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Main actors behind the endometrial receptivity and successful implantation

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ABSTRACT

Embryo implantation occurs during a short period of time, the implantation window, in the mid-secretory phase of the menstrual cycle. The cross-talk between the endometrium and the embryo, at the stage of blastocyst, is a necessary condition for successful implantation. Till now, no single molecule or receptor has been identified to play an essential role on embryo implantation but a huge number of mediators, including cytokines, lipids, adhesion molecules, growth factors, and others, are reported to support the establishment of pregnancy.

Therefore, the aim of this review is not only to describe the different actors involved in the implantation process, but also to try to characterize the relationships between these factors as well as their time-regulated activation. Moreover, the availability of *in vitro* culture systems to study the interactions between embryo and endometrium as well as the paracrine communication regulated by exosomal vesicles will be investigated, as an innovative approach for a more precise characterization of the interactions between the different molecules involved in this process.

The in-depth knowledge of all these complex mechanisms will allow to address the reasons of implantation failure and infertility, thus providing new avenues for promoting the successful establishment of a pregnancy.

1. Introduction

Successful embryo implantation requires a receptive endometrium, a functional embryo at the blastocyst stage and a synchronized communication between the maternal tissue and the embryo. The endometrium undergoes a series of cyclic changes and remains receptive for a limited period of time, known as "implantation window", 6–8 days after the ovulation, maintaining the receptivity for almost four days (20-24th day of a regular cycle) (Bergh et al., 1992; Wang et al., 2017). The implantation process consists of three phases: (i) apposition, (ii) adhesion, and (iii) invasion (Bazer et al., 2009) (Fig. 1). During these stages, a large number of molecular mediators, coordinated by ovarian steroid hormones, are involved in the initial maternal-fetal interaction. These mediators include adhesion molecules, cytokines, growth factors, lipids and others (Fitzgerald et al., 2016; Zhao et al., 2010). Throughout the apposition, which occurs when the embryo stops to move freely inside the uterine cavity, embryo shows signs of polarity (Ebner et al., 2012). The trophoblast cells adhere to the luminal epithelium of the receptive endometrium, to proceed with the anchoring at the basal layer and extracellular matrix. At this point the adhesion takes place, characterized by a stable interaction between the trophectoderm and the epithelial cells of the endometrium. This interface is mediated by the adhesion molecules (Cellular Adhesion Molecules - CAM), expressed at the apical surface, like integrins, cadherins, selectins and immunoglobulins (Lessey et al., 1995; Lipari et al., 2008). The final step of the implantation process is the invasion: it consists of a progression of the trophoblast throughout the epithelial cells until the underlining stroma. This process is highly invasive and is mediated by proteases, that allow the anchoring of trophectoderm cells at this site and the access at the maternal vascular system for nutrition (Yamakage et al., 2020).

Endometrial pathologies such as endometriosis, endometrial fibromatosis, adenomyosis, chronic endometritis and congenital defect negatively impact the integrity and receptivity of the endometrium, as well as the implantation of the blastocyst (Bouet et al., 2016; Galliano et al., 2015; Luddi et al., 2020a, 2019a; McQueen et al., 2015). Recurrent implantation failure also represents the major limiting factor in the success rates of *in vitro* fertilization even though the clinical and technological advancement act in last years for improving assisted reproductive technology (Bashiri et al., 2018). For a long time, the reason behind this failure was attributed to the low quality of *in vitro* gametes and embryo, therefore, the researchers focus has been on the improvement of the quality of the oocyte and on the selection of the best embryo

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to transfer. However, embryo implantation remain the "black box" of reproductive medicine, and it is in fact, the main cause of infertility in healthy women (Lucas et al., 2020; Quenby et al., 2007) and cannot be excluded as a main reason of failure in an assisted reproductive cycle.

From here, takes rise the huge interest in studies related to the embryo implantation and the characterization of the endometrial role in this important interaction. In this review we will present the data, even though limited, obtained from animal models and *in vitro* models, in order to shed light to the bidirectional cross-talk between the endometrium and the embryo, necessary until the implantation takes place. The whole phenomenon is attributed to the paracrine signaling by extracellular vesicles and molecular pathways presented both by the embryo and the endometrium, with two main objectives, to find an optimal implantation site (about 95 % of implantation site is found at the end of the uterus) (Saravelos et al., 2016), and to reprogram the immunity system in such way to induce the immunological tolerance.

2. The role of the endometrium on implantation

Endometrium is formed by an epithelium which consists in a single layer of columnar cells, under which a stroma is constituted by abundant fibrillar connective tissue, with stromal components, fibroblasts and immune cells (Cornillie et al., 1985; Lawn et al., 1971). Functionally, endometrium is defined as a highly dynamic tissue, distinguished by a functional layer, which results in the implantation site of the embryo and is completely shed during menstruation, and a basal layer, which is crucial for the successful reconstruction of the epithelium after the menstrual cycle (Yamaguchi et al., 2021; Yoshimasa and Maruyama, 2021).

Estrogens and progesterone are the key hormonal modulators of these changes in the endometrium, preparing it for the implantation of the blastocyst (Ma et al., 2003; Wang et al., 2017). The two structural components of the endometrial mucosa, luminal and glandular epithelium with their surrounding stroma, express specific receptors for progesterone and estrogens. Under the influence of estrogens, produced by the granulosa cells of the ovarian follicles, the basal layer of the endometrium undergoes a quick development at the level of both glands and stroma (proliferative phase). Estrogen induces glandular proliferation by stimulating the stromal cells to produce growth factors, like the IGF1 and EGF, which act on the receptors expressed by epithelial tissue (Robertshaw et al., 2016). After the ovulation, the corpus luteum starts to produce progesterone and estrogens, stimulating the secretory activity of the endometrial glandular epithelium. Progesterone inhibits the proliferation of epithelial and stromal components, thus promoting the proliferation of the glands and modifying the stroma. It is interesting to highlight that an increase in the production of the progesterone brings a decrease in the expression of the estrogen receptors in the glands and in the stroma, decreasing the sensitivity of the endometrium to the estrogen produced by the ovaries (Wang et al., 2017). If the fertilization does not take place, the degeneration of the corpus luteum at the 24–28th day and the deep decrease of progesterone, bring to a shed of the functional layer, which marks the start of the menstrual cycle. In addition to the estrogens and progesterone, other hormonal modulators play a role in the regulation and maturation of the endometrium.

Even androstenedione and testosterone induce important morphological and functional changes having an impact on the implantation process (Kodaman and Taylor, 2004).

The main morphological modification that marks the endometrial receptivity is absolutely the presence of pinopodes, bleb-like structures at the apical surface of the endometrial epithelium, presenting different lengths, projected into the uterine lumen, just above the microvilli level of the surrounding cells. The adhesion of the blastocyst to the

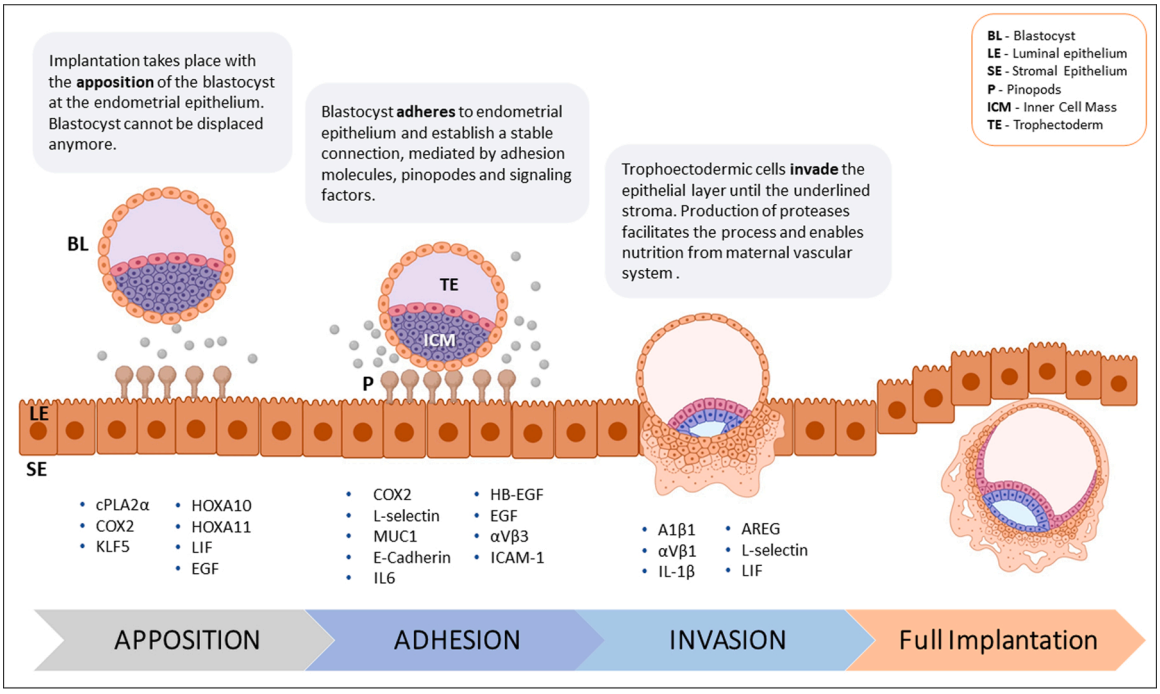

Fig. 1. Illustration of the three phases that take place during the embryo implantation process.(i) APPOSITION: the blastocyst contacts the implantation site of the endometrium, about 2-4 days after the morula enters the uterine cavity; (ii) ADHESION: the trophoblast cells of the blastocyst attach to the receptive endometrial epithelium; (iii) INVASION: the invasive trophoblast cells cross the endometrial epithelial basement membrane, invade the endometrial stroma and migrate into the maternal decidua.

Implantation occurs during the putative "implantation window", in which the maternal endometrium is ready to accept the blastocyst, which also plays a specific role. The process is complex and requires interplay of many molecules. (cPLA2α denotes cytosolic phospholipase A2α; COX2, cyclooxygenase-2; KLF5, Kruppel-like factor 5; HOXA 10/11, homeobox A 10/11; LIF, leukemia inhibitory factor; EGF, epidermal growth factor; MUC1, Mucin 1; IL6, interleukin 6; HB-EGF, Heparin-binding epidermal growth factor-like growth factor; αVβ3, integrin αVβ3; ICAM-1 Intercellular Adhesion Molecule 1; α1β1, integrin α1β1; αVβ1, integrin αVβ1; IL1β, interleukin 1 β; AREG, amphiregulin).

endometrium occurs at the top of the pinopods (Usadi et al., 2003). The presence of endometrial pinopodes is limited to a short period of time, corresponding to the implantation window and therefore they are identified as morphological markers for the endometrial receptivity (Adams et al., 2002).

3. Molecular mechanisms and main molecules involved in endometrial-embryo cross-talk

A successful implantation requires the establishment of a dynamic interaction, throughout the implantation window, between the blastocyst and the receptive endometrium. This interaction is orchestrated by paracrine signaling mediated by several key molecules and its alteration is often attributable to a suboptimal embryonic quality; alterations in this paracrine signaling can also adversely affect placentation and fetal development (Guzeloglu-Kayisli et al., 2009). The main molecules reported so far to have a role in embryo implantation are described in the following paragraphs.

3.1. Prostaglandins

Prostaglandins (PG) exert several responses in female reproductive tract. PG cascade has been evaluated in several patients with repeated implantation failure, showing alteration in expression of two major enzymes $cPLA_{2\alpha}$ (Phospolipase A2) and COX-2 (Cyclooxygenase 2). $cPLA_{2\alpha}$, expressed in both endometrium and blastocyst. Achace and coworkers demonstrated that 85 % of the patient experiencing repeated implantation failure, shown to have decreased levels of $cPLA_{2\alpha}$, as a result, reduced PG synthesis (Achache et al., 2010). Similarly, the inactivation of $cPLA_{2\alpha}$ in mice, exhibited inappropriate mechanism responsible for the spacing of implanting blastocysts in uterus, that were reversed by PG supplementation (Song et al., 2002). Concordantly, PLA secreted by the embryo activates CXCR4 as a result of P13 K/ERK1/2 pathway, involved in early embryo implantation (Banerjee et al., 2009). Elevated expression of CXCR4 were demonstrated in endometrial stromal cells throughout the implantation window (Dominguez et al., 2003), so it is hypothesized that apposition, adhesion and invasion are influenced by PGE2 throughout CXCR4.

COX-2 enzyme mediates the conversion of arachidonic acid to PGI2, claiming this way it is role in PG signaling pathway. PGI2 is the most secreted prostaglandin at the implantation site, exerting vasoactive properties and facilitating the initial interaction between blastocyst and luminal epithelium. The time specific expression levels of the inducible COX2 has a key role in fertilization, implantation and decidualization in mice (Lim et al., 1997). Interestingly, this enzyme also interacts with different phospholipids and lysophospholipids involved in a wide range of cellular processes (Ye et al., 2005).

3.2. Transcription factors

Krüppel-like factors (KLFs) are zinc finger-containing transcription factors with diverse biological functions, including proliferation, differentiation, apoptosis and development. KLF5 shows a distinct expression throughout the implantation window, during the first days of pregnancy iis present in luminal and glandular epithelium, while move to stromal cells at the start of the attachment. Sun et al. (2012) demonstrated the essential role of KLF5 in embryo implantation as a result of alteration of the apoptosis process in luminal epithelium (Sun et al., 2012). Infertility in KLF5 null mice, comes as a consequence of a failure of the degeneration of the epithelium at the apposition site. (Sun et al., 2012). Moreover, the levels of COX2 were also absent in the KLF5 inactivated mice, highlighting the pivotal role of this enzyme in numerous pathways in this process.

Another family of transcription factors, HomeBox containing transcription factors (HOX) have been studied for their role in early pregnancy. Expression of HOXA10 in epithelial and stromal cells increase at the implantation window (Satokata et al., 1995). This transcription factor mediates stromal cell proliferation, by influencing the responsiveness of these cells to progesterone; indeed, HOXA $10^{-}/^{-}$ mice showed impaired responsiveness of stromal cells to this hormone (Lim et al., 1999). Therefore, the inactivation of this factor causes implantation failure. Despite the fact that estrogen levels were unaffected by alteration in HOXA10 expressions, it was observed a reduced number pinopodes at the surface of endometrial epithelium, confirming this transcription factor deep involved in uterine receptivity (Bagot et al., 2001).

Expression levels of HOXA11, another homeobox gene, reach a peak at implantation time in stromal cells (Gendron et al., 1997). Deletions of this gene induce an alteration in stromal cell proliferation and Leukemia Inhibitory Factor (LIF)expression in response to ovarian steroids.

3.3. Adhesion molecules

The major groups of Cellular Adhesion Molecules (CAMs) involved in the embryo-endometrium crosstalk are integrins, cadherins, selectins, immunoglobulins and mucins. These surface ligands are mostly glycoproteins that mediate the adhesion between cells. Their main functions are the maintenance of the structural integrity of the tissue, cellular migration and tumor metastasis.

3.3.1. Integrins

Integrins are transmembrane glycoproteins with a heterodimeric structure formed by two subunits, α and β, involved in cell-matrix adhesion, cell-cell adhesion and other physiological processes like embryo development, hemostasis, thrombosis, wound healing, immunological defense mechanisms and oncogenic transformation. (Lustig and Denduchis, 1993). Different types of integrins are expressed by the endometrium in correspondence of the implantation window, especially from the surface of the luminal endometrial epithelium, which interacts at first with the trophoblast. In this regard it is claimed that several integrins act as important endometrial receptors for the adhesion of the blastocyst (Lessey et al., 1992). Integrin alpha V beta 3 (αvβ3) has been described as a biomarker of endometrial receptivity, appearing at the endometrial epithelial cells at the time of the implantation window. αvβ3 binds to osteopontin, a ligand expressed both by the trophoblast and the endometrium (He et al., 2016). This interaction is highly impacted by steroid hormones and growth factors; osteopontin levels are dependent on progesterone, whereas αvβ3 expression is down-regulated by E2 and induced by growth factors (Apparao et al., 2001). Meanwhile, it was been shown that the integrins α1β1 and αvβ1 are also expressed from the trophoblast cells at the time of implantation and their expression increases analogously to the differentiation of the cytotrophoblast cells towards extravillous phenotype (Achache and Revel, 2006; Wang and Armant, 2002). It was hypothesized that integrins of the endometrial epithelium and those expressed by the trophoblast are connected to specific components of the extracellular matrix, allowing the embryo adhesion according to a “sandwich” model (Achache and Revel, 2006).

3.3.2. Selectins

Selectins transmembrane glycoproteins, part of the CAM family, include P-selectin, E-selectin and L-selectin (Smalley and Ley, 2005). Selectins interacting to their ligands mediates the migration of trans-endothelial leucocytes, a process known as leucocytes ‘rolling’. This process resembles the apposition of the blastocyst at the maternal endometrium, that is mediated by L-selectin as well. The interaction between L-selectin, expressed by trophoblast cells and the respective oligosaccharides ligands, expressed by receptive endometrium during the implantation window can guide the blastocyst to adhere to an optimal implantation site (Fazleabas and Kim, 2003).

3.3.3. Cadherins

Cadherins are a group of transmembrane glycoproteins, responsible for the mechanism of calcium-dependent homophilic cell adhesion. The expression of E-cadherins, localized at the adherent junctions between the plasma membrane of the epithelial cells, significantly increases during the luteal phase. Progesterone, acts indirectly on the expression of the cadherins by regulating calcitonin levels. Calcitonin is described as a modulator of the endometrial receptivity. By increasing the intracellular calcium, adhesiveness and polarity are altered as a consequence of CAM redistribution. *In vitro* evidences confirmed the correlation between the rise of the intracellular calcium and the suppression of E-Cadherin (Niessen et al., 2011). These results, affirm the double role of Cadherins, which maintain adhesion between epithelial cells prior to implantation while, at the time of implantation, their under-expression allows separation of epithelial cells (Fukuda and Sugihara, 2012; Li et al., 2002).

3.3.4. Immunoglobulins

The immunoglobulin superfamily is a large protein superfamily of CAMs. They are cell surface and soluble proteins, involved in the recognition, binding, or adhesion processes. Intercellular adhesion molecule-1 (ICAM-1) mediates the cell-cell adhesion acting as a ligand for the β2 integrins. This cellular interaction plays a major role on the transendothelial migration of leukocytes, cells that in physiological conditions play a part on decidualization. ICAM-1 is localized to the apical surface of the luminal and glandular epithelium, also in the membrane of stromal cells, during the whole menstrual cycle (Thomson et al., 1999). The expression of ICAM-1 both in epithelium and stroma demonstrates the significant role it plays in physiopathology of the endometrium (Defrère et al., 2005). Basigin, also known as CD147, is expressed at the lateral epithelial surface and has a significant role on implantation, throughout the regulation of matrix metalloproteinases activity, which contribute to the remodeling of the uterus lining. Studies performed on animal models, confirmed an alteration on implantation rates in bsg(-/-) mice (Chen et al., 2009; Igakura et al., 1998).

3.3.5. Mucins

Mucins are glycoproteins with a high molecular weight, abundant in mucous secretion. When it is highly expressed by the cell surface, MUC-1 interferes with the cell-cell adhesion and cell-matrix adhesion, generated by sterically hindering. In the endometrium, MUC-1 is extended beyond the glycocalyx and is probably the first molecule interacting with the embryo, pushing it in such a way, in order to find the correct implantation site. High levels of progesterone probably reduce the endometrial expression of MUC-1, facilitating the interaction with the embryo. MUC-1 results to be overexpressed at endometrial levels during the time that passes from the proliferative to the intermediate secretory phase (Marzieh et al., 2009). This represents a paradox considering the anti-adhesion function of MUC-1, but at the contrary, it presents evidences regarding the existence of local agent mechanism to remove the barriers of the MUC-1 in order to guarantee the embryo implantation (Thathiah and Carson, 2004). It was in fact, demonstrated that MUC-1 is not present at the surface of pinopodes, the elective site for the blastocyst implantation (Horne et al., 2002). Tumor Necrosis Factor-α (TNFα), a proinflammatory cytochine secreted by the endometrium and the blastocyst, increases the expression of ADAM-17 (A Disintegrin and Metalloprotease 17), a transmembrane zinc protease part of the Adamalysine superfamily, which plays a role in the local removal of the MUC-1 from the implantation site. MUC-1 expression was studied in women with recurrent pregnancy loss and compared to the control group of fertile women., resulting down-regulated in women experiencing recurrent pregnancy lost; therefore it was supposed that levels beneath a cut-off value may alter the immune system and affect the endometrial function during the implantation, therefore impacting the latter one (Bastu et al., 2015).

3.4. Cytokines

Cytokines include a group of proteins which modulate several cellular functions, like cellular proliferation and differentiation. They play an important role in regenerative and inflammatory–like processes which are verified at every menstrual cycle in human endometrium and are implicated also in specific events like reproduction, ovulation and implantation (Guzeloglu-Kayisli et al., 2009).

3.4.1. LIF

Leukemia inhibitory factor is a cytokine, secreted by the luminal and glandular epithelium, as well as stromal cells. The secretion of LIF is gradually increased from the 18th to the 28th day of the menstrual cycle, having its peak at the 20th day.(Chen et al., 1995) IL-1α, TNF, Platelet-derived Growth Factor (PDGF), TGFβ, activin A are all potential inducers of LIF, with exception of IFNα which inhibits the secretion (Warshamana et al., 2001). The increased expression of LIF from the decidual leukocytes leads to a hypothesized interaction between the leukocytes of the maternal decidua and the trophoblast cells in invasive phase (Charnock-Jones et al., 1994). The embryo can also induce the secretion of LIF by the release of precocious mediators, in a dose-dependent manner (d'Hauterive et al., 2005). Although it is known that LIF has an important role in implantation process, its mechanism is still unknown. LIF activates the Jak/Stat signal transduction pathway, therefore phosphorylating Stat3, member of the Stat family. Inhibition of Stat3 phosphorylation in mice identified a critical role of this peptide, which affected the expression of LIF-regulated genes such as *Irg1*, a lipopolysaccharide-inducible gene, essential for embryo attachment (Catalano et al., 2005).

3.4.2. IL1

Interleukin 1 is a key mediator in the immunological and inflammatory responses, whose importance is verified by the capacity of this molecule to induce the increase of the β3 integrin, necessary to promote the implantation of the blastocyst (Simon, 1997). Although its production by the stromal and glandular cells continues throughout the whole menstrual cycle with its peak at the luteal phase, the significant diminution of the endometrial expression of IL1 during the implantation window suggests the presence of specific regulatory mechanisms which, by the inhibition of the IL-1 antagonist, promotes the pro-implantation action of IL1 (Boucher et al., 2001).

3.4.3. IL6

Interleukin 6 is a pleiotropic cytokine, whose endometrial levels rise progressively during the intermediate secretory phase, followed by a decline in the late one. The receptor of IL6 is expressed both by blastocyst and the endometrium, under the influence of IL1β which induces the expression in a time/dose dependent manner, demonstrating an autocrine/paracrine role of this cytokine in the implantation event (Achache and Revel, 2006).

3.5. Chemokines

Chemokines are a superfamily of low molecular weight proteins, structurally and functionally related to cytokines, sharing their ability to induce direct chemotaxis in nearby responsive cells. They interact with specific receptors expressed at leukocytes membranes, increasing their adhesiveness, promoting this way the transendothelial migration (Graves and Jiang, 1995). The stromal cells of human endometrium, during the intermediate secretory phase, produce distinct chemokines, among which Macrophage-derived Chemokine (MDC), fractalkina, Macrophage Inflammatory Protein 1β, fundamental for the recruitment of the Natural Killer (NK) cells and macrophages, characteristic components of the decidua (Jones et al., 2004; Kitaya et al., 2003; Watanabe et al., 2006). NK cells start to infiltrate the endometrium at the LH + 3 day, surrounding the spiral arteries and the decidualized stroma,

creating an immunologically favorable environment for the blastocyst invasion (Moffett-King et al., 2002). The receptors for the CCR2B and CCR5 chemokines were identified at the trophoectoderm and endometrial epithelium during the implantation window (Dominguez et al., 2003). As a conclusion, chemokines, released in uterine lumen or expressed by the endometrial glandular epithelium, play an important role in the apposition and adhesion phases of the blastocyst.

3.6. Growth factors

The superfamily of Transforming Growth Factors β (TGF- β), include a large number of proteins with an important role in several cellular process, like cellular differentiation, neural growth, bone morphogenesis, wound healing, reproductive function, motility, adhesion and cellular development (Gordon and Blobe, 2008). In human endometrium, TGFβ is expressed both by glandular epithelium and stroma, reaching elevated levels at a late secretory phase (Kane et al., 2008; Lu et al., 2021; Ni and Li, 2017). It has an indirect role in implantation by the induction of other growth factors, like Vascular Endothelial Growth Factor (VEGF), Insulin Growth Factor-1 (IGF-1) and Epidermal Growth Factor (EGF), or by the induction of adhesion of trophoblastic cells at the extracellular matrix (Guzeloglu-Kayisli et al., 2009). Among EGF family, amphiregulin (AREG) and heparin-binding EGF-like growth factor (HB-EGF) expression indicates a role in uterine receptivity. *AREG* has been identified as a specific gene for the implantation process in the uterus. The expression on *AREG*-mRNA is dispersed throughout the glandular and luminal epithelial cells before the implantation takes place specifically when the blastocyst attaches to the endometrium. In fact amphiregulin become highly abundant in the luminal epithelium surrounding the blastocyst and slightly reduced far from the implantation site (Das et al., 1995). HB-EGF, expressed both on epithelial and stromal cells, is highly dependent by estrogen and progesterone. HB-EGF paracrine signaling regulates the expression of endometrial proteins with a relevant role on implantation. Furthermore, HB-EGF not only acts as an active growth factor, but also as a juxtacrine factor, mediating the adhesion between the blastocyst and surface epithelium (Lessey et al., 2002).

4. Role of immune system in implantation

The prerogative of the immune system is the recognition and the control towards the nonself. The embryo is considered a semi-allograft, since 50 % of its genome is paternally derived; so it is vulnerable to the attack from the maternal immune system. Therefore, an immunologic tolerance is necessary to prevent a precocious rejection of the blastocyst (Piccinni, 2010). It is estimated that in over 20 % of the couples experiencing idiopathic infertility, the implantation failure is attributed to immune system alterations (Ali et al., 2018; Murata et al., 2021).

Cytochines homeostasis in the female reproductive tract, is crucial for the induction of an immunologic tolerance (Piccinni et al., 2016). The recruitment at the implantation site, of adequately active immunological cells like the Natural Killer (NK) cells, T regulators lymphocytes (T*reg*) and Th17/Th2 lymphocytes have a relevant role in immune tolerance. Even before the apposition of the embryo at the epithelial surface, the immune cells modulate their proper function in order to allow the successive adhesion and invasion of the underlining stroma (Saito et al., 2010; Trowsdale and Betz, 2006).

T helper lymphocytes (CD4+) can be classified in Th1, Th1, Th17 and T*reg*, according to the cytokines released by them. Lymphocytes Th17 and Treg alternate their phenotype in response to the modification of the environmental conditions, meanwhile the differentiation into lymphocytes Th1 and Th2 is an irreversible process (Zhou et al., 2009).

Th1 lymphocytes produce mainly pro-inflammatory cytokines, like IL2, TNFβ and INFy, while the population of the Th2 lymphocytes produces anti-inflammatory cytokines, like IL4, IL13 and other important cytokines to promote the lymphocyte B development, like IL5. It has been proved that the cytokines like Th1, which promote the immune system rejection, can be harmful for the pregnancy, meanwhile those as Th2, which has an inhibitory effect on Th1 response, can promote the tolerance to the semi-allogeneic fetus, allowing this way the pregnancy to take place (Zabrodskii et al., 2007) (Piccinni et al., 2015).

Lymphocytes Th17, similar to Th1 cells, seem to have a role in the tissue rejection. Recent studies have shown that the number of CD4+ cells that produce IL17 is significantly higher in women with recurrent miscarriage in respect to the women undergoing a normal pregnancy (Wang et al., 2010). Interestingly. in normal pregnancies, high levels of the T CD4+ cells producing both IL4 and IL17A have been reported, while in case of recurrent abortion dominate the CD4+ that secrete only IL17A or a combination of IFNγ with IL17a. Therefore, it appears that the combination of IL17A with IL4 is beneficial in order to maintain pregnancy, meanwhile IL17A with IFNγ, or the production of IL17A can induce precocious fetal rejection (Nakashima et al., 2010).

In Fallopian tubes, far away from the implantation site, can be observed a predominance of the Th17 and Th17/Th1 cells, meanwhile the Th17/Th2 are located only in at the implantation site (Cosmi et al., 2010). These data suggest that lymphocytes Th17/Th2 have a role in implantation, in the vital immune tolerance and in guaranteeing the protection from extracellular pathogens, potentially responsible for spontaneous abortion.

Progesterone is a potential inductor in the production of IL4 and IL17A from CD4+ lymphocytes. Another component that can induce the production of IL4 is HLA-G5, which is secreted by extravillous cytotrophoblast. HLAG5 can be responsible for the switch from T CD4+ cells into Th17/Th2 cells, necessary for the successful implantation (Logiodice et al., 2019)(Lombardelli et al., 2013; Piccinni et al., 1995).

Treg lymphocytes are anti-inflammatory agents with immunosuppressive function. They inhibit the proliferation of T cells and the production of cytokines, suppress the proliferation of B cells and the production of antibodies. Furthermore, they inhibit the cross-presentation of antigens by the dendritic cells and the maturation and activation of macrophages and lastly, inhibit the cytotoxicity of NK cells (Ali et al., 2018; Murata et al., 2021). Treg cells start to accumulate in uterus at the moment of ovulation, in response to a rise in estrogen levels, which results in chemotaxis by the chemokines. Meanwhile, the increased levels of progesterone in the luteal phase not only induces the conversion of the T cells in Treg but at the same time induces the immunosuppressive function of Treg (Mao et al., 2010).

T*reg* lymphocytes interact with other leucocytes inside the endometrium, including the APC (antigens presenting cells) like macrophages and dendritic cells. These cells are highly expressed by the endometrial stroma and uterine myometrium (Schulke et al., 2008). A rise in the estrogen levels stimulates the surge of macrophages in uterus. After the implantation of the blastocyst, their percentage increases from 15 % to 20 % of the overall stromal components. Uterine macrophages are implemented in local defensive action and tissue remodeling. Once activated, they produce immunosuppressive cytokines, like TGFβ and IL10, which are essential for the immune tolerance induction at the implantation site. Moreover, they contribute on the trophoblast invasion and placental development (Renaud and Graham, 2008).

The NK cells are also involved into the complex system of the immunological cells present at the maternal-fetal interface. Other than having a key role in defense mechanisms, they are involved in tissue remodeling and endometrial receptivity. The ones localized in uterine stroma express a unique phenotype which varies throughout the menstrual cycle. Progesterone plays an important role on the NK cells (King et al., 2010).

5. Role of extracellular vesicles in embryo-endometrial interactions

Extracellular vesicles (EVs) have become a field of interest for the scientific community, with a large focus on their role in intercellular

communication. The communication between different cell types is generally maintained through secretory soluble molecules, but in the last years data from literature indicate that EVs produced by cells are important actors in this process (Raposo and Stoorvogel, 2013). In fact, EVs have been observed to transfer information to other cells, by well-characterized molecules, regulating cellular activities of the target cells.

EVs are nano-sized vesicles, membrane-enclosed, classified as exosomes, microvesicles and apoptotic bodies according to their sizes, biogenesis and secretion. EVs are released in the extracellular microenvironment by all kind of cells, which can transport a variety of bioactive molecules like lipids, proteins, DNA and RNAs (Andronico et al., 2019).

EVs can be released by the endometrial epithelium into the uterine cavity, so they can transfer specific information to the trophectodermal cells or to endometrial epithelial cells, contributing this way to the cross-talk between the endometrium and the embryo, essential to the implantation process (Andronico et al., 2019; Gurung et al., 2020; Mishra et al., 2021). Studies of last years have identified the presence of exosomes at the level of apical surface of the epithelial endometrial cells throughout the whole menstrual cycle (Salamonsen et al., 2009). Future studies can extend our knowledge regarding the importance of the exosome specific micro (mi)RNA, released into the uterine cavity by a receptive endometrium.

Endometrial EVs have been isolated from different biological sources as uterine flushes, mucus, cultured endometrial epithelial cells, stromal and mesenchymal cells, from human and other animal species. Almost all the data on endometrial EVs are derived from *in vitro* cultured cells. EVs from endometrial epithelial cells can increase adhesion/invasion of the blastocyst and promote embryo development. EVs from stromal/decidual cells promote the angiogenesis and increase the invasion. EVs from embryonic trophoblast cells are able to modulate the immune system and increase embryo attachment. EVs from endometrial mesenchymal stromal cells can increase blastomere division, and promote endometrial angiogenesis and vascularization (Mishra et al., 2020)

The possibility of using EVs recovered from human uterine flushes and from a cervical brush, as surrogates for invasive endometrial biopsies, was reported by Luddi in 2019. Starting with a reliable protocol for EVs isolation, with subsequent characterization of isolated vesicles, the author demonstrated the presence of specific mRNAs, known to be expressed in the endometrium. EVs showed the presence of typical endometrial markers *PAEP*, *ESR1*, and *PGR* mRNA (Luddi et al., 2019b). In particular, glycodeline A (GdA), encoded from *PAEP* gene, is a glycoprotein secreted specifically from the endometrial glands and decidual glandular epithelium, expressed in a cycle-dependent manner (Focarelli et al., 2018).

The knowledge of endometrial EVs is still largely unknown and need to be more investigated. These studies will be crucial in the discovery of other undiscovered aspects of the fetal and maternal cross-talk, optimizing this way our capacities on infertility treatments and increasing the success rate of the implantation in Assisted reproductive technology (ART).

6. New *in vitro* model to study embryo implantation

The knowledge of the human implantation process is compromised by both ethical issues, that not allow to study this process *in vivo*, and by the accuracy and reproducibility of *in vitro* models of human endometrium. Effective and reliable embryo implantation model is necessary in order to mimic the molecular event cascade that occurs *in vivo*; many steps were done towards a model that is reliable and reproducible. Despite the enormous importance of *in vivo* models and the fact that they represent an invaluable asset for many aspects, these are laborious and expensive and do not yet address the human translational medicine issues represented by interspecies variability. Human *in vitro* models have been successfully developed by many groups with the goal of obtaining effective tools to explore the complexity of this process (Rosner et al., 2021; Stern-Tal et al., 2020; Teklenburg et al., 2010; Weimar et al., 2013).

For these reasons human primary cells exhibit a great homology with the real *in vivo* condition resulting in a suitable model to use. In fact, they allow to evaluate the role of embryo secretome in modifying the molecular profile in endometrial organoids mimicking the implantation window, for example to test if the use of embryo-conditioned culture medium at the time of the embryo transfer may increase *in vitro* fertilization outcome (Luddi et al., 2021).

6.1. Monolayer culture cells

Endometrial stromal and epithelial cells have both been employed to study in a two-dimensional (2D) environment the early feto-maternal interactions since 1985 (Lindenberg et al., 1985) and trophoblast spheroids were added to mimic human blastocyst to investigate molecular events beyond the luminal epithelium-embryo attachment and endometrium dysfunction in reproductive failure (Aplin and Ruane, 2017; Huang et al., 2017; Lee et al., 2015; Weimar et al., 2013). Decidualized stromal endometrial cells were found to be selectively migrating towards high quality human embryos and negatively affected by the presence of poor-quality embryos or conditioned media in term of implantation associated genes (Berkhout et al., 2018; Brosens et al., 2014; Teklenburg et al., 2010). Monolayer cell culture is a simple and reliable model although have the disadvantage to lack of resident immune cells environment and tri-dimensional (3D) architecture.

6.2. Co-cultures

Firsts approaches to a 3D culture model, was to seed stromal and endothelial cells on hydrophilic polymer as a scaffold used to resemble the structural properties of the extracellular matrix (Bentin-Ley et al., 1994; Evron et al., 2011). To study trophoblast invasion transwell-co culture approaches were employed, seeding endometrial epithelial cells on top of the Matrigel TM -coated insert prior to adding throphobast cells and stromal cells (Arnold et al., 2001; Gellersen et al., 2010; Pierro et al., 2001). However, these models lack of glandular structures, necessary to maximally reproduce the physiological environment surrounding the implantation.

6.3. Organoids

Endometrial Organoids (EOs) are self-organized 3D-tissue cultures derived from endometrial biopsies. First mention of organoids-like structure was in 1988 by Rinehart (Rinehart et al., 1988). Recently, organoids protocols were established to generate and comprehensively analyze functions of 3D endometrial epithelial organoids (EEOs) (Boretto et al., 2017; Turco et al., 2017).

Furthermore, these studies demonstrated that, EEOs also exhibit physiological hormone responsiveness showing that these can retain intrinsic properties of the diseases and resemble human endometrium when undergoes extensive remodeling during the menstrual cycle that is chiefly regulated by the ovarian steroid hormones estradiol and progesterone (Boretto et al., 2017; Fitzgerald et al., 2019; Luddi et al., 2020b; Turco et al., 2017).

Single-cell RNA sequencing analysis of organoids reported a stem cell population that decreased when they were treated with estrogen, progesterone and cyclic AMP (cAMP) (Fitzgerald et al., 2019). To investigate whether these markers are specific for cells with stem cell/progenitor potential, reporter organoid lines for these genes could be generated using gene editing approaches, using the CRISPR/Cas9 technology (Artegiani et al., 2020). These genetically modified organoid lines would allow the investigation of pathways regulating proliferation/regeneration and perhaps interaction with embryos.

A major step forward in the generation of more complex *in vitro*

models using an animal mode of reproductive tissues has been the application of microfluidic technologies (Xiao et al., 2017).

This 'Multi-Organ' microfluidic system of the human menstrual cycle over 28 days incorporate explants of the murine ovary, human fallopian tube, uterus, cervix and liver with steroid hormones released from ovarian follicles. Another approach is a dual reproductive organ-on-a-chip system that allows the bidirectional crosstalk between the ovaries and the endometrium. This model recapitulates the multicellular complexity of both tissues as the ovarian compartment contains granulosa and theca cells, whilst the endometrial compartment includes fibroblasts, vascular epithelial cells, immune cells, and endometrial stem cells (Park et al., 2020).

Recent advances were made with the establishment of endometrial *assembloids*, incorporating stromal cells (Rawlings et al., 2021) based on their single cell transcriptomic analysis these assembloids contains several subpopulation of both stromal and epithelial cells that secrete implantation factors. Therefore, organoids and assembloids cultures represent a powerful tool for precision medicine.

7. Conclusion and future perspectives

Endometrial receptivity during the implantation window requires a complex interaction by multiple agents. Even though a lot of these factors are identified, their role remains still unclear. These limitations depend on the ethical implication which inhibit the research of these processes *in vivo* and on the shortage of *in vitro* models to study this process. The major part of the data comes from animal models or from *in vitro* cultured cells which contribute to a limited amount of information in confront of the real complex role that these agents play *in vivo*.

The comprehension of the mechanisms that regulate the implantation remains a requisite in order to achieve better results regarding the treatments of female infertility. Only by understanding and optimizing these complex machinery involved, will be possible to target the pathway involved in embryo implantation that implicate the infertility.

In conclusion, by an accurate and detailed identification of the implantation markers, due to the latest techniques of molecular medicine, will be possible, in the future, to identify the unknown obstacles of implantation and predict a positive pregnancy outcome. The future perspective to use the precision medicine and to build up a patient-specific biobank of implantation markers will represent a great opportunity for each woman.

Author contributions

Laura Governini, Francesca P. Luongo, Alesandro Haxhiu: Investigation; Writing - original draft. Alice Luddi, Paola Piomboni: Conceptualization; Supervision, Writing - review & editing.

Declaration of Competing Interest

We have no conflict of interest.

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