and 39-wk term gestation (F) were processed for immunohistochemistry using IL-10 mAb or control IgG as described in Materials and Methods. Magnification, ×40 in A–D and ×100 in E–G.
nuclei are visible as a result of hematoxylin counterstaining. Magnification, 3 C
IL-10R Ab; IL-10 mAb. As shown in Fig. 2, Immediately after isolation, cytotrophoblasts were stained using a second trimester and term pregnancies “before” the onset of labor. Cytotrophoblasts represented second trimester or term (no-labor) gestational ages.

Methods

For these questions, cytotrophoblasts were isolated as described in Materials and Methods from placental tissues (n = 7) representing second trimester and term pregnancies “before” the onset of labor. Immediately after isolation, cytotrophoblasts were stained using an IL-10 mAb. As shown in Fig. 2, E and F, IL-10-positive immunostaining was much weaker in term cytotrophoblasts compared with second trimester cytotrophoblasts. These results confirm that IL-10 production is down-regulated in term cytotrophoblasts. Because syncytiotrophoblasts represent differentiated fused cytotrophoblasts and usually contain or express proteins biosynthesized in their undifferentiated precursors (55), these results demonstrate that both cytotrophoblasts and syncytiotrophoblasts are main sources of IL-10 in the placenta and that this expression is down-modulated at term. There was no immunostaining in cytotrophoblasts with isotype-matched control IgG (Fig. 2G).

IL-10 expression is transcriptionally reduced at term

To delineate whether diminished IL-10 production in term cytotrophoblasts is due to transcriptional regulation, we evaluated the presence of IL-10 mRNA in cytotrophoblasts from second trimester and no-labor or labor term deliveries. For positive controls, IL-10-specific transcription was analyzed in an AIDS-associated B cell lymphoma cell line, HBL-1 (lane 2), from IL-10 expressing normal human B cells (lane 6), and from cytotrophoblasts representing placental tissues from term with labor (lane 3), term no labor (lane 4), and second trimester (17-wk gestation; lane 5). Lane 1 represents a 100-bp DNA ladder.

From these results, we determined that RT-PCR for IL-10 expression was performed using total RNA isolated from an IL-10-positive AIDS-associated lymphoma cell line, HBL-1 (lane 2), from IL-10 expressing normal human B cells (lane 6), and from cytotrophoblasts representing placental tissues from term with labor (lane 3), term no labor (lane 4), and second trimester (17-wk gestation; lane 5). Lane 1 represents a 100-bp DNA ladder.

FIGURE 3. Expression of IL-10 mRNA at different gestational ages. RT-PCR for IL-10 expression was performed using total RNA isolated from placental tissues (Fig. 3, lane 7) representing second trimester (17-wk gestation; lane 5) and term no labor (lane 6). In contrast, cytotrophoblasts from term before labor (40-wk gestation) failed to give rise to any detectable product (Fig. 3, lane 4). RNA isolated from term cytotrophoblasts after the onset of labor had a low abundance of IL-10-specific product (Fig. 3, lane 3). Lanes 2 and 6 represent IL-10-positive signal from HBL-1 and normal B cells, respectively. An equal intensity of GAPDH signal through the lanes validates that approximately equal amounts of RNA were used in each reaction. IL-10 and GAPDH products were of 360-bp and 220-bp sizes, respectively, as determined by comparison with the 100-bp ladder (Fig. 3, lane 1). These results support the data presented in Figs. 1 and 2 and suggest that IL-10 transcription is developmentally regulated during gestation.

IL-10R expression in placental tissues and on cytotrophoblasts

To demonstrate that the diminished production of IL-10 is a unique event and not a general phenomenon associated with the end of pregnancy, we investigated the expression of the IL-10R in second trimester and term placental tissues as well as on isolated cytotrophoblasts from these tissues using an IL-10R mAb or control IgG. As shown in Fig. 4, IL-10R-positive staining was quite similar in cytotrophoblasts isolated from both second trimester (Fig. 4A) and term (Fig. 4B) placental tissues. Control IgG failed to stain cytotrophoblasts from term placental tissues (Fig. 4C). Similar results were obtained when placental tissue sections were used, and the cytotrophoblast layer, but not other cells inside the mesenchyme, stained positive for IL-10R (data not shown). Thus, down-regulation of IL-10 at term may be a physiological event and is independent of the expression of its receptor.

IL-4 is not a major cytokine produced by the human placenta

Although IL-4 is mainly expressed by T cells and mast cells (56, 57), its production has been shown in murine feto-placental tissue. This information is significant in that IL-4 is a key regulator of Th2 immunity and possesses anti-inflammatory characteristics, albeit they are less potent than IL-10 (58). To evaluate whether IL-4 or its signaling receptor is expressed in placental cells, we performed immunohistochemical analysis of placental tissues representing first trimester or term with no labor. As shown in Fig. 5, a very weak staining for IL-4 was detected in placental tissues from first trimester (Fig. 5B), whereas no positive staining was observed in term placental tissues (Fig. 5C). Curiously, IL-4R α-chain was expressed exclusively in cytotrophoblasts and in cells of the villous core in first trimester placental tissue blocks (Fig. 5D), whereas only the villous core cells stained positive in term placental tissues, suggesting that syncytiotrophoblasts lack expression of IL-4R (Fig. 5E). Control IgG, matched isotypes to Abs against IL-4, and its receptor gave no positive staining. Moreover, placental tissue
blocks from different stages of the pregnancy did not exhibit significant staining with Abs against human IFN-γ or IL-2 (data not shown). Taken together, these data confirm the results in Fig. 1 that IL-4 was not secreted by placental explants from any gestational age.

Cytokine production in activated PBMCs during pregnancy

To correlate systemic cytokine production with that in the placenta, peripheral blood samples from first trimester and term pregnancies were obtained from the same subjects as the placental tissue samples. Blood was also collected from age-matched healthy nonpregnant women. PBMCs were isolated and activated in vitro as described in Materials and Methods. Culture supernatants were collected and assayed for cytokine concentration using specific ELISA kits. Production of IL-10 by activated PBMCs from first trimester was higher than that from nonpregnant women (p < 0.05). However, these levels dropped to approximately prepregnancy levels at the end of pregnancy (Fig. 6). In contrast, IL-4 concentration did not statistically differ between nonpregnant and pregnant women. IFN-γ production was mildly down-regulated at the end of pregnancy compared with nonpregnant women (Fig. 6). IL-2 production was also not modulated in a statistically significant manner.

Labor-related regulation of IL-10 production in the placenta

Our data on diminished IL-10 production at term “before” the onset of labor warrants its further analysis in placental tissues collected after spontaneous labor. Placental explants from labor or

![Figure 5](http://www.jimmunol.org/)

![Figure 6](http://www.jimmunol.org/)
TNF-α expression was localized only to the inner part (cytotrophoblasts) of the trophoblast layer in second trimester placental tissues. However, its expression in term placental tissues appeared to be mainly localized to the villous core (Fig. 5), suggesting that the cytotrophoblast could act as a target of IL-4 produced by the decidua.

Several lines of evidence suggest that IL-10 may play a major role in influencing the activity of the placental trophoblast, which has been proposed as a key cell type in regulating the fetal immunoprotection (32, 39, 63). The placenta produces proinflammatory cytokines, which are thought to be associated with trophoblast apoptosis, protease production, and stimulation of several uterotonins (prostaglandins, etc.), usually detected in increased levels at the time of spontaneous or preterm labor (64–67). Significantly, IL-10 displays a potent bioactivity in down-regulating the expression and activities of proinflammatory cytokines and uterotonins (41, 68). Furthermore, IL-10 is a potent inhibitor of cell-mediated immunity, which has been shown to be immunologically incompatible with establishment of the feto-placental unit in mice (11). Given these observations, we speculate that down-regulation of IL-10 at term may serve as one of the initial signals in a complex regulatory scheme necessary to ensure up-regulation of proinflammatory cytokines and uterotonins at parturition. This contention is supported by our observations (Fig. 7) demonstrating up-regulation of TNF-α and IL-1β only in association with labor. It is important to point out that up-regulation of inflammatory cytokines at term may not be restricted to trophoblasts in placental tissue. Thus, to further elucidate the IL-10-proinflammatory cytokines two-way signaling at term, it will be necessary to evaluate expression profiles of cytokines and their receptors in different cell lineages in both the placenta and the decidua before and after the onset of labor.

The regulatory role of IL-10 is supported by the observations that this cytokine successfully blocks LPS-induced preterm delivery in mice (69). Importantly, it modifies the activity and expression of prostaglandin dehydrogenase in cultured term human villous trophoblast and chorion trophoblast cells (68). Our results on re-expression of IL-10 mRNA after labor (Fig. 3) suggest that it might be a part of delayed negative feedback mechanism. It has been suggested that TNF-α, IL-12, and prostaglandin E2 induce IL-10 to autoregulate their own production (70, 71). Importantly, the data presented in Fig. 4 clearly show that the IL-10R continues to be expressed on trophoblast cells even at term, suggesting that these cells remain IL-10 responsive. Although the physiological role of IL-10 in pathologic pregnancies is not well defined, we have data suggesting that placental tissues or isolated cytotrophoblasts from 26-wk and 33-wk preterm labor deliveries lack expression of IL-10 (our manuscript in preparation). Furthermore, it is noteworthy to point out that IL-10 has been shown to reverse experimental fetal growth restriction and demise (72). Thus, IL-10 may be critical in normal fetal development and down-regulation of inflammatory responses in the placental microenvironment.

Of interest is the observation that in early and mid-pregnancy samples, the major site of placental IL-10 production was the trophoblast layer, inclusive of both the cytotrophoblasts and syncytiotrophoblasts (Fig. 2). In contrast, the trophoblast layer at term failed to exhibit significant IL-10-specific immunostaining (Fig. 2), suggesting that syncytiotrophoblasts, the major cell component of the trophoblast layer at term, harbor altered characteristics. Does this reflect a syncytiotrophoblast-specific phenomenon or a regulated process that is intrinsic of diminished population of cytotrophoblasts at term? Our findings in Figs. 2 and 3 indicate that IL-10 expression was primarily down-regulated in freshly prepared term cytotrophoblasts. However, it has been demonstrated that term cytotrophoblasts when cultured in the presence of serum are induced to express IL-10 (39). Thus, it is important to use freshly isolated, noncultured placental tissues or cytotrophoblasts for further studies on this topic.
from different gestational ages to gain insights into the ontogenic pattern of cytokine production. Culture conditions appear to signifi-
cantly modulate the natural expression profiles of these mole-
cules (73). Moreover, IL-13 has been shown to be expressed only in first trimester placenta (36), suggesting cytokines might indeed be subjected to gestational age-dependent regulation. In this re-
gard, cytokine gene loci may provide crucial model systems to study intrinsic differences between early and late gestation regu-
latory paradigms.

The data presented here further show that both pro- and anti-
flammatory cytokines, including IL-2, IL-4, IL-10, and IFN-γ, are expressed in activated PBMCs throughout pregnancy (Fig. 6), in contrast to their placental production. It can be implied that competence of the maternal immune system is not severely compromised during normal pregnancy. This follows the argument that there should not be a significant dampening of immune responses during pregnancy to avoid generalized immunosuppression (74–77). Nevertheless, there was statistically significant up-regulation of IL-10 during early pregnancy (Fig. 6), which may be sufficient to exert moderate Th2 dominance. On the other hand, there might be a more profound systemic Th1 balance in pathologic pregnancies, as recently observed (78). Thus, because IL-10 is a potent regulator of anti-inflammatory immune responses as well as of intrauterine mediators, its ontogenic participation in the placental microenvironment is of fundamental importance in delineating the molecular mechanisms underlying normal or pathologic human gestation.

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