International Journal of
Molecular Sciences

MDPI

Review

Molecular signaling regulating endometrium-blastocyst crosstalk

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Received: date; Accepted: date; Published: date

Abstract: Implantation of the embryo into the uterine endometrium is one of the most finely regulated processes that leads to the establishment of a successful pregnancy. A plethora of factors are released in a time-specific fashion to synchronize the differentiation program of both the embryo and the endometrium. Indeed, blastocyst implantation in the uterus occurs in a limited time-frame called the "window of implantation" (WOI), during which the maternal endometrium undergoes dramatic changes, collectively called "decidualization". Decidualization is guided not just by maternal factors (e.g. oestrogen, progesterone, thyroid hormone), but also by molecules secreted by the embryo, such as chorionic gonadotropin (CG) and interleukin-1β (IL-1β), just to cite few. Similarly, once reached the uterine cavity, the embryo orients correctly toward the uterine epithelium, interacts with specialized structures, called uterodomes, and begins the process of adhesion and invasion; all these events are guided by factors secreted by both the endometrium and the embryo, such as leukaemia inhibitory factor (LIF), integrins and their ligands, adhesion molecules, Notch family members, metalloproteinases and their inhibitors. Aim of this review is to give an overview of the factors and mechanisms regulating implantation, with a focus on those involved in the complex dialogue between the blastocyst and the endometrium.

Keywords: implantation; endometrium; blastocyst; embryo; chorionic gonadotropin; progesterone; Notch; cytokines

1. Introduction

Infertility is considered a pathological condition of the reproductive system. The WHO has designated infertility as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" [1,2]. Infertility is one of the main health issues in all societies worldwide, with a prevalence of 3.5-16.7% in developed countries and 6.9-9.3% in developing countries [3,4]. Causes of infertility may be various. Male infertility is responsible for 20–30% of cases, while 20–35% of cases are due to female infertility, and 25–40% are due to combined problems in both partners [5]. In 10–20% of cases, infertility is unexplained [5]. Regarding the female, causes of infertility are diverse, such as lack of regular ovulation, blocked or damaged fallopian tubes, endometriosis, and endometrial problems [6]. This last situation leads to defects in blastocyst implantation in the maternal uterus, causing implantation failure, which is a common cause of impaired fertility [7]. The term "implantation failure" refers to the lack of implantation after the transfer of good quality embryos, following assisted reproduction techniques (ARTs). However, the term "implantation failure" actually implies a series of conditions

in which the embryo does not implant in the maternal endometrium after both spontaneous and *in vitro* fertilization [8]. In spontaneous conception 30% of pregnancies are lost before implantation [7]. On this premise, it is conceivable that implantation is a rather inefficient process, also considering that the estimated implantation rate in humans is 30% per cycle [9,10].

The inefficiency in blastocyst implantation may be explained by the fact that implantation is a complex process involving the simultaneous development of an embryo able to implant and an endometrium able to respond to embryonic signals. Implantation is defined as the process by which the floating blastocyst adheres to the endometrium and invades the stroma, leading to the formation of the placenta. Implantation requires a complex crosstalk between the endometrium and the blastocyst, which is highly regulated by a variety of factors, such as soluble growth factors, hormones, prostaglandins, adhesion molecules and the extracellular matrix (ECM) [11-15]. These factors, produced by the receptive endometrium in response to the presence of the blastocyst and viceversa, are able to synchronize the development of the embryo to the blastocyst stage and differentiation of the uterus to the receptive state [16,17].

The present review describes and discusses the molecular mechanisms underlying the implantation process, focusing on factors implicated in the complex blastocyst-endometrium crosstalk, which are crucial for successful implantation. Further research for new factors involved in the dialogue between the blastocyst and the endometrium would allow to reduce the current rates of implantation failure, allowing many couples with infertility problems to reach a successful pregnancy.

2. Preparation of the endometrium to implantation

Interaction between the uterus and the blastocyst can only occur during a limited defined period, known as the "window of implantation" (WOI) [18-20]. In humans, this defined period corresponds to the mid-secretory phase, occurring between the 20th and the 24th day of the menstrual cycle, or 6-10 days after the luteinizing hormone (LH) peak [18,21-23]. In this timeframe, the molecular program regulating growth and differentiation of the embryo synchronizes with the molecular program regulating endometrial receptivity. Failure in such synchronization results in failure of the blastocyst to implant. Given the relevance of this stage for the establishment of a successful pregnancy, the WOI is regulated by a wide variety of cytokines, growth factors, prostaglandins, enzymes and adhesion molecules [24-26].

During the WOI, the uterine endometrium is affected by changes which allow blastocyst implantation [27]. The epithelial cells present vacuoles to a supranuclear position and glands become more irregular with a papillary appearance, but the major changes take place in the stroma. The endometrial stromal cells undergo the decidual reaction, in which they proliferate and differentiate from fibroblast-like to epithelial-like cells, which will form the maternal decidua. Decidual cells progressively increase in size and number throughout pregnancy, starting from 9.8% of stromal cells in early pregnancy and arrive to 57.8% at term [28]. The acquisition of the epithelial-like phenotype by stromal cells consists of an increase in size, rounding of the nucleus with an increase in number of nucleoli, accumulation of glycogen, lipid droplets and secretory granules in cytoplasm, and expansion of rough endoplasmic reticulum and Golgi apparatus [29]. The term "decidua" derives from Latin "de cadere" and means to fall down, so it refers to the fact that the decidualized uterine tissue is lost after parturition. Decidua is mainly formed by decidualized endometrial stromal cells, but also contains hematopoietic cells, macrophages, uterine natural killer and monocytes [30,31]. Decidualization starts in the luteal phase, with stromal cells surrounding the spiral arteries in the upper two-thirds of the endometrium, regardless of whether or not the blastocyst is present [32]. Differently from most mammals, decidualization in humans occurs before the embryo reaches the uterine cavity and is driven by the postovulatory rise in progesterone levels and local increase of cyclic adenosine monophosphate (cAMP) production, occurring way before the embryo is ready to implant. In the absence of pregnancy, progesterone levels decrease, and menstrual shedding and cyclic regeneration of the endometrium occur. Decidualization is responsible for embryo quality

control, promoting implantation and development, or facilitating early rejection in case, for example, of chromosomally abnormal human embryos [33].

Estrogen and progesterone guide the structural and functional remodeling occurring during decidualization. The estrogen receptor (ER) exists in two isoforms, ERα and ERβ, but only ERα is essential for implantation since ERα knockout mice are infertile, while those knockout for ERβ appear fertile [34]. During the proliferative phase, high levels of estrogen induce proliferation of the epithelial, stromal, and vascular endothelial cells [35,36]. In ARTs estradiol priming results in endometrial proliferation and induction of PRs. Subsequently, progesterone acts on these receptors, thus opening the WOI [37]. Decidualization is guided by progesterone, which starts to increase during the secretory phase of the menstrual cycle and remains elevated in case of pregnancy. PR exists in two isoforms, PR-A and PR-B, and only PR-A is essential for implantation since mice knockout for both PR-A and PR-B are infertile, while those knockout for PR-B only are fertile [38,39].

The role of the various factors that regulate decidualization has also been clarified by *in vitro* experiments. In these models, decidualization of human endometrial stromal cells (HESCs) is induced by different treatments. Most of them requires the use the steroid hormones, progesterone or progesterone and estradiol [40,41], but with higher efficiency if steroid hormones are used in combination with cAMP [42,43]. cAMP alone can induce decidualization of HESCs but for few days only [44-46], since for the stabilization of the process is necessary the presence of both cAMP and progesterone [43]. As already discussed, decidualization is also induced by stimulation of stromal cells with CG. [47-52].

Once the WOI is opened, a variety of factors, activating multiple signaling pathways, allows the establishment of the complex crosstalk at the blastocyst-maternal interface, indispensable for implantation and pregnancy. Chorionic gonadotropin (CG) is produced by the embryo very early and it is one of the main players in this communication. The ovaries respond to CG, which acts as an agonist of LH, by maintaining the corpus luteum, thus producing the progesterone necessary for the establishment and progression of pregnancy. The responses of the endometrium are multiple, but basically refer to the inhibition of apoptosis, which usually occurs at the end of the menstrual cycle, by activating anti-apoptotic genes as B-cell lymphoma 2 (BCL-2) [53,54], and the induction of the decidualization process [54-56]. Both epithelial and stromal cells possess the LH/CG receptor LHCGR, a seven transmembrane G protein-coupled receptor, which shows the highest expression during the secretory phase of the menstrual cycle [47,48,55]. Endometrial epithelial cells respond to CG by expressing cyclooxygenase-2 (COX2) and prostaglandin E synthase (PGES), through the activation of extracellular signal-regulated protein kinases 1/2 (Erk1/2) signaling pathway. The increased production of prostaglandin E2 (PGE2) [48-50] induces cAMP in endometrial stromal cells and promotes their decidualization [50,51]. COX-derived PGE2 plays an important role in the increase of endometrial vascular permeability, which characterizes the inflammatory reaction typical of implantation [57,58]. In endometrial stromal cells CG activates Erk1/2 signaling pathway, thus increasing the expression of the progesterone receptor (PR) and regulating the expression of genes controlling endometrial receptivity [47]. Moreover, in primates, endometrial stromal cells respond to CG and progesterone by activating NOTCH1 pathway, as discussed later. NOTCH1 induces the expression of α-smooth muscle actin (α-SMA), which positively regulates remodeling of cytoskeleton and the initial changes typical of the decidualization process [59]. Subsequently, a decrease in CG and NOTCH1 levels is necessary for the completion of decidualization, which is accompanied by an increase in the expression of insulin-like growth factor binding protein-1 (IGFBP1) and prolactin (PRL), markers of decidualization [60-62], and a downregulation of LHCGR [56,63-65].

In response to progesterone uterodomes (also konown as pinopods), apical cell membrane protrusions of the endometrial luminal epithelium, appear. The specific temporal and spatial expression of uterodomes [66] coincides with the WOI, so it has been proposed as a marker of endometrial receptivity [18,67]. The function of uterodomes is not entirely clear. Some authors suggest that uterodomes are responsible of pinocytosis and endocytosis of uterine fluid and macromolecules, which facilitates adhesion of the blastocyst to the endometrium, by inter-digitating with microvilli on the apical trophectodermal surface of the blastocyst [68-72]. In a study by Nikas *et*

al. it has been demonstrated that, in humans, the presence of uterodomes correlated with the success of embryo implantation. In fact, patients with abundant uterodomes became pregnant, while those with a moderate number of uterodomes showed a pregnancy rate lower than 50%, and patients with few uterodomes did not achieve pregnancy [68]; however, the validity of uterodomes as markers of endometrial receptivity is dabeted [73]. More recently it has been proposed that uterodomes might be responsible of the secretion of leukaemia inhibitory factor (LIF) [74], which is indispensable for blastocyst implantation, as discussed later in this review.

Uterine receptivity is also regulated by members of the epidermal growth factor (EGF) family, whose expression pattern in the peri-implantation uterus has been widely investigated in murine models [75-82]. Amon the EGF family members, amphiregulin (AREG) has been identified in the luminal epithelium exclusively at the site of blastocyst apposition and its expression appears to correlate first with the increase of progesterone levels and then with the attachment reaction [77]. Similarly, the expression of heparin binding-EGF (HB-EGF), which is under the control of both estrogen and progesterone [80], requires the presence of competent blastocysts and it occurs in the luminal epithelium when the uterodomes are fully formed at the sites of blastocyst apposition [75,81], while epiregulin (EREG) is expressed in both the luminal epithelium and stroma during blastocyst attachment [78]. This unique expression pattern suggests a role for AREG, HB-EGF, and EREG in uterine receptivity and subsequent embryo adhesion. The role of HB-EGF in blastocyst adhesion to the uterus has been further demonstrated *in vitro* in a co-culture of a mouse cell line synthesizing transmembrane human HB-EGF (TM HB-EGF) and mouse blastocysts. Cells synthesizing TM HB-EGF adhered to mouse blastocysts more than parental cells or cells synthesizing a constitutively secreted form of HB-EGF [83]. These results were confirmed in a more recent study using HB-EGF mutant mice which demonstrates that maternal deficiency of HB-EGF limits pregnancy success [82].

NOTCH signaling pathway is involved in the regulation of various cellular processes such as cell proliferation, invasion, adhesion, survival, apoptosis and differentiation [84-87]. All four NOTCH receptors, the ligands Jagged1 (JAG1) and Delta-like 4 (DLL4) and the target genes hairy enhancer of split (HES) and Hes-related 1 (HEY1) are known to be expressed by the endometrium [88-91]. Several ligands and receptors of the NOTCH signaling pathway are expressed in both the inner cell mass (ICM) and trophectoderm of the human blastocyst [92-94]. NOTCH1 plays an important role in the process of decidualization, by inducing pro-survival signals in the endometrium, thus avoiding apoptosis normally occurring at the end of the menstrual cycle. Hess et al. showed that blastocyst-conditioned medium induces an increase in the expression of NOTCH family members in decidual cells, suggesting a role for this pathway in decidualization [95]. Moreover, it has recently been shown that NOTCH signaling pathway is dysregulated in the endometrium of women with unexplained recurrent pregnancy loss [96]. Activation of NOTCH1 pathway in the endometrium is stimulated by CG and progesterone and leads to increased expression of α-SMA and Forkhead box protein O1 (FOXO1) [11,59,97]. FOXO1, in turns, induces expression of PRL and IGFBP1 and it is essential for the decidualization process [98-102]. NOTCH1 is involved in the inhibition of cAMP/protein kinase A (PKA) signaling pathway [103], so that NOTCH1 needs to be downregulated to allow cAMP response of stromal cells. Similar to what described for α-SMA and LHCGR expression, a downregulation of NOTCH1 is necessary for the induction of IGFBP1 and the completion of decidualization [42,56,59].

Interleukin-1β (IL-1β) is another important factor supporting blastocyst-endometrium dialogue, playing a fundamental role in decidualization of stromal cells and in successful blastocyst implantation. IL-1β is secreted by cytotrophoblast cells isolated from first trimester placenta, while its expression is lower in cultures from second and third trimester placenta [104]. In endometrial stromal cells IL-1β induces the expression of COX2 and PGE2, known to increase the levels of cAMP, which are necessary for decidualization, as above described [105,106]. Moreover, *in vivo* infusion of IL-1β and CG promotes the expression of IGFBP1 in apical surface stromal cells [64]. It has been demonstrated that inhibition of COX2 in human and baboon endometrial stromal cells is able to block the decidualization induced by IL-1β in the presence of steroid hormones, suggesting that IL-1β acts upstream of COX2 [105]. On the contrary, inhibition of COX2 does not affect decidualization induced

by cAMP and steroid hormones, suggesting that cAMP acts downstream of COX2 and PGE2 [105]. Interestingly, cAMP is able to block decidualization induced by IL-1β, indicating a negative feedback between IL-1βand cAMP [105,107]. In baboon, IL-1β positively regulates the expression of matrix metalloproteinase 3 (MMP3) in endometrial stroma, thus inducing degradation of the ECM. Considering that disruption of the ECM might reflect in cellular cytoskeleton remodeling, IL-1β may play an important role in the decidualization also by promoting cytoskeleton changes typical of this process [108,109]. All these data clearly indicate that IL-1β plays a relevant role in blastocyst-endometrium crosstalk.

Endometrial receptivity is regulated also by thyroid hormone (TH). Both thyroid hormone and thyroid-stimulating hormone receptors (TR and TSHR, respectively) are expressed in the endometrium with variations during the menstrual cycle [110]. Two of the isoforms of TR, TRα1 and TRβ1, are expressed during the mid-luteal phase in glandular and luminal epithelium, showing an increase during the secretory phase, followed by a drastic decrease. Interestingly, the expression of TRα1 and TRβ1, and also of TRα2 and TSHR, in endometrial cells is concomitant to the appearance of the uterodomes and the establishment of endometrial receptivity. The expression of TRα1, TRβ1, TRα2 and also of type 2 deiodinase (DIO2) is regulated by progesterone. In fact, the administration of mifepristrone, an anti-progestinic drug that makes the endometrium unreceptive and induces menstrual bleeding, reduces the expression of TRα1 and TRα2, while it up-regulates TRβ1 and DIO2 expression, suggesting a role for progesterone in regulating molecules involved in TH synthesis and metabolism [111]. The role of TH pathway in endometrial function is also demonstrated by the observation that hypothyroidism is able to reduce uterine endometrial thickness, and also interferes with estrogenic response of the endometrium [112]. TH regulates endometrial receptivity also by acting on LIF pathway, since TSH induces increased expression of LIF and LIF receptor (LIFR) in endometrial stromal cells obtained from human endometrial biopsy samples, suggesting a major role for TSH in the implantation process [110].

A role for the immune system in embryo implantation has been widely investigated for obvious reasons. The decidua plays a fundamental role in ensuring immune tolerance toward the semi-allogenic conceptus, protecting it from the mother's immune system. Regulatory T cells (Tregs) are CD4+CD25+ T cells, having the role to suppress the immune response [113]. During early pregnancy, in the decidua there is an increase in Tregs, which produce immunosuppressive cytokines, such as IL-10, for inducing immune tolerance [114-117]. Uterine natural killer (uNK) are a particular type of NK cells, which lose their cytotoxic functions during pregnancy. uNK cells play a supportive role by enhancing angiogenesis and induce immune tolerance, by reducing inflammation through interferon-γ (IFN-γ) [118] and inhibiting the function of T cells through the expression of immunomodulatory molecules such as galectin-1 and glycodelin A [119].

Recently, a customized endometrial receptivity array (ERA), containing 238 genes related to endometrial receptivity, was created [120]. These genes, differentially expressed in the receptive phase, encode for factors involved in several biological processes, such as processes relating to the immune system, circulation, response to external stimulus, behavior, cell cycle, cell adhesion, anatomical structure development, cell–cell signaling, and mitotic cell cycle. ERA represents a useful tool for clinicians to choose the best time for blastocyst transfer during ART procedures [120,121]. *In vitro* fertilization (IVF) cycles often fail since it is difficult to identify potential dysregulations of the many factors involved in implantation. High throughput screening, as ERA technology, might allow identification of molecular alterations responsible for recurrent implantation failures (RIF) which are not currently evaluated in routine workup. Thus, ERA could suggest clinicians a possible therapy, leading to an increase in the success of the ART procedures.

3. Implantation of the competent blastocyst

Implantation is a crucial event of mammalian reproduction, during which the embryo makes contact with the maternal uterus for the first time. It is defined as "a series of events initiated by fertilization of the ovum which ultimately leads to the embedding of the blastocyst in the endometrium" [13]. So, implantation starts with the fertilization of the ovum, in the ampulla of the

Fallopian tube within 24 to 48 hours after ovulation and ends with the formation of the primitive placenta.

3.1. Transport, orientation and hatching

After fertilization, the embryo, encased in a non-anchored glycocalix, the so-called zona pellucida, which prevents ectopic implantation, descends the Fallopian tube and reaches the uterine cavity while undergoing profound morphological changes ending in the formation of the blastocyst [122,123].

For a successful implantation into the maternal tissues, a correct orientation of the blastocyst towards the uterine wall is needed. In most eutherian mammals, at the time of first contact of the blastocyst to the endometrial epithelium, the ICM of the various embryos has an almost constantly specific orientation toward the uterus. In humans, the ICM faces the uterine wall. This positioning of the ICM usually correlates with the site of trophectoderm attachment to the endometrium, as well as with subsequent development of the fetal membranes and placental structures [124,125]. Why, within most species, the ICM of the blastocyst, or the placenta, should be positioned consistently in the same way with respect to the uterine wall is not completely understood. Moreover, how the blastocyst becomes correctly oriented [126,127] or what directs the process has not been well clarified, for even the most commonly studied mammals.

Embedding of the blastocyst into the maternal endometrium requires hatching from the zona pellucida, which otherwise would prevent adhesion of the embryo to the uterine wall. Blastocyst hatching exposes the trophectoderm and allows the blastocyst to implant in the maternal uterus. The crucial event for blastocyst hatching is the formation of a nick into the zona pellucida, and proteases, such as serine-, cysteine- and metallo-proteases have been proposed to play a major role in this event depending on the species [128-133]. Cathepsins, belonging to the ubiquitous cysteine proteases family [134], have been demonstrated to be involved in blastocyst hatching and zona lysis in mice: the expression of cathepsin L and P (mRNA and protein) and their natural inhibitor, Cystatin C, has been demonstrated in mouse peri-hatching blastocysts [135]. Treatment of golden hamster embryos with Cystatin C is able to block blastocyst hatching [131]. The process of murine blastocyst hatching from the zona pellucida is also regulated by two mouse-specific proteinases, Strypsin (ISP1) and Lysin (ISP2). ISP1 and ISP2 are two related S1-family serine proteinases, which are tandemly localized in a cluster of tryptase genes [136,137]. The ISPs are co-expressed in the mouse preimplantation embryos and in the mouse uterine endometrium during the WOI, indicating that they could play a role in the process of blastocyst implantation [136,138]. Expression of ISP genes is positively regulated by progesterone and TH [129,133,136] and ISPs are secreted by the blastocyst and the endometrial glands into uterine fluid just prior to implantation [139]. The use of antibodies against ISP1/ISP2 abrogate murine embryo hatching and outgrowth, ascribing a crucial role for ISPs in this process [138]. This is further supported by our recent observations using mouse blastocysts cultured in the presence of TH, with or without endometrial cells used as the feeder layer. In the presence of endometrial feeder cells, TH is able to anticipate blastocyst hatching (Figure 1) by upregulating the expression of blastocyst produced ISPs, and to enhance blastocyst outgrowth by upregulating endometrial ISPs and MMPs. On the contrary, in the absence of the endometrial feeder layer, TH does not affect blastocyst hatching, suggesting that TH is one of the players involved in the bidirectional crosstalk between the blastocyst and the endometrium during the WOI [133]. Human homologs of ISPs have not been so far identified, and it is possible that other proteases might be involved in blastocyst hatching in humans.

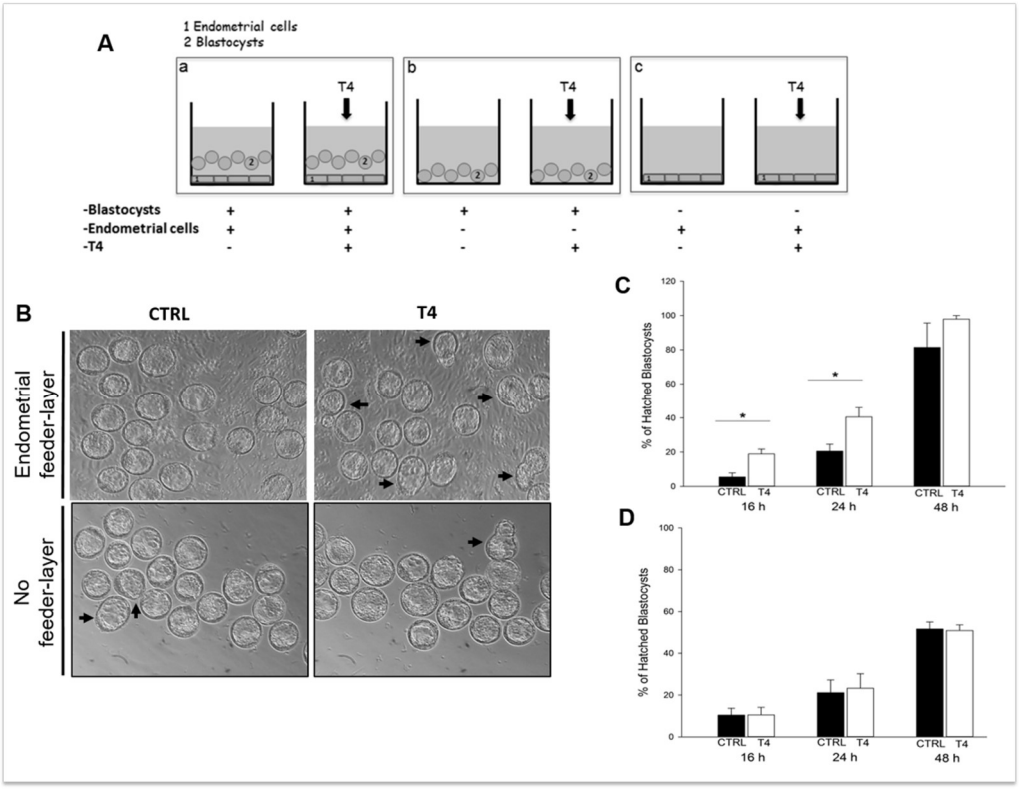

Figure 1. Thyroid hormone (TH) supplementation stimulates mouse blastocyst hatching *in vitro*. (**A**) Schematic representation of the *in vitro* model developed to assess TH role in implantation. (**a**) Co-culture of murine blastocysts and endometrial primary cells as the feeder layer; (**b**) blastocysts cultured on plastic; (**c**) endometrial cells cultured without blastocysts. (**B**) Representative images of the cultures. Scale bar 50μm. (**C**, **D**) Graphs summarizing the results shown in B: percent of hatched blastocysts after co-culture on endometrial cells (**C**) or on plastic (**D**). Reproduced with permission from Piccirilli *et al*. [133].

3.2. Apposition

Histological analysis of uteri of pregnant women allows to recognize three different levels of blastocyst adhesion to the uterine wall, which correspond to the three stages of blastocyst implantation (Figure 2) [140,141].

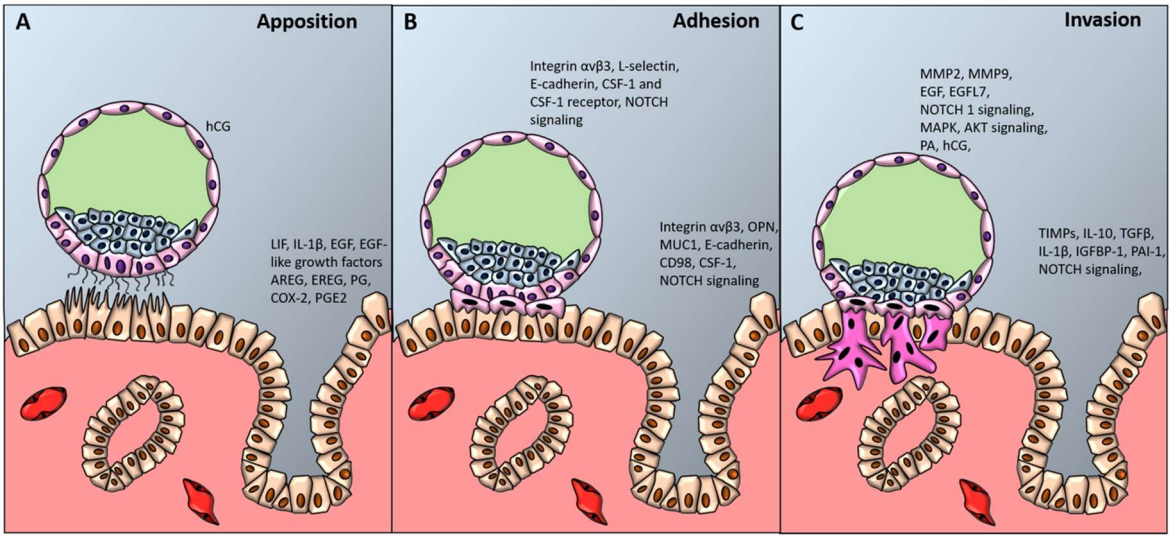

Figure 2. Blastocyst apposition, adhesion and invasion. The diagram shows a preimplantation-stage (**A**, **B**) and invading (**C**) blastocyst (about 9 to 10 days after conception) and the processes and factors required for uterine receptivity and blastocyst apposition, adhesion (**A**, **B**) and invasion (**C**). hCG denotes human chorionic gonadotropin, LIF leukemia inhibiting factor, IL-1β interleukin-1 beta, EGF-like growth factors epidermal growth factor-like growth factors, AREG amphiregulin, EREG epiregulin, PG progesterone, COX-2 cyclooxygenase-2, PGE2 prostaglandin E2, CSF-1 colony stimulating factor-1, OPN osteopontin, MUC-1 mucin-1, MMPs metalloproteinases, EGFL7 epidermal growth factor-like domain 7, MAPK mitogen activated protein kinase, AKT protein kinase B, PA plasminogen activator, TGFβ transforming growth factor beta, TIMPs tissue inhibitor of metalloproteinases, PAI-1 plasminogen activator inhibitor-1.

Blastocyst apposition is the initial stage representing the first physical contact between the blastocyst and the endometrium, in which the blastocyst finds a site for implantation, guided by the maternal endometrium [142,143]. The site of implantation in the human uterus is usually in the upper and posterior part in the midsagittal plane. During blastocyst apposition, the microvilli placed on the apical surface of trophectoderm interdigitate with the uterodomes localized on the apical surface of the uterine epithelium (Figure 2A). These specialized structures support a stable binding between trophoblast and uterine epithelial cells, so that the plasma membranes of these cells are parallel and separated by a distance of 20 nm [144]. The uterodomes secrete LIF [74]. LIF is a cytokine of the IL-6 family, which in the uterus activates the Janus kinases (JAK) - signal transducer and activator of transcription protein (STAT) pathway, and therefore phosphorylates STAT3, whose activation is required for implantation [145,146]. LIF is indispensable for blastocyst implantation. Mice knockout for LIF are infertile since, although they are able to develop blastocysts, these fail to implant, however successfully implant in surrogate female mice [147]. In Lif-null mice the expression of EGF-like growth factors, such as HB-EGF, AREG and EREG, which, as previously mentioned, are normally expressed by the luminal epithelium adjacent to the blastocyst and are essential for successful pregnancy, is abolished [148]. Since the defects in decidualization caused by the absence of LIF can be rescued by intrauterine administration of EGF ligand [149], it has been hypothesized that LIF favors blastocyst invasion by reducing the expression of cell-cell junction molecules and proliferation of the stromal cell through activation of EGF signaling pathway [150]. In fertile women, LIF expression increases in the endometrium around the time of implantation, while infertile women express low levels of this factor [151,152]. Once a competent blastocyst takes contact with the maternal endometrium, a dialogue made of signals and responses between them occurs. One of the most important factors secreted by trophoblast cells is CG. CG is expressed very early by the embryo, since its mRNA can be detected starting from the 6-8 cell stage, but the secreted protein becomes measurable starting from the late blastocyst stage [153]. During pregnancy, CG is firstly detectable in maternal blood during implantation and then rapidly increases [154]. As discussed before, CG plays a fundamental role in inducing the production of progesterone and the decidualization process, thus allowing implantation of the blastocyst.

3.3. Adhesion

Following apposition, stable adhesion of the blastocyst to the endometrium occurs, mediated by the interaction between several receptors and ligands (Figure 2B). Over the last decades, several of these ligands and receptors have been identified. It has been observed that both the uterodomes of the endometrial epithelium and the trophectoderm of the blastocyst express the integrin αvβ3, together with the endometrial expression of its ligand the glycoprotein osteopontin (OPN). Their expression at the WOI suggests a role in implantation [80,155,156], and the binding between integrin αvβ3 and its ligand OPN might mediate the stable adhesion between the blastocyst trophoblast and the endometrium [157]. Using an *in vitro* model of implantation, Genbacev *et al*. suggested that trophoblast adhesion to the uterine wall is also mediated by L-selectin expressed on the surface of the trophoblast cells, and uterine epithelial oligosaccharide ligands, such as HECA-452 and MECA-79 [158,159]. More recently it has been also demonstrated that the transmembrane glycoprotein Mucin 1 (MUC1), abundantly expressed at the apical surface of uterine epithelium under the control

of progesterone, acts as a scaffold mediating the binding between L-selectin and their ligands [160]. The adhesion of the blastocyst to the endometrium is also promoted by the expression of adhesion molecules, such as cadherins. The presence of endothelial cadherin (E-cadherin) in both the trophoblasts and endometrial epithelium, regulated by progesterone, indicates that it may play an important role in blastocyst adhesion to the endometrium [161]. As trophoblast cells proliferate, differentiate and invade the stroma, they downregulate E-cadherin and increase osteoblast cadherin (OB-cadherin) [162,163]. The expression of OB-cadherin in the endometrial epithelium suggests that this adhesion molecule later mediates trophoblast–endometrium interactions. Blastocyst adhesion is also favored by the expression of the glycoproteic receptor CD98 on the surface of endometrial cells, which is normally involved not only in amino acids transport but also in cell fusion [164,165]. Using two human endometrial cell lines characterized by low and high receptivity, Dominguez *et al*. demonstrated that CD98 receptor is significantly associated with the receptive phenotype. In human endometrial samples, they found that CD98 expression was spatially restricted to the apical surface of endometrial cells and temporally restricted to the WOI. Treatment of primary endometrial epithelial cells with hCG, 17-β-estradiol, LIF or EGF increases expression of CD98, greatly enhancing murine blastocyst adhesion, while its siRNA-mediated depletion reduced blastocyst adhesion rate [166]. The expression of NOTCH receptors and ligands in the trophectoderm of the blastocyst and that of NOTCH1, DLL4 and JAG1 in the apical surface of the endometrial epithelium during the mid-secretory phase [90,167] would suggest a role for NOTCH signaling in the adhesion of the blastocyst to the epithelium. Indeed, it has been demonstrated that blastocyst-conditioned medium regulates NOTCH1 and JAG1 expression in endometrial epithelium [167], suggesting that the blastocyst is able to activate NOTCH signaling in the endometrium, thus possibly regulating endometrial receptivity. This is reinforced by the fact that women with primary infertility show a reduced or absent immunostaining for JAG1 in the luminal endometrial epithelium during the mid-secretory phase [167]. As already mentioned, adhesion of the blastocyst to the endometrium is regulated by several different factors. A role for colony-stimulating factor-1 (CSF-1) in implantation has been proposed. Indeed, supplementation of CSF-1 in cultures of human trophoblast cells promotes their differentiation in syncytiumtrophoblast cells and leads to the production of placental lactogen [168], while supplementation of CSF-1 to cultures of murine blastocyst induces trophoblast outgrowth [169]. However, using osteopetrotic mutant mice, which lack CSF-1, it has been shown that a maternal source of CSF-1 is not necessary for pregnancy, and possibly the fetus can provide a source of CSF-1 which compensate for the absence of maternally produced CSF-1 [170].

3.4. Invasion

Finally, in the third stage, invasion occurs starting with the penetration of highly invasive trophoblast cells in the uterine epithelium (Figure 2C), followed by infiltration in the basement membrane and in the stromal compartment, a process known as "interstitial invasion" [143,171,172]. Besides invading the endometrial stroma, trophoblast cells also migrate down the lumen of maternal spiral arteries, replace the vascular endothelial lining and become embedded in the arterial walls. This process of "endovascular invasion" allows to replace small-caliber, high-resistance vessels with large-caliber, low-resistance vessels, ensuring an adequate blood supply to the fetoplacental unit [173,174]. Defects in trophoblast endovascular invasion of maternal spiral arteries can seriously impair placental function, leading to significant complications of advanced gestation, such as intrauterine growth restriction (IUGR) and preeclampsia [175]. The huge invasive ability of the fetal trophoblast is due to a high production of activated gelatinases, in particular MMPs 2 and 9 [176-178]. Trophoblastic MMPs are regulated in response to IL-1β, tumour necrosis factor alpha (TNFα), IL-1α, macrophage colony-stimulating factor (MCSF), transforming growth factor β (TGFβ), IGFBP1, leptin, hCG, EGF [104,179-183], which are secreted from different cell types at the feto-maternal interface, such as trophoblasts themselves and endometrial cells, promoting trophoblast invasion. As already mentioned above, the expression of MMPs involved in endometrial invasion by trophoblast cells is also under the control of TH, as TH positively regulates MMP expression by endometrial cells [133]. Recently, we demonstrated that the migration and invasion of trophoblast cells is regulated by

the secreted factor Epidermal growth factor-like domain 7 (EGFL7), which activates NOTCH1, MAPK and AKT signaling pathways [184]. Activation of the NOTCH pathway is important in both interstitial and endovascular invasion by trophoblast cells. *In vitro* functional assays show that invasion of Matrigel by trophoblast cells is impaired in the presence of a γ-secretase inhibitor, normally used to inhibit NOTCH activation [175,184]. NOTCH appears to be also involved in trophoblast endovascular invasion, since uNK, involved in the disruption of endometrial spiral arteries integrity, express NOTCH1 and 2 and maternal cells surrounding spiral arteries express Delta-like 1 (DLL1) [175], and NOTCH activation may lead to arterial wall disruption. These results are further confirmed by the fact that NOTCH pathway is dysregulated in placenta of women affected by preeclampsia [175,185-191], a common pregnancy disorder characterized by an insufficient trophoblast invasion and an inadequate vascular remodeling. In women affected by preeclampsia, the alteration of NOTCH pathway is accompanied by a concomitant altered expression of NOTCH ligand EGFL7, in both placenta and maternal circulation [185,192].

In all the placental species the extent of endometrial decidualization is proportional to the invasiveness of the embryo. The human placenta is the most invasive one known so far, and it has been suggested that the unique invasiveness of the human trophoblast could due to its high production of hyperglicosylated CG isoform, which is maximal in the first weeks of pregnancy [193,194]. In order to limit the extent of trophoblast invasion, both trophoblast and endometrium balance the expression of growth factors, cytokines, and enzymes. As an example, maternal endometrium increases the production of tissue inhibitors of MMPs (TIMPs), due to a spatial and temporal regulation of cytokines and growth factors, such as IL-10 [195], TGFβ and IL-1α [179]. While IL-1α significantly increases the activity of MMP-9 and MCSF increases MMP-9 immunoreactivity, TGFβ inhibits total gelatinolytic activity, MMP-9 activity and immunoreactivity [179]. TIMP-3, which is up-regulated by progesterone, plays a major role in limiting trophoblast invasion by limiting ECM degradation. It has been detected in the fetal extravillous trophoblasts, as well as in the maternal endometrial cells [196,197]. On the contrary, by in situ hybridization in implanting mouse embryos no expression was observed for TIMP-1 or TIMP-2 in the embryo proper, trophoblasts, or in the decidua. Weak signals were demonstrated for TIMP-1 only in the circular layer of myometrial smooth muscle and in some uterine stroma cells distant from the site of embryo implantation. Moreover, the expression of TIMP-1 and TIMP-2 is not dependent on the stage of the menstrual cycle [197]. Trophoblast invasion is promoted by the action of the plasminogen activator (PA) system since it is able to promote trophoblast invasion, by converting plasminogen into the active serine protease plasmin, which in turn, degrades ECM [198]. In endometrial cells, TGFβ regulates trophoblast invasion up-regulating the expression of plasminogen activator inhibitor-1 (PAI-1), which is the main inhibitor of urokinase-type plasminogen activator (uPA) [199-201], and decorin, a decidua-derived TGFβ binding proteoglycan, which inhibits proliferation, migration and invasion of trophoblast cells [202]. The blastocyst is completely embedded in the uterine stroma 8 days after fertilization and the site of entry is covered by fibrin, over which the uterine epithelial cells grow [143,203,204].

4. Conclusions

Human reproduction is a rather inefficient process, with a chance to achieve pregnancy of 15% per cycle [205]. ART procedures help several couples to have a baby, but only 25% of transferred embryo will successfully implant [206]. Implantation is a critical process, finely regulated by a variety of molecules and hormones secreted by both the blastocyst and the endometrium. Considering this, it is difficult to identify the alteration of those factors that determine the lack of embryo implantation. This also occurs probably because at present these factors are not sufficiently evaluated in routine clinical screening. Poor knowledge of the factors that regulate implantation and therefore not sufficient clinical screening exams are responsible of the high incidence of unexplained infertility cases (25%). A more in-depth knowledge of the mechanisms involved in the early stages of pregnancy, leading to increased efficiency of ART techniques, will definitely improve the diagnosis and treatment of infertility.

458 **Author Contributions:** conceptualization, L.C. and M.M.; writing—original draft preparation, M.M.; writing—
459 review and editing, L.C., C.T. and R.R.; visualization, V.L. and F.L.C.; supervision, L.C.; project administration,
460 L.C. and M.M.

461 **Funding:** This work has been supported by the Grant for Fertility Innovation 2017, funded by Merck

462 **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the
463 study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
464 publish the results.

465 ## Abbreviations

WOI	Window of implantation
CG	Chorionic gonadotropin
IL	Interleukin
LIF	Leukemia inhibitory factor
ARTs	Assisted reproduction techniques
ECM	Extracellular matrix
LH	Luteinizing hormone
cAMP	Cyclic adenosine monophosphate
BCL-2	B-cell lymphoma 2
COX2	Cyclooxygenase-2
PGES	Prostaglandin E synthase
Erk1/2	Extracellular signal-regulated protein kinases 1/2
PGE2	Prostaglandin E2
PR	Progesterone receptor
α-SMA	α-smooth muscle actin
IGFBP1	Insulin-like growth factor binding protein-1
ER	Oestrogen receptor
PRL	Prolactin
EGF	Epidermal growth factor
AREG	Amphiregulin
HB-EGF	Heparin binding epidermal growth factor
EREG	Epiregulin
JAG1	Jagged1
DLL4	Delta-like 4
HES	Hairy enhancer of split
HEY1	Hes-related 1
ICM	Inner cell mass
FOXO1	Forkhead box protein O1
PKA	Protein kinase A
MMP	Matrix metalloproteinase
TH	Thyroid hormone
TR	Thyroid hormone receptor
TSHR	Thyroid-stimulating hormone receptor
DIO2	Type 2 deiodinase
LIFR	LIF receptor
ERA	Endometrial receptivity array
IVF	In vitro fertilization
RIF	Recurrent implantation failures
HESCs	Human endometrial stromal cells
Tregs	Regulatory T cells
uNK	Uterine natural killer
IFN-γ	Interferon-γ
ISP1	Strypsin
ISP2	Lysin
JAK	Janus kinases

STAT	Signal transducer and activator of transcription protein
OPN	Osteopontin
MUC1	Mucin 1
E-cadherin	Endothelial cadherin
OB-cadherin	Osteoblast cadherin
CSF-1	Colony-stimulating factor-1
IUGR	Intrauterine growth restriction
TNFα	Tumor necrosis factor α
MCSF	Macrophage colony-stimulating factor
TGFβ	Transforming growth factor β
EGFL7	Epidermal growth factor-like domain 7
DLL1	Delta-like 1
TIMPs	Tissue inhibitors of MMPs
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
uPA	Urokinase-type plasminogen activator

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