4

Immune Regulation of Human Embryo Implantation by Circulating Blood Cells

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1. Introduction

Mammalians have developed the part of the female genital tract, namely the uterus, to be a specific site that can receive embryo implantation. As a result, the mother must interact with the implanting embryo in the uterus, reconstruct maternal tissues during placentation and adapt maternal whole organs to accept embryo parasite. To induce the uterine environment to be favorable for embryo implantation, mammalian females in the reproductive stages periodically construct a unique endocrine organ, corpus luteum (CL), from the ovulated follicle. This newly constructed endocrine organ produces abundant amount of progesterone and induces the estrogen-primed endometrium to become a further differentiated stage that is suitable for embryo implantation (Yen, 1991). When the developing embryo enters the uterine cavity, a direct cross-talking of the embryo and the maternal endometrium is considered necessary to achieve a subsequent successful implantation of the embryo (Simón *et al*., 2001). However, the precise mechanisms of the initial step of human embryo implantation remain unknown. Recently, accumulating evidence suggests that local immune cells at the implantation site actively contribute to embryo implantation (Lea and Sandra, 2007; Yoshinaga, 2008). In this chapter, we introduce new mechanisms by which circulating blood immune cells contribute to embryo implantation by inducing endometrial differentiation and promoting embryo-maternal cross-talk.

2. Regulation of ovarian function and differentiation by circulating immune cells

2.1 Endocrine regulation of human CL of pregnancy

In each menstrual cycle, human CL function continues only for 14 days. However, when becoming pregnant, the implanting embryo secretes human chorionic gonadotropin (HCG). This hormone is produced by the developing trophoblasts of the embryo and

secreted into maternal blood circulation immediately after embryo invasion within the endometrium. HCG shares a receptor with luteinizing hormone (LH) and stimulates the function of CL of menstrual cycle in the ovary. This hormone also induces its transformation into CL of pregnancy to further produce progesterone, which in turn maintains embryo implantation in the uterus. Accordingly, there is a systemic crosstalk between the implantation embryo and mother through blood circulation system from the very early stages of human pregnancy. In this process, CL of pregnancy is an essential organ and it has been believed that HCG is a major regulator of human CL of pregnancy (Yen, 1991)(Fig. 1).

However, there are several lines of basic studies and clinical evidence to suggest that human CL of pregnancy is also regulated by different mechanisms (Rao *et al*., 1977; Shima *et al*., 1987). For example, in patients with ectopic pregnancy or natural abortion, despite a high HCG level in blood, progesterone production by the CL decreases and abortion proceeds (Alam *et al*., 1999). Text books say that rapid and continuous increase in HCG concentration is necessary to maintain CL of pregnancy. However, LH/HCG receptor system does not need such a high concentration for its activation. On the other hand, chorionic gonadotropin is only detected in mare and primates (Murphy and Martinuk, 1991). Accordingly, regulatory mechanisms for CL of pregnancy are completely different among mammals. However, although many researchers have investigated additional essential hormones, no soluble factor other than HCG has been identified and the precise regulatory mechanisms remain unknown (Kratzer and Taylor, 1990).

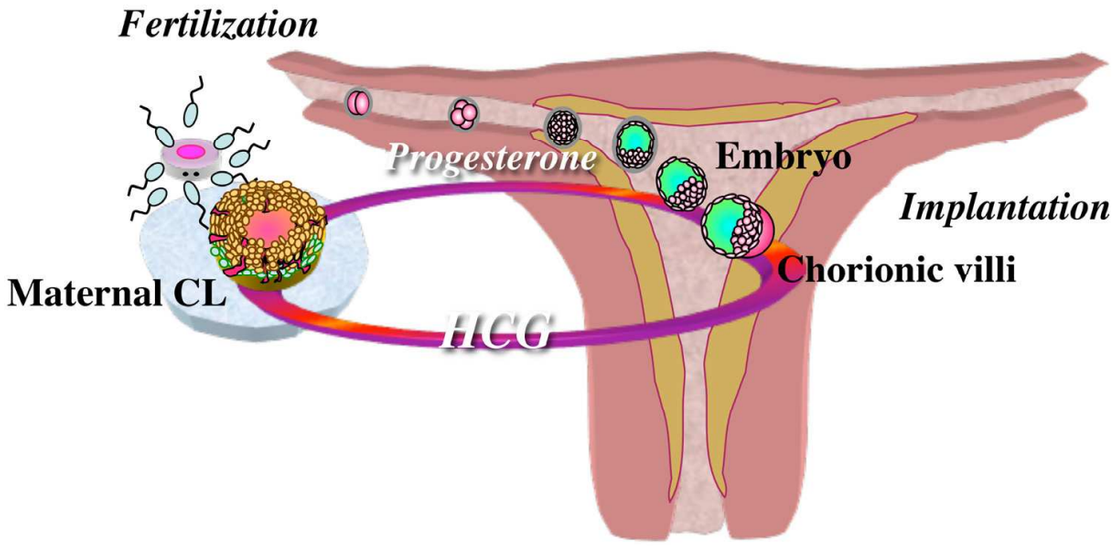

Fig. 1. Endocrine regulation of human early pregnancy

When being pregnant, human chorionic gonadotropin (HCG) that shares a receptor with LH, is secreted from the invading embryo and transmitted to the maternal ovary from the uterus by systemic blood circulation. Then, HCG stimulates corpus luteum to produce progesterone and this ovarian hormone is in turn transmitted to the uterus to maintain embryo implantation. Accordingly, there is a cross-talk between embryo and mother by endocrine system from the very early stage of human pregnancy.

2.2 Immune regulation of human CL of pregnancy

Based on the above background, we tried to identify the molecules that are expressed in human CL of pregnancy in order to clarify regulatory mechanism(s) that influence CL function and pregnancy. We found that several molecules that mediate interaction with T-lymphocytes were expressed on the luteal cell surface in the human CL of pregnancy (Fujiwara *et al*., 1993; Hattori *et al*., 1995). In general, immune cells are considered to enhance CL regression (Pate and Keyes, 2001). However, since these molecules such as HLA-DR and leukocyte functional antigen (LFA)-3/CD58 appear during CL formation, we speculated that interaction with immune cells plays some role in the functional and morphological transition from CL of menstrual cycle to CL of pregnancy.

After hatching, the human embryo takes an apposition, facing inner cell mass toward luminal epithelial layer, and then attaches to endometrial epithelial cells via trophectoderm layer. During this process, trophectoderm is activated to acquire invasive property and the attached embryo invades the endometrium as a mass through the epithelial layer, becoming buried within endometrial stromal tissue within 8-9 days after ovulation. Thereafter, trophoblast invasion transiently slows down and the lacunar spaces, which will become the intervillous spaces, are formed within the trophectoderm layer. Maternal blood gently flows in these spaces and then returns to the maternal systemic circulation (Boyd JD, Hamilton, 1970). At this stage, HCG is abundantly produced by the trophectoderm and the secreted HCG is transmitted to the ovary through the blood circulation, stimulating corpus luteum to produce progesterone via the LH/HCG receptor. During formation of the lacunar spaces, maternal blood cells including peripheral blood mononuclear cells (PBMC: lymphocytes and monocytes) are also considered to infiltrate here and these cells also return to the maternal systemic circulation including those in the ovary.

Taken together with the findings that the human CL expresses several molecules that can mediate direct interaction with T lymphocytes, we hypothesized that circulating immune cells contribute to the systemic crosstalk between embryo and mother (ovary) via blood circulation. It is theoretically sound to speculate 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, via not only soluble factors, but also circulating cells (Hattori *et al*., 1995) (Fig. 2).

The maternal immune system recognizes the presence of the developing and implanting embryo in the Fallopian tube and the uterus by embryo- and species-specific signals such as degraded products of zona pellucida glycoprotein and/or HCG. Then, effector immune cells move to the ovary and the endometrium via blood circulation to regulate CL function and induce endometrial differentiation. The local immune cells at the implantation site also contribute to induction of embryo invasion, secreting chemoattractants by HCG stimulation.

To prove this hypothesis, we investigated the effects of PBMC derived from pregnant women in early pregnancy on progesterone production by human luteal cells in culture. We found that PBMC derived from women in early pregnancy promoted progesterone production as much as that by HCG stimulation, suggesting that circulating blood immune cells in early pregnancy enhance CL function (Hashii *et al*., 1998). Based on these findings, we extended our hypothesis to the further concept that circulating immune cells transmit information about the presence of the developing embryo to various organs throughout the

whole body and induce adequate functional change or differentiation in these organs to facilitate embryo implantation (Fujiwara, 2006).

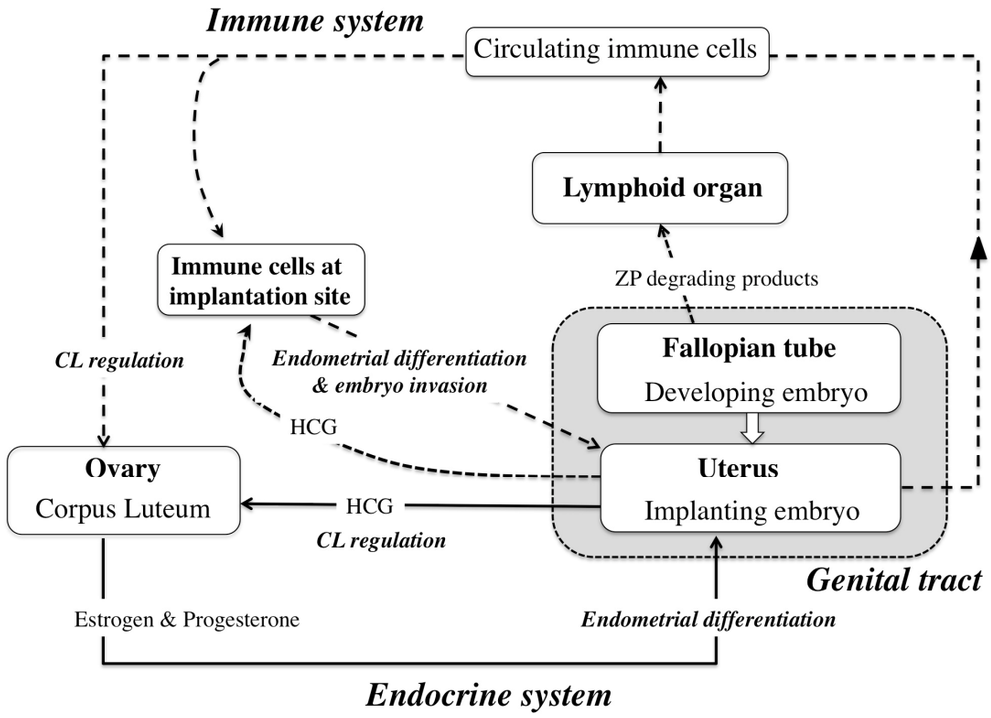

Fig. 2. Dual regulation of human embryo implantation by hormones and circulating immune cells

3. Regulation of endometrial differentiation by circulating immune cells

In human CL, LH/HCG receptor expression was already disappeared on 14th day after ovulation (Takao et al., 1997). When becoming pregnant, serum HCG arise around 11 days after ovulation. However, at this stage CL regression has already started. So, to achieve smooth transformation into CL of pregnancy, it is reasonable to expect that immune cells transmit information of the developing embryo to CL before embryo implantation. To support this speculation, HLA-DR and leukocyte functional antigen (LFA)-3/CD58 that can mediate direct interaction with T lymphocytes were expressed on human CL during corpus luteum formation.

3.1 Immune regulation of endometrial differentiation in mice

To investigate the above idea, we first examined the effects of circulating immune cells on endometrial differentiation and embryo implantation using mouse implantation experiments. When blastocysts were transferred into the uterine cavity of pseudopregnant recipient mice that had been mated with vasectomized male mice, successful implantation was only achieved during 3-5 days after ovulation when the endometrium was adequately differentiated. This period is called the "implantation window" (Psychoyos, 1993; Dey, 1996). However, when spleen cells, stocked circulating immune cells, were obtained from

pregnant day 4 mice (before attachment stage) and were administered to pseudopregnant mice, embryo implantation was induced prior to the implantation window (1-2 days after ovulation) when embryos cannot normally be implanted (Takabatake et al., 1997a). Then we examined direct effect of spleen cells and observed that T-cell-rich population more effectively promoted embryo implantation (Takabatake et al., 1997b). In contrast to pregnant mice, spleen cells derived from diestrus or pseudopregnant mice had no effects. These findings indicated that before hatching stage, immune cells in early pregnancy have changed their functions to promote embryo implantation. This also suggests that developing embryo is necessary for functional change of immune cells.

In order to examine the direct effects of the splenocytes on endometrial differentiation, we used a delayed implantation model in which pseudopregnant mice were treated with daily progesterone supplementation following an oophorectomy on post-ovulatory day 3. In this model, in the absence of ovarian estrogen, blastocysts that are transferred into the uterine cavity remain floating in the luminal spaces, and the exogenous administration of estrogen induces expression of leukemia inhibitory factor (LIF) in the endometrium, restarting embryo implantation (Bhatt et al., 1991). Interestingly, instead of estrogen, intravenous administration of splenocytes derived from early pregnancy restarted embryo implantation along with induction of LIF expression (Takabatake et al., 1997b). These results indicated that circulating immune cells could encourage early endometrial differentiation that was necessary for subsequent embryo implantation. These findings suggest that endometrial differentiation just prior to embryo attachment can be achieved by dual control of the endocrine and immune systems (Fig. 2).

Notably, it was demonstrated that thymocytes from non-pregnant immature mice, especially CD8-negative population, could promote embryo implantation along with induction of LIF expression in the uterus. This indicates that there is a certain immune cell population that can induce endometrial differentiation and embryo implantation even in non-pregnant mice (Fujita et al., 1998).

3.2 Immune regulation of human endometrial receptivity

Implantation window is also supposed to exist in women. However, there has been no study to directly prove this window. Therefore, we then examined whether or not human endometrial receptivity really changes during menstrual cycle and immune cells can affect endometrial receptivity. To examine this issue, we first developed an attachment assay using a human choriocarcinoma-derived BeWo cell mass that mimics a human blastocyst and a human endometrial epithelial cell monolayer culture. In this assay, high attachment rates were observed in endometrial culture derived from women in the mid-luteal phase, supporting that there is an implantation window in human endometrium. Importantly, when these endometrial cells were co-cultured with autologous PBMC, attachment rates significantly increased in the culture derived from women in the late proliferative and early secretory phases, showing that autologous PBMC promote human endometrial cell receptivity *in vitro* (Kosaka *et al*., 2003). These findings led us to think about the possible clinical application of autologous circulating immune cells to treatment for patients suffering with repeated implantation failures.

4. Maternal recognition of the human developing embryos by the immune system

4.1 HCG as a specific embryonal signal to the immune system

In order to receive information of the presence of the human developing embryo, the immune system must recognize the embryo-specific signals from the developing embryo in the female genital tract. First, we focused on the embryo-specific hormone, HCG, which is secreted from the developing and implanting human embryo. In invasion assays using murine embryo and BeWo cells, PBMC derived from women in early pregnancy promoted murine trophectoderm and BeWo cell invasion more than those obtained from non-pregnant women. It was also shown that this effect was exerted by soluble chemoattractive factors that were secreted by PBMC. Importantly, when PBMC derived from non-pregnant women were incubated with HCG, HCG-treated PBMC promoted invasion more than non-treated PBMC (Nakayama *et al*., 2002; Egawa *et al*., 2002). These findings suggest that HCG can change PBMC functions to facilitate embryo implantation.

Several decades ago, HCG crudely purified from urine was reported to suppress immune reactions (Adcock *et al*., 1973). However, it was later shown that highly purified HCG had no effect on lymphocyte function (Muchmore and Blaese, 1997). Accordingly, the effects of HCG on immune cell function have been controversial for a long time. Ten years ago, we found that recombinant-HCG enhanced IL-8 production by human monocytes at relatively high concentrations via activation of NF-κB. HCG shares a receptor with LH to commonly access the LH/HCG receptor. However, the so-called LH/HCG receptor was not detected on the cell surface of monocytes. Therefore, it was speculated that there was a different pathway besides the LH/HCG-R system, which could respond to high HCG concentration. It should be noticed that HCG is an evolutionarily current hormone that is detected in primates (Cole, 2007). The most important difference between LH and HCG is the presence of abundant sugar chains at the C-terminal of the HCG β-subunit. Notably, binding of HCG on the cell surface of the monocytes and HCG-induced IL-8 production were inhibited by an exogenous excess of mannose, suggesting that HCG can regulate PBMC function through sugar chain receptors, which is a primitive regulatory mechanism in the immune system (Kosaka *et al.*, 2002). It should also be noted that the sugar chains of purified HCG are largely cleaved before urine production (Cole, 2009).

A high concentration of HCG is locally produced at the embryo implantation site. It is well known that the initial change around the implantation site is an increase in vascular permeability, leading to recruitment of certain immune cells to the area. Recently, it was reported that human trophoblasts invading the implantation site produce hyperglycosylated HCG, and that the hyperglycosylated HCG up-regulates trophoblast invasion in humans (Handschuh *et al*., 2007). Therefore, it is reasonable to speculate that HCG stimulates endometrial immune cells to produce chemoattractants and vasodilators and that these cytokines in turn induce embryo invasion (Fujiwara, 2006).

To support our proposal, Kane et al. reported that HCG induced proliferation of uterine natural killer cells via the mannose receptor (Kane *et al*., 2009). In addition, Schumacher et al., demonstrated that high HCG levels at very early pregnancy stages ensure regulatory T cells to migrate to the site of contact between paternal antigens and maternal immune cells and to orchestrate immune tolerance toward the fetus (Schumacher *et al*. 2009). Furthermore,

Wan et al. proposed that HCG contributed to the maternal-fetal tolerance during pregnancy by inducing dendritic cells toward a tolerogenic phenotype (Wan *et al*., 2008).

4.2 Maternal recognition of the developing embryo in the Fallopian tube by the immune system

The findings from mouse implantation experiments suggest that functional changes in the immune system have already occurred in the early stage of pregnancy when the developing embryo passes through the Fallopian tube. In support of this speculation, it was also reported that intraepithelial lymphocytes of human oviduct, which were identified as CD8-positive T lymphocytes, expressed both estrogen receptor-β and membrane progesterone receptor. The number of Ki-67- and estrogen receptor-positive intraepithelial lymphocytes fluctuated during menstrual cycle in the normal oviducts and significantly increased in tubal pregnancy oviducts, suggesting their involvement in immune reactions during early pregnancy (Ulziibat et al, 2006).

In order to accurately identify the embryo, the maternal immune system must distinguish non-self tissues belonging to the same species from those of other organisms. The immune system must also discriminate between developing embryos and unfertilized eggs. However, since the embryo is surrounded by the zona pellucida, immune cells cannot directly interact with the embryo in the Fallopian tube. Therefore, it is speculated that the developing embryo actively releases species-specific and embryo-specific factors into the Fallopian tube. However, it appears difficult for the embryo to produce a sufficient amount of such soluble factors to successfully activate the maternal immune system at such an early stage in its development.

Since it was shown that the sugar chains of the HCG effectively activate immune cells, we then paid attention to the zona pellucida that contains abundant glycoproteins. It is well known that the zona pellucida is composed of glycoproteins that mediate species-specific interaction between spermatozoa and oocytes (Florman and Ducibella, 2006). Accordingly, the zona pellucida can be considered an abundant store of species- and oocyte-specific glycoproteins. In addition, in contrast to unfertilized oocytes, the developing embryo actively degrades the zona pellucida. During fertilization, the zona pellucida of fertilized oocytes can be a target for acrosomal enzymes of sperm and cortical granules of oocytes (Oura and Toshimori, 1990; Tanii *et al*., 2001) and developing embryos further degrade the zona pellucida in order to achieve hatching. Thus, degradation products of zona pellucida glycoproteins may be released from fertilized oocytes/developing embryos into the Fallopian tube. Accordingly, we hypothesized that degradation products of zona pellucida glycoproteins can be an embryonal signal that transmits information about the presence of the developing embryo to the immune system in the female genital tract (Fujiwara *et al*., 2009).

Taken together, we propose that degraded products of zona pellucida glycoprotein and HCG are important candidates for embryo- and species-specific signals for maternal recognition by the immune system (Fig. 1).

5. Clinical application

As a growing clinical problem in reproductive medicine, increasing attention has been paid to repeated implantation failure in infertile patients who had undergone in vitro fertilization

(IVF) therapy. Unfortunately, no effective therapy had been developed. Based on our original findings, we developed a novel therapy using autologous PBMC. In this treatment, autologous PBMC are pre-incubated with HCG and then administrated into the uterine cavity prior to blastocyst transfer in order to induce endometrial differentiation that facilitates subsequent embryo implantation. We applied this therapy to patients who had experienced implantation failure in IVF therapy and found that PBMC treatment effectively improved pregnancy and implantation rates (Yoshioka *et al*., 2006). Several possible mechanisms relevant to this procedure can be demonstrated as follows. 1) PBMC may induce endometrial differentiation that facilitates embryo attachment. 2) Although PBMC are autologous cells from the patient, the induction of PBMC by themselves is expected to evoke favorable inflammatory reactions in the uterine cavity *in vivo*. 3) PBMC can secrete proteases that may effectively change the function or structure of surface molecules expressed on endometrial luminal epithelial cells. 4) PBMC can move from the uterine cavity toward the endometrial stromal tissue, creating a leading pathway for subsequent embryo attachment and invasion (Yoshioka *et al*., 2006; Fujiwara *et al*., 2009b).

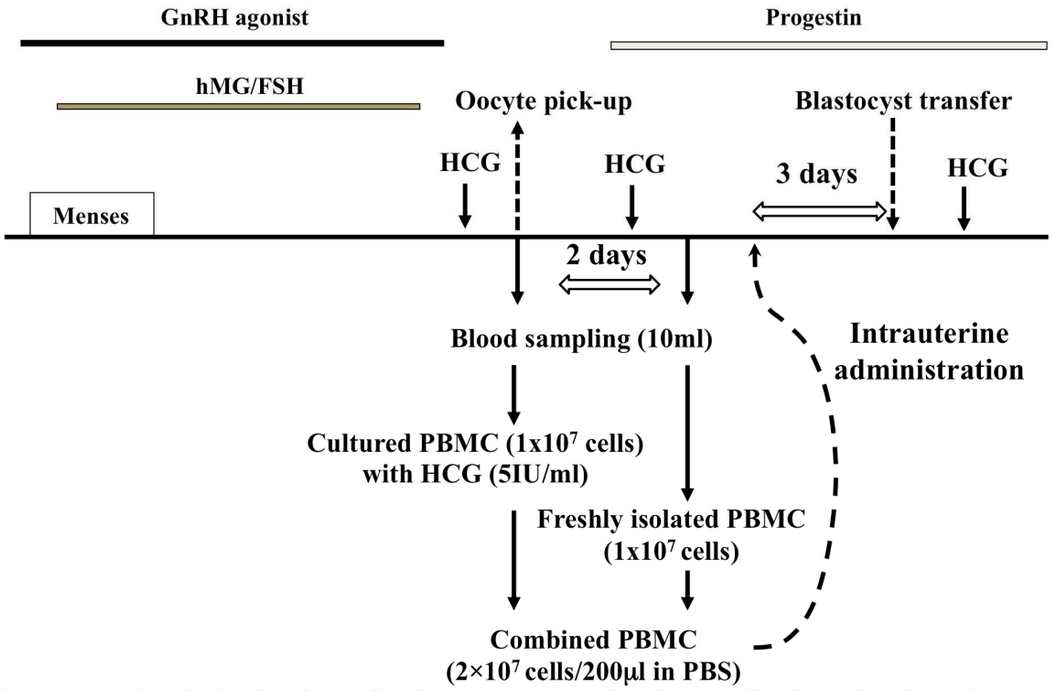

Fig. 3. Clinical protocol of PBMC treatment in IVF therapy

In our clinical protocol, PBMC are isolated at the time of oocyte pick-up, and activated with HCG. After 2-day incubation, PBMC are isolated again and the freshly collected PBMC are combined with the cultured PBMC and they are administrated into uterine cavity 3 days before blastocyst transfer.

In accordance with these clinical findings, Ideta et al. reported that administration of autologous PBMC in the uterine cavity increases pregnancy rates in bovine embryo transfer (Ideta et al., 2009). In addition, it was also observed that HCG-non-treated autologous PBMC were effective for patient with repeated implantation failures in IVF therapy

(Okitsu et al., 2011). These findings suggest that the application of autologous PBMC is an effective therapy for infertile patients suffering from repeated implantation failures.

6. Conclusion

In conclusion, we described a novel concept that immune cells receive information about the presence of a developing embryo and transmit this information through blood circulation to the whole body, inducing functional changes or differentiation in various organs, which facilitate human embryo implantation in cooperation with the endocrine system. Importantly, when the endocrine mechanism does not adequately operate, alternative mechanisms involving the immune system can be applied to infertility therapy. In the future, further clarification of the precise mechanisms for maternal recognition of the developing embryo by the immune system will contribute to our understanding the physiology of human embryo implantation and to developing more effective therapies using autologous immune cells along with further improvement in the breeding of domestic animals.

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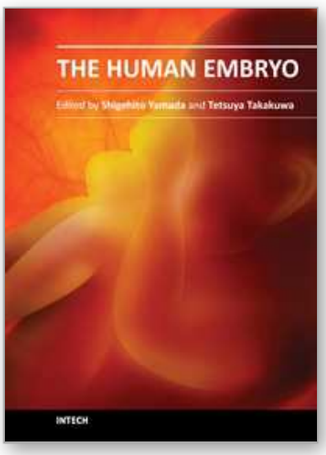

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