Dual positive regulation of embryo implantation by endocrine and immune systems
- Step-by-step maternal recognition of the developing embryo -

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Abstract

In humans, HCG secreted from the implanting embryo stimulates progesterone production of the corpus luteum to maintain embryo implantation. Along with this endocrine system, current evidence suggests that the maternal immune system positively contributes to the embryo implantation. In mice, immune cells that have been sensitized with seminal fluid and then the developing embryo induce endometrial differentiation and promote embryo implantation. After hatching, HCG activates regulatory T and B cells through LH/HCG receptors and then stimulates uterine NK cells and monocytes through sugar chain receptors, to promote and maintain embryo implantation. In accordance with the above, the intrauterine administration of HCG-treated PBMC was demonstrated to improve implantation rates in women with repeated implantation failures. These findings suggest that the maternal immune system undergoes functional changes by recognizing the developing embryos in a stepwise manner even from a pre-fertilization stage and facilitates embryo implantation in cooperation with the endocrine system.

Introduction

The mammalian female evolved to utilize the uterus to receive embryo implantation. Consequently, it had to evolve to interact with the implanting embryo in the female genital tract and adapt maternal organs to accept the embryo in the uterus. To achieve this adaptation, a new endocrine organ developed, the corpus luteum (CL), which is periodically transformed from the ovulated follicles in the ovary, and utilizes a CL-produced hormone, progesterone, that induces suitable endometrial differentiation for embryo implantation[1].

On the other hand, since Wegmann proposed the immunotrophic hypothesis[2], it has been gradually accepted that there are certain immune cell populations that positively play an important role in embryo implantation from the very early stage of pregnancy. Current evidence suggests that immune cell contribution at the implantation site is important for successful embryo implantation[3-5]. We have also provided evidence to support that immune cells in the blood circulation positively regulate the CL function and endometrial differentiation to promote subsequent embryo implantation[6]. In this article, we describe dual regulation by endocrine and immune systems, focusing on the positive contribution of the immune system to embryo implantation in cooperation with the endocrine system.

Dual positive control of CL function by endocrine and immune systems

In 1994, Espey proposed that ovulation is an inflammatory reaction caused by various proteolytic enzymes such as serine proteases and metalloproteases[7]. After ovulation, human granulosa cells transform into large luteal cells, undergoing luteinization to produce progesterone, while theca interna cells become small luteal cells, producing androgen that is converted into estrogen. We previously confirmed the differential functions of luteinized granulosa and theca interna cells *in vitro*[8], and the individual differentiation pathways, identifying differentiation markers[9-11]. Along with the luteinizing process, endothelial cells migrate into the granulosa cell layer and construct a new vascular network[12]. Luteinizing granulosa cells were demonstrated to produce several angiogenic factors such as vascular endothelial growth factor (VEGF), angiogenin, endocrine gland-VEGF, and angiopoietin[13-16]. A suitable synchronization between luteinization and neovascularization is necessary to establish a mature CL, and this maturation process should be further synchronized with endometrial differentiation in order to timely receive and support the implantation of the

developing embryo. The functional lifespan of the human CL continues only for 14 days when embryo implantation does not occur[17]. However, when implantation is successfully achieved, the CL of the menstrual cycle is further transformed into the CL of pregnancy to supply progesterone.

Luteinizing hormone (LH) and human chorionic gonadotropin (HCG) are key hormones to regulate the CL formation, transformation to the CL of pregnancy, and maintenance of the function. Both hormones share the same receptor, the LH/HCG receptor. We immunohistochemically confirmed that LH/HCG receptor expression on luteal cells is maintained in the CL of pregnancy, while its expression disappears in the regressing CL[18]. HCG promotes progesterone[8] and VEGF production[19] by luteal cells, indicating that LH/HCG is a major factor to induce luteinization and neovascularization in the CL (Figure 1).

Previously, we reported that interleukin-1 inhibits LH-induced luteinization of porcine granulosa cells *in vitro*[20], proposing a dual control of the cyclic ovarian function by endocrine and immune systems[21]. Later, we demonstrated that PBMC, especially T lymphocytes, enhance progesterone production by cultured human luteinizing granulosa cells[22], and that cytokines regulate their luteinization[23]. Furthermore, these cytokines, but not HCG, were shown to enhance the luteinization-associated expression of dipeptidyl peptidase-IV that can degrade chemokines on the cell surface[24]. These findings suggest that the circulating immune cells physiologically contribute to the luteinization process of human granulosa cells during CL formation[25].

After successful implantation, HCG secreted from the implanting embryo induces transformation of the CL of the menstrual cycle into the CL of pregnancy. In contrast, clinical evidence suggests the presence of different mechanisms to regulate the human CL of pregnancy[26]. However, no soluble factor other than HCG has been identified[27]. To identify new mechanisms, we raised monoclonal antibodies and found that HLA-DR, LFA-3/CD58, and ALCAM/CD166, which mediate interaction with T lymphocytes, were expressed on the human luteal cells[28,29]. These findings led us to the new concept that immune cells are involved in the transition into the CL of pregnancy and its functional regulation. Accordingly, we hypothesized that signals from the developing embryo in the genital tract are transmitted to the ovary by not only the endocrine system, but also the immune system, in other words, not only soluble factors, but also circulating immune cells[6,29].

To evaluate the above theory, we examined the effects of PBMC on progesterone production by the luteal cells isolated from the human CL. In contrast to the long-standing

concept that immune cells enhance CL regression[30], PBMC isolated from pregnant women were shown to promote progesterone production. In the same co-culture, the production of Th-2 cytokines, IL-4 and IL-10, was increased and these cytokines significantly enhanced progesterone production by human luteal cells[31], suggesting that circulating blood immune cells during early pregnancy promote CL function[32] (Figure 1).

In the cow, it was reported that the populations of residual $CD8\alpha\alpha^+$ and $\gamma\delta^+CD8\alpha\alpha^+$ T cells were significantly increased during CL regression along with a decrease in the proportion of $FOXP3^+$ lymphocytes. In contrast, there was an increase in the $CD8\alpha\beta^+$ and $\gamma\delta^+CD8\alpha\beta^+$ populations within the CL of early pregnancy[33]. Currently, it is being proposed that immune cells modulate both luteotropic and luteolytic processes[34].

Dual positive control of endometrial function and differentiation by endocrine and immune systems

Recently, gene knockout techniques confirmed that progesterone critically induces endometrial differentiation and decreases the contractility of uterine smooth muscle cells to promote embryo implantation[1,35]. By the sequential administration of estradiol and progesterone, waves of human uterine contraction from the fundus to cervix were induced by estradiol, but were immediately diminished after the administration of progesterone[36], providing supporting evidence that progesterone is an essential hormone to maintain the pre-implantation embryo in the uterine cavity.

In humans, endometrial decidualization is induced by progesterone. In 1991, we reported that interleukin-1 inhibits progesterone-induced decidualization on human endometrial stromal cells, being the first to demonstrate that cytokines can regulate human endometrial differentiation[37]. Thereafter, accumulating evidence demonstrated that cytokine networks play important roles in endometrial differentiation and embryo-maternal cross-talk[38-42].

Recently, Treg cells were demonstrated to promote maternal immune tolerance and play critical roles in embryo implantation and fetal development[43]. In mice, seminal fluid and sperm cause the expansion of a CD4(+)CD25(+)FOXP3(+) Treg cell population in the para-aortic lymph nodes after coitus and the subsequent accumulation of Treg cells in the uterus before embryo implantation[44]. It was also reported that Treg cells were rapidly recruited to para-aortic lymph nodes and activated in the first few days after embryo

implantation[45]. Importantly, the exposure of the maternal immune system to seminal fluid was demonstrated to promote embryo implantation by activating inflammatory and inducing immunological changes in the female reproductive tract that facilitate embryonic development and endometrial receptivity[46,47].

Intriguingly, we found that the systemic administration of splenocytes derived from early pregnant mice (pregnancy day 4) induced successful implantation in pseudopregnant recipient mice that received blastocyst transfer on pseudopregnant day 2[48]. To examine the effect on endometrial differentiation, we used a delayed implantation model in which pseudopregnant mice were treated with daily progesterone supplementation after oophorectomy on post-ovulatory day 3. In this model, intrauterine transferred blastocysts remain floating in the uterine cavity. The subsequent administration of estrogen induces blastocysts to restart implantation along with the expression of leukemia inhibitory factor (LIF) in the uterus[49], which is an essential cytokine for embryo implantation[50,51]. Notably, the intravenous administration of splenocytes derived from early pregnancy induced LIF expression in the uterus and restarted embryo implantation without estrogen administration[52]. These findings indicate the presence of the dual control of endometrial differentiation before embryo attachment via the endocrine and immune systems (Figure 1).

In contrast to pregnant mice, splenocytes derived from pseudopregnancy day 4 mice did not significantly expand the implantation window[48,52]. Since the immune system of pseudopregnant recipient mice that had been mated with vasectomized male mice was already sensitized with the seminal plasma component of seminal fluid, it is reasonable to speculate that the presence of developing embryos in the Fallopian tube and uterus induce additional changes in the maternal immune function to facilitate embryo implantation. Although the key signals from the developing embryo remain under investigation, several factors such as early pregnancy factors that inhibit T-cell-induced rosette formation, just after fertilization[53], embryo-specific peptides, pre-implantation factors (PIF), from the two-cell stage[54], and degradation products of zona pellucida glycoprotein, from fertilization to hatching[55], have been proposed as embryonal signals conveyed to the maternal immune system from the developing embryo before implantation stages. Taken together, it is reasonable to propose that there are step-by-step mechanisms to recognize pregnancy by the maternal immune system prior to embryo implantation (Figure 2).

Endocrine control of immune cell function and differentiation

It is generally accepted that ovarian sex steroids are important regulators of the function and population of immune cells in the maternal uterus[3]. The immune-endocrine interaction is considered to suppress adverse maternal immune responses and promote immunotolerance pathways, contributing to fetal survival[56].

Progesterone regulates the immune cell function by producing mediators such as the progesterone-induced blocking factor (PIBF) that induces Th2-dominant cytokine production[57] and glycodelin A that protects the embryo from maternal immune attack by reducing the monocyte function[58]. Nuclear progesterone receptor knockout mice revealed that progesterone antagonizes the estrogen-induced recruitment of macrophages and neutrophils into the uterus[59]. In humans, CD56(high+)CD16(-) uterine natural killer (NK) cells are the predominant population in the decidual tissues during the late secretory phase of the menstrual cycle and early pregnancy. Uterine NK cells were reported to interact with extravillous trophoblasts and contribute to the remodeling of endometrial spiral arteries[60,61]. Since the expression level of progesterone receptors on uterine NK cells is very low[62], the progesterone-induced endometrial environment is a key factor for the in situ proliferation or differentiation of uterine NK cells and reprogramming of their chemokine receptor profiles[63,64]. Progesterone has also been reported to reduce the antigen-presenting capacity of dendritic cells, monocytes, and macrophages and induce the recruitment of regulatory T (Treg) cells, contributing to the local accumulation of pregnancy-protective cells[60] (Figure 1).

More than 40 years ago, crude HCG purified from urine was demonstrated to suppress immune reactions, leading to the proposal of the involvement of HCG in maternal immune tolerance to the fetus[65]. However, immunosuppressive effects were not observed in highly purified HCG[66], and the effects of HCG on the immune cell function subsequently remained controversial for a long time. Previously, we found that recombinant-HCG induced the activation of NF-κB and promoted IL-8 production by human CD14-positive monocytes at relatively high concentrations of more than 10 IU/mL[67]. However, the cell surface expression of LH/HCG receptors on monocytes was not observed. Intriguingly, this HCG-induced IL-8 production was inhibited by an exogenous excess of D-mannose. These findings suggest that HCG regulates the PBMC function through sugar chain receptors, which is a primitive regulatory mechanism in the immune system[67]. In addition, HCG stimulated human PBMC to produce chemoattractants and enhance invasion by BeWo cells, a cell line derived from human choriocarcinoma-derived cells[68]. This hormone also stimulated human PBMC to enhance murine blastocyst attachment and spreading[69]. From these findings, we

proposed that HCG stimulates maternal immune cells at the implanting site through a lectin–glycan interaction, which, in turn, promotes embryo attachment and invasion based on cooperation between the endocrine and immune systems[32].

Later, a high concentration of HCG was demonstrated to regulate uterine NK cell proliferation. This effect was mediated via mannose receptors rather than by LH/HCG receptors that were not expressed[70]. The sugar chains of HCG purified from urine are largely cleaved before urine production[71]. Accordingly, this may explain the previous discrepancy in the effects on immune cells between crude HCG and highly purified HCG[67]. This mechanism can partially explain the reason why such a high concentration of HCG is necessary to maintain a normal pregnancy[67].

On the other hand, it was reported that LH/HCG receptors are expressed on Treg cells, and that HCG secreted from human trophoblast induces Treg cell migration to the trophoblast[60]. Based on the subsequent experiments, HCG was proposed to play a central role in pregnancy-induced immune tolerance[72]. Recently, CD19+CD24(high+)CD27+ regulatory B cells were reported to increase in the first trimester of pregnancy and that the population in patients suffering from spontaneous abortions remain as low as in non-pregnant women[73]. This type of regulatory B cells was also demonstrated to express LH/HCG receptors and produce IL-10 on stimulation with recombinant HCG, suggesting the positive effects of HCG on regulatory B cells[73,74].

Accordingly, HCG is currently considered to play an important role as a modulator of the immune function during pregnancy[75]. Considering that the sugar chain system needs a high concentration of HCG, it is also speculated that there are two-step processes of immune cell sensitization by HCG, where the maternal immune system firstly responds to a normal range of HCG through LH/HCG receptors, and then responds to a high concentration of HCG, which is a reliable sign indicating the adequate development of the implanting embryo, through sugar chain receptors[6,67] (Figure 2).

In accordance with the above concept, we observed that an excessive reaction of immune cells to HCG could induce the high-level local production of VEGF, leading to ovarian hyperstimulation syndrome (OHSS)[76]. Consequently, this novel mechanism should also be investigated from a pathological perspective.

Clinical approach to infertile patients applying positive immune control mechanism

To examine the direct roles of immune cells in human endometrial receptivity, we developed an embryo attachment assay. This assay showed high attachment rates between a human choriocarcinoma-derived BeWo cell mass and endometrial epithelial cells derived from the mid-luteal phase, and that co-culture with autologous PBMC increased attachment rates in the endometrial epithelial cells derived from late proliferative and early secretory phases[77]. These findings suggest that autologous PBMC derived from non-pregnant women contain populations that promote endometrial cell receptivity. In accordance with these findings, we observed that thymocytes derived from immature non-pregnant female mice induce LIF expression and embryo implantation in the delayed implantation model[78], indicating that even with a non-pregnant status, certain T-cell populations can induce endometrial differentiation and promote embryo implantation.

Recently, implantation failure has become one of the most difficult problems that must be overcome in IVF therapy. It should be noted that IVF therapy skips a large part of the maternal immune recognition process involving the developing embryo in the female genital tract. Accordingly, we supposed that supplementation of the recognition process by the immune system can improve the implantation outcome, and have developed a novel therapy using autologous PBMC. Briefly, PBMC are isolated from the patients and incubated with HCG in order to activate them for 2 days. Thereafter, the activated PBMC are administered into the uterine cavity to induce adequate endometrial differentiation. Three days later, blastocysts are transferred into the uterine cavity. We applied this treatment to patients with 4 or more repeated IVF therapy failures and found that PBMC treatment effectively improved the pregnancy and implantation rates[79]. Later, the intrauterine administration of autologous PBMC without HCG treatment was also demonstrated to improve implantation rates of patients with no less than 3 repeated implantation failures[80]. However, this therapy did not improve the outcome in the population without repeated implantation failures. On the other hand, this approach was applied to domestic animals, and was found to improve embryo implantation rates[81]. In addition, the intrauterine administration of PBMC derived from non-pregnant mice prior to embryo implantation was reported to enhance endometrial receptivity and promote embryonic implantation in mice with the dysfunction of embryo implantation[82]. Very recently, the intrauterine administration of autologous PBMC was shown to improve clinical outcomes of patients with repeated implantation failures[83]. Furthermore, a recent study showed that the intrauterine administration of

corticotropin-releasing hormone (CRH)-treated autologous PBMC can improve clinical pregnancy rates of women with repeated implantation failure[84].

Several mechanisms relevant to this procedure can be proposed as follows. Firstly, PBMC may induce endometrial differentiation that facilitates embryo attachment. Secondly, although PBMC are autologous cells from the patient, the induction of PBMC by themselves is expected to lead to favorable inflammatory reactions in the uterine cavity *in vivo*. Thirdly, PBMC can secrete proteases that may effectively change the function or structure of surface molecules expressed on endometrial luminal epithelial cells. Fourthly, PBMC can move from the uterine cavity toward the endometrial stromal tissue, creating a leading pathway for subsequent embryo attachment and invasion. Finally, HCG or CRH-treated PBMC contribute to creating a favorable immune environment for embryo implantation[85].

A recent study suggested that patients with repeated implantation failure have pre-conceptional endometrial deregulations, including cell-mediated immune response[86]. Consequently, it is an important issue to develop an effective pre-evaluation method to accurately select patients who will benefit from immune therapy.

Conclusion

In conclusion, we described a current novel concept whereby immune cells receive information about the presence of a developing embryo in the female genital tract and transmit this information through the blood circulation to the uterus, inducing endometrial functional changes or differentiation, and facilitating embryo implantation in cooperation with the endocrine system (Figure 1). Accumulating evidence has led to a novel concept that there are step-by-step mechanisms to recognize pregnancy by the maternal immune system from prior to fertilization to after embryo implantation (Figure 2). When the endocrine mechanism does not adequately operate, alternative mechanisms by the immune system can be clinically applied to infertility therapy. Although the efficacy of this clinical approach needs more accurate evaluation, further clarification of the mechanisms of maternal immune cell recognition of the developing embryo will contribute to creating more effective immune therapies.

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Figure Legends

Figure 1. Proposed dual positive regulation of CL function and endometrial differentiation by the endocrine and immune systems.

The maternal immune system recognizes the presence of a developing and implanting embryo in the Fallopian tube and uterus due to embryo- and species-specific signals such as seminal fluid, early pregnancy factor, degraded products of zona pellucida glycoprotein, pre-implantation factors, and/or HCG. Affector immune cells transmit this information to the lymphoid organs, and then effector immune cells move to the ovary and endometrium via blood circulation to regulate the CL function and induce endometrial differentiation.

Figure 2. Step-by-step recognition of the embryo by the maternal immune system.

Although the key signals from the developing embryo remain under investigation, several factors such as seminal fluid (directly after intercourse), early pregnancy factors (EPF, directly after fertilization), pre-implantation factors (PIF, from the two-cell stage), degradation products of zona pellucida glycoprotein (ZP-DP, from fertilization to hatching), HCG through LH/HCG receptors (HCG-R, after hatching), and HCG through sugar chain receptors (SC-R, after implantation) have been proposed as embryonal signals conveyed to the maternal immune system from the developing embryo before implantation stages. Proposals of the above signals have given rise to a novel concept that there are step-by-step mechanisms to recognize pregnancy by the maternal immune system prior to embryo implantation.

Fig. 1

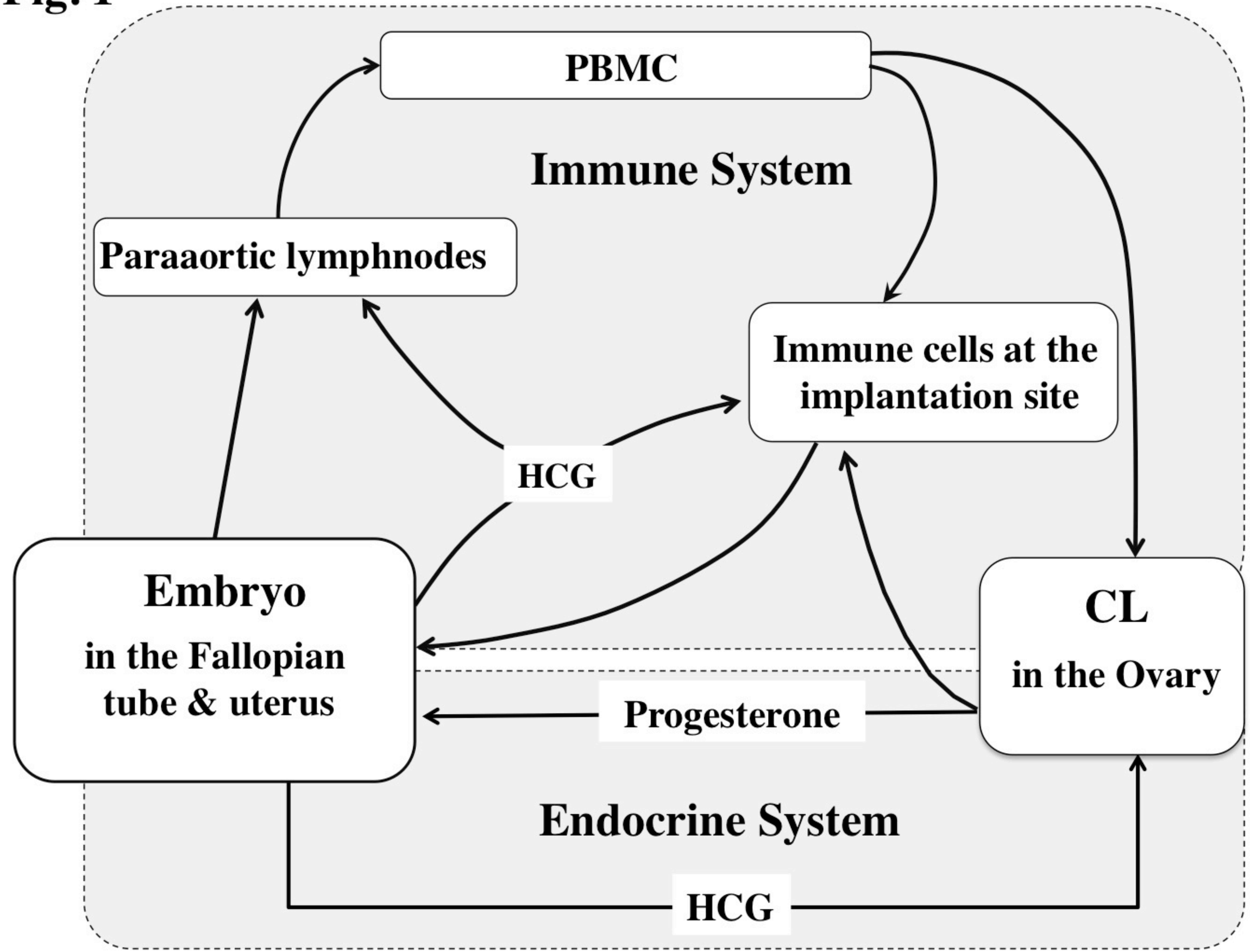
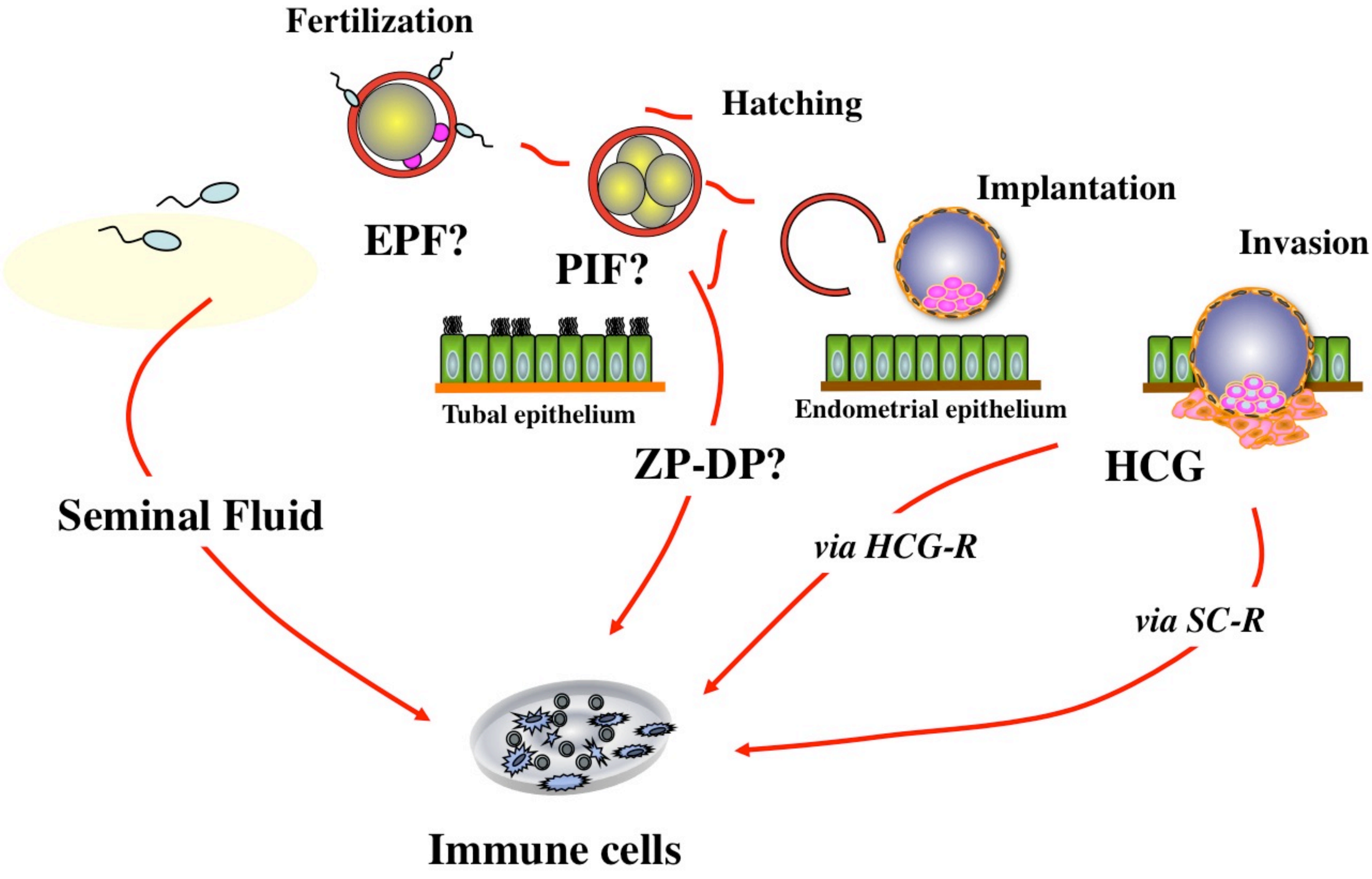

PBMC
Immune System
Paraaortic lymphnodes
Immune cells at the implantation site
HCG
Embryo
in the Fallopian tube & uterus
CL
in the Ovary
Progesterone
Endocrine System
HCG

Fig. 2


Fertilization
Hatching
Implantation
Invasion
EPF?
PIF?
Tubal epithelium
Endometrial epithelium
ZP-DP?
HCG
Seminal Fluid
via HCG-R
via SC-R
Immune cells