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SYMPOSIUM: IMPLANTATION REVIEW

In-vitro model systems for the study of human embryo—endometrium interactions

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Abstract Implantation requires highly orchestrated interactions between the developing embryo and maternal endometrium. The association between abnormal implantation and reproductive failure is evident, both in normal pregnancy and in assisted reproduction patients. Failure of implantation is the pregnancy rate-limiting step in assisted reproduction, but, as yet, empirical interventions have largely failed to address this problem. Better understanding of the mechanisms underlying human embryo—endometrium signalling is a prerequisite for the further improvement of assisted reproduction outcomes and the development of effective interventions to prevent early pregnancy loss. Studying human embryo implantation is challenging since in-vivo experiments are impractical and unethical, and studies in animal models do not always translate well to humans. However, in recent years in-vitro models have been shown to provide a promising way forward. This review discusses the principal models used to study early human embryo development and initial stages of implantation *in vitro*. While each model has limitations, exploiting these models will improve understanding of the molecular mechanisms and embryo—endometrium cross-talk at the early implantation site. They provide valuable tools to study early embryo development and pathophysiology of reproductive disorders and have revealed novel disease mechanisms such as the role of epigenetic modifications in recurrent miscarriage.

KEYWORDS: co-culture, embryo, endometrium, implantation, in vitro, model

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Introduction

Compared with other mammalian species, human reproduction may be considered highly inefficient. Even among fertile couples, the probability of achieving a pregnancy within one menstrual cycle, defined as the monthly fecundity rate, is only 20–30%. In contrast, the monthly fecundity rate may be as high as 80% in baboons and 90% in rabbits (Chard, 1991; Foote and Carney, 1988; Stevens, 1997). In humans, one in every six couples suffers from subfertility, and in around 25% of those investigated no clear cause is identified. Assisted reproduction technology offers many subfertile couples effective treatment, but implantation failure remains the rate-limiting step, and only around 25% of transferred embryos will successfully implant (Boomsma et al., 2009b; Edwards, 2006; Ferraretti et al., 2012). Knowledge of the aetiology of implantation failure is in its infancy and requires a better understanding of embryo implantation.

The two key components in successful embryo implantation are the competent embryo and the receptive endometrium that together undertake intimate bilateral communication (**Figure 1A**) (Cha et al., 2012; Norwitz et al., 2001; Paria et al., 2002; Quenby and Brosens, 2013). Both poor embryo quality and poor endometrial receptivity contribute significantly to the occurrence of implantation failure (Diedrich et al., 2007; Evans et al., 2012; Urman et al., 2005). The role of embryo quality in implantation success is discussed in more detail in the accompanying article by Montag et al. (2013, this issue) (see also Campbell et al., 2013a,b; Aparicio et al., 2013, this volume; Futures in Reproduction Symposium, in press).

The human endometrium undergoes cyclic changes in receptivity, encompassing the postovulatory process of decidualization in preparation for embryo implantation. In the human, this process of endometrial remodelling is driven primarily by progesterone secreted by the corpus luteum and, to a lesser extent, by oestrogen and relaxin (Gellersen et al., 2007). Decidualization results in changes in all cell types that make up the endometrium, and these cellular components should be considered when establishing an in-vitro model for embryo implantation. Endometrial stromal cells differentiate from fibroblast-like cells into secretory, epithelioid and receptive decidual endometrial stromal cells (Gellersen et al., 2007; Tang et al., 1993). Endometrial stromal cells have been shown to provide a receptive substrate for trophoblast invasion and to support embryo implantation by providing defence mechanisms against oxidative stress and by aiding the immunological tolerance of the allogeneic fetus (Weimar et al., 2013). Endometrial epithelial cells can be characterized as either glandular or luminal epithelial cells (Lim et al., 2002; Niklaus and Pollard, 2006). Glandular epithelial cells undergo secretory transformation, thereby sustaining the embryo with histiotrophic nutrition (Burton et al., 2002; Cheong et al., 2013). Luminal epithelial cells start to locally express cell adhesion receptors and ligands that promote embryo attachment (**Figure 1A**) (Lessey et al., 1992). As a result, the endometrium is receptive to embryo implantation but

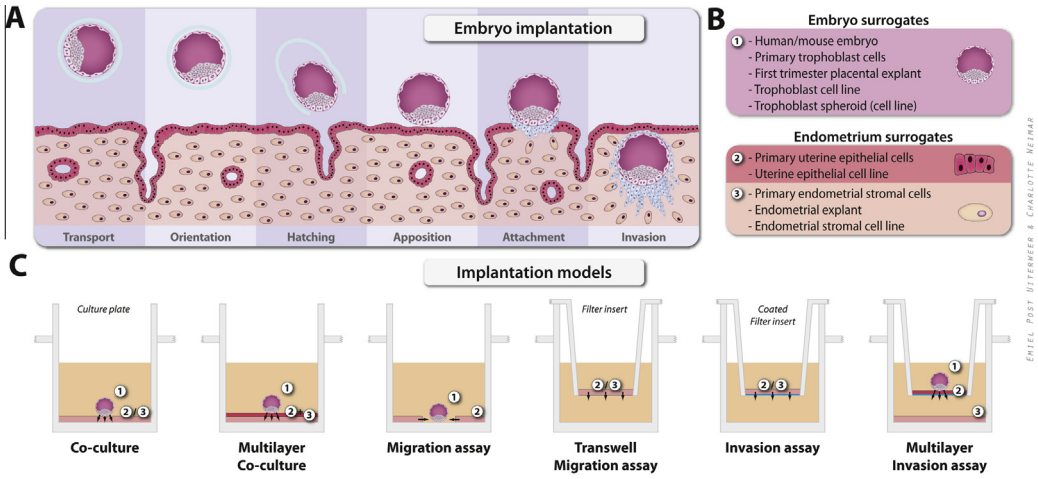

Figure 1 Schematic representation of the phases of human embryo implantation and the in-vitro models of human embryo implantation. (A) Illustration of a human embryo transported through the Fallopian tube and arriving in the uterine cavity around day 5 or 6 after fertilization. The inner cell mass shows orientation towards the endometrial lining and the embryo hatches from the zona pellucida (light blue). The embryo, now in close contact with the endometrium (apposition), starts to attach to the uterine epithelium via the polar trophectoderm and will eventually invade the decidualizing endometrium. (B) In-vitro models utilizing human embryos and primary endometrial tissue cultures would most closely mimic the in-vivo implantation environment. As these materials may be difficult to acquire, or simplified models are less complex to study, surrogates have been used for both groups. (C) Different in-vitro models allow for the study of the various stages of implantation. Each model is illustrated by a cross-section of the culture plate and a schematic representation of the cellular ingredients. The blue line in the last two assays represents a Matrigel extracellular matrix coating, other colours and numbers correspond to those in B. 1 = human embryo or surrogate; 2 = human endometrial epithelial cells; 3 = human endometrial stromal cells, or their surrogates. Arrows represent the direction of migration/invasion. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

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only for a relative short time span, for 6–10 days after the LH surge (Paria et al., 2001). This is often referred to as the putative 'window of implantation'. A key difficulty that is directly relevant to use of in-vitro models of implantation is the current inability to define the receptive endometrium at the molecular level. Current progress to develop biomarkers of receptive endometrial epithelial and stromal cells are discussed by Salamonsen et al. (2013b, this issue). Decidualization is also associated with a remarkable increase in endometrial leukocytes, especially uterine natural killer cells. Uterine natural killer cells acquire a unique phenotype and are suggested to become key regulators at the implantation site, with functions ranging from immunological tolerance of the genetically distinct embryo to regulation of trophoblast invasion and induction of vascular growth and remodelling (Erlebacher, 2013; Hanna et al., 2006; Lee et al., 2011; Quenby and Farquharson, 2006). Decidualization is further characterized by the initial phase of vascular remodelling involving both vascular smooth muscle cells and endothelial cells of the spiral arteries, which eventually leads to transformation of the spiral arteries into low-resistance, high-capacitance vessels, ensuring unimpeded blood flow to the villous placenta later in pregnancy (Cartwright et al., 2010).

The embryo encounters the endometrium about 5 days after fertilization, when it has reached the blastocyst stage and has hatched from the zona pellucida (**Figure 1A**) (Chen et al., 2005; Hamatani et al., 2006; Watson et al., 2004). Embryo implantation is a three step process: (i) apposition; (ii) attachment to the luminal epithelial surface of the endometrium; and (iii) invasion through the basement membrane into the underlying stromal cell compartment (Simon et al., 2000, 2001; Meseguer et al., 2001). During the invasion phase, the embryonic cytotrophoblast interacts with maternal endometrial stromal cells and the extracellular matrix and begins to differentiate into villous cytotrophoblast, syncytiotrophoblast and extravillous trophoblast, forming the embryonic part of the placenta. Extravillous trophoblasts invade deep into the uterus, thereby further orchestrating the development of the maternal side of the placenta, characterized by vascular remodelling. Embryo–endometrium interactions during these phases are tightly regulated and include both cell–cell and cell–extracellular matrix interactions (Dominguez et al., 2002).

The physiological and molecular mechanisms underlying successful embryo implantation remain only partially understood (Cha et al., 2012). However, in recent years a major body of literature has emerged in which genome-wide RNA expression and proteomic analysis of endometrial tissue, secretions and flushings have identified key molecular processes and potential biomarkers of endometrial receptivity (Boomsma et al., 2009a; Bourdiec et al., 2013; Brosens and Gellersen, 2010; Burney et al., 2007; Casado-Vela et al., 2009; Dominguez et al., 2003; Hannan et al., 2011; Horcajadas et al., 2007; Petracco et al., 2012; Salamonsen et al., 2013a; Scotchie et al., 2009; Talbi et al., 2006). In addition, the secretory products and key signalling factors produced by in-vitro generated embryos are being elucidated (Campbell et al., 1995; Dominguez et al., 2003, 2008; Scott et al., 2008; Katz-Jaffe et al., 2009; Cortezzi et al., 2011, 2013; Cheong et al., 2013). In a clinical setting, embryo quality seems to be a major cause of implantation problems, which is discussed in detail by Montag et al. (2013, this issue). While a comprehensive discussion of these studies is beyond the scope of this review, it is clear that this work on endometrial and embryo biology has led to important advances in the understanding of the mechanisms which determine successful human implantation. However, in order to interrogate the complex embryo–endometrium interactions that occur during implantation, studies with representative functional models, ideally including both embryo and endometrial components, are also needed.

Studying human embryo implantation is challenging since in-vivo human experiments are practically and ethically unfeasible. Alternative in-vivo and in-vitro approaches have therefore been required. In-vivo animal studies include non-human primate models which have led to significant advances in the understanding of the processes that mediate embryo implantation (Banerjee and Fazleabas, 2010; Genbacev et al., 2003; Illera et al., 2000; Nyachieo et al., 2007; Paria et al., 1981; Slayden and Keator, 2007; Wang and Dey, 2006). However, because of differences in the reproductive tract between the human and animal models, including the murine and primate, the extent to which these models represent conditions in the human may be limited (Lee and DeMayo, 2004). For instance, in contrast to the mouse, decidualization of the human endometrium commences without the presence of an embryo (Lopes et al., 2004) and is differently regulated, as progesterone receptor expression in the female reproductive tract varies greatly between humans and mice (Teilman et al., 2006). In addition, the monthly fecundity rate in murine species is around 3-times higher, with an average litter size of around 10–12 in mice (Evers, 2002; Foote and Carney, 1988). As an alternative to in-vivo animal models, a series of in-vitro human models has been developed that mimic the early and later stages of human embryo implantation.

In-vitro models

In-vitro models aim to mimic certain aspects of the in-vivo human implantation environment, enabling the study of the apposition, attachment and invasion phases of implantation. In combination with contemporary molecular techniques, including genomic microarrays, reverse-transcription PCR (RT-PCR), multiplex assays and proteomics by mass spectrometry, these models provide potentially powerful tools to address the knowledge gap for human implantation. In this review, the reported in-vitro models are described in three groups: A, B and C (**Figure 1** and **Table 1**). Models discussed in group A permit study of early stages of implantation and focus on the interactions of the endometrial epithelium with the embryo. Group B models focus on the stage of implantation following breach of the luminal epithelium and generally employ co-culture systems of endometrial stromal cells and embryos. Finally, the more complex (3D) co-culture systems addressed in group C

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Table 1 Selected models for studying embryo–endometrium interactions.

Implantation stage	*Model and components*	*References*
Apposition, attachment, invasion	**Co-culture**	
	Endometrial epithelial cell + human embryo	Lindenberg et al. (1985), Simon et al. (1997, 1999), Meseguer et al. (2001), Caballero-Campo et al. (2002), Dominguez et al. (2003), Gonzalez et al. (2011)
	Endometrial epithelial cell + human embryo surrogate	Mouse embryo: Domínguez et al. (2010)
		Trophoblast spheroid: Galan et al. (2000), Hohn et al. (2000), Heneweer et al. (2003, 2005), Mo et al. (2006), Uchida et al. (2007), Aboussahoud et al. (2010), Liu et al. (2011), Ho et al. (2012), Holmberg et al. (2012), Xiong et al. (2012)
	Endometrial stromal cell + human embryo	Carver et al. (2003), Grewal et al. (2008), Teklenburg et al. (2010b, 2012), Weimar et al. (2012)
	Endometrial stromal cell + human embryo surrogate	Mouse embryo: Shiokawa et al. (1996), Hanashi et al. (2003), Grewal et al. (2010), Estella et al. (2012)
		Trophoblast spheroid: Harun et al. (2006), Gonzalez et al. (2011), Holmberg et al. (2012), Weimar et al. (2012), Gellersen et al. (2013)
		First-trimester placental explant: Popovici et al. (2006)
Apposition, attachment, invasion	**Multilayer co-culture**	
	Rabbit endometrium + rabbit embryo	Glenister (1961)
	Endometrial stromal cell + Matrigel + endometrial epithelial cell + human embryo	Bentin-Ley et al. (2000), Petersen et al. (2005)
	Endometrial epithelial cell + Matrigel + endometrial stromal cell	Park et al. (2003)
	Endometrial stromal cell + RL95-2 + JAr spheroids	Evron et al. (2011)
	Endometrial stromal cell + endometrial epithelial cell + JAr spheroids	Wang et al. (2012)
Migration (as part of invasion)	**Migration assay**	
	Endometrial stromal cell+ embryo (surrogate)	Weimar et al. (2012)
Migration (as part of invasion)	**Trans-well migration assay**	
	Endometrial stromal cell + embryo surrogate	Gellersen et al. (2010, 2013)
Invasion	**Invasion assay**	
	Endometrial stromal cell + embryo surrogate	Gellersen et al. (2010), Estella et al. (2012)

utilize endometrial tissue explants or multilayered culture systems, which allow the study of both the early and later stages of embryo implantation. As the use of human embryos is restricted in many countries and papers describing human embryo–endometrium interactions are therefore scarce, relevant studies that describe the use of embryo surrogates such as trophoblast or blastocyst-like spheroids are also considered.

A. Early-phase implantation models

Using embryos

One of the first reports of an in-vitro model described pre-implantation interactions between endometrial epithelial cells and human embryos (Lindenberg et al., 1985)

(Table 1). A similar model was subsequently developed with the aim of supporting embryo development *in vitro* (Figure 1C). In this co-culture system, preimplantation embryos of women with implantation failure undergoing IVF treatment were cultured from day 2 until day 6 of gestation (blastocyst stage) on an autologous endometrial epithelial cell monolayer. On day 6, the embryo was transferred to the uterus. Increased blastocyst formation and implantation rates after exposure to endometrial epithelial cells were reported compared with embryos cultured without (Simon et al., 1999). Using this approach, the embryonic regulation of chemokine receptors on endometrial epithelial cells was also investigated. The presence of a blastocyst was shown to up-regulate the expression of *CXCR1*, *CXCR4* and *CCR5* in endometrial epithelial cells (Dominguez et al., 2003), as well as interleukin (IL) 8 (Caballero-Campo et al., 2002). Moreover, this co-culture model has also provided evidence for the embryonic regulation of cell surface molecules believed to be key to successful apposition, such as mucin 1 (MUC1), integrins and leptin receptors (Gonzalez et al., 2000; Meseguer et al., 2001; Simon et al., 1997). These data show that viable preimplantation embryos are not only further nurtured in the presence of endometrial epithelial cells, but themselves induce pro-apposition changes in endometrial epithelial cell surface characteristics and chemokine networks. Embryo-derived signals to the endometrium include human chorionic gonadotrophin (HCG), IL-1 and other secreted factors (Dey et al., 2004; Teklenburg et al., 2010b). While in-vivo experiments that validate these in-vitro observations are difficult in humans, a study in baboons showed that embryo-derived HCG up-regulates receptivity markers such as leukaemia inhibitory factor (LIF), providing further support for the concept that embryo-derived signals enhance endometrial epithelial cell receptivity (Sherwin et al., 2007).

Because the isolation of the two endometrial epithelial cell types (glandular and luminal) is technically challenging, a mix of glandular and luminal epithelial cells is present in most studies employing an endometrial epithelial cell–embryo in-vitro model. This structure may not fully represent the cellular uterine epithelium interacting with the apposing embryo *in vivo*. An alternative to primary cells is the use of an endometrial epithelial cell line as an endometrium surrogate. The use of cell lines has a number of attractions: they are more easily handled; there are no limitations on supply of material; they are less expensive to procure; and they bypass some of the ethical concerns associated with the use of primary human or animal tissues. Cell lines also provide a purer source of cells, providing a consistent sample and reproducible results. Practically, they are easier to use for gene silencing approaches (Cervero et al., 2007). Despite being a powerful tool to study embryo implantation, immortal cell lines also have several drawbacks. Since cell lines have undergone significant mutations to become immortal, this may alter their phenotype, original functions and their sensitivity to stimulation. Consecutive passage of cell lines can further induce genotypic and phenotypic changes. The other major concern using cell lines is contamination with other cell lines or mycoplasma. A recent study to verify the identity of several of the most commonly used endometrial epithelial cell lines indicated that several were contaminated with other cells, suggesting that this problem is serious (Korch et al., 2012). Cell lines should display and maintain functional features as close to the primary cells that they are used to model (Apps et al., 2011). The most common cell lines used for the study of embryo implantation are described in detail by Hannan et al. (2010).

Using embryo surrogates

In order to compensate for the limited availability of human embryos, a number of studies have employed embryo surrogates (Figure 1B). Although a few studies have been conducted with mouse blastocysts (Cervero et al., 2007; Singh et al., 2010), most studies have used trophoblast spheroids (cluster of cells that form spheres) derived from cell lines as an alternative to human embryos, making the model more readily available and much simpler to manipulate.

The creation of trophoblast spheroids can be achieved by the culture of an immortal trophoblast cell line in either low-attachment plates (Gonzalez et al., 2011; Weimar et al., 2012) or in rotating glass tubes (Holmberg et al., 2012). Trophoblast spheroid formation generally occurs between 24 h and a few days in culture, depending on the technique applied. In a study that compared the two methods, spheroids generated in low-attachment plates were shown to be more consistent regarding both size and structure (Holmberg et al., 2012). The most commonly employed trophoblast cell line for creation of spheroids is the JAr cell line (John et al., 1993), as they are readily generated and the cells express placental hormones HCG and progesterone. This choriocarcinoma cell line develops directly from trophoblastic placental tumour tissue and has both villous and extravillous characteristics (Hannan et al., 2010; Mandl et al., 2006; White et al., 1988).

Models employing trophoblast spheroids have been used successfully to study embryo attachment with various human endometrial epithelial cell-like cell lines. These include the glandular epithelial cell line RL95-2, derived from moderately differentiated adenosquamous carcinoma cells (Galan et al., 2000; Liu et al., 2011; Xiong et al., 2012), and the endometrial cell line with luminal epithelial cell characteristics ECC-1, from adenocarcinoma origin (Heneweer et al., 2003; Ho et al., 2012; Hohn et al., 2000; Holmberg et al., 2012; Mo et al., 2006). As trophoblast cell lines and mouse blastocysts are known to be highly adhesive to RL95-2 cells, this cell line is widely used as a model for receptive endometrial epithelial cells. Other groups use another endometrial cell line to study embryo attachment, the 'Ishikawa' adenocarcinoma cell line. Expressing both glandular and luminal epithelial characteristics, it is used to study endometrial epithelial cell attachment in a co-culture model with JAr trophoblast spheroids (Heneweer et al., 2005; Uchida et al., 2007). Hombach-Klonisch et al. (2005) immortalized human primary endometrial epithelial cells isolated from normal proliferative-phase endometrium by stably transfecting the catalytic subunit (hTERT) of the human telomerase complex and named the resulting cell line hTERT-EEC. These cells express many features of uterine luminal epithelial cells and show superior oestrogen and progesterone responsiveness compared with other endometrial epithelial cell lines Hombach-Klonisch et al. (2005).

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This cell line was used to establish an optimized co-culture implantation model for studying the influence of intrauterine infection on the attachment of JAr trophoblast spheroid attachment. Bacterial flagellin was shown to suppress attachment and this supression was dependent on toll-like receptor 5 (TLR5; Aboussahoud et al., 2010). However, the recent typing of endometrial cell lines using DNA microsatellite short tandem repeats suggests that the hTERT-EEC cell line has been contaminated by the breast cancer line MCF7 (Korch et al., 2012). This finding illustrates a key problem with the use of cell lines.

The RL95-2-JAr trophoblast spheroid combination has been used to investigate the Sialyl Lewis X (sLEX)/l-selectin adhesion system and its role in epithelial cell apoptosis at the implantation site, one of the events induced by the embryo following attachment (Galan et al., 2000). Indeed, the sLEX/l-selectin adhesion system effectively induced endometrial epithelial cell apoptosis, as was shown by the sLEX/l-selectin-induced up-regulation of FAS (or FASL, a cytokine belonging to the tumour necrosis factor family) in RL95-2 cells (Liu et al., 2011). The same model was applied to investigate the effect of calcitonin on uterine receptivity (Xiong et al., 2012).

Besides the JAr cell line, other cell lines have been used to generate trophoblast spheroids with different attachment rates. When the rate of attachment of spheroids derived from three different trophoblast cell lines, JAr, BeWo and Jeg-3, onto a monolayer of RL-95 endometrial epithelial cells was evaluated (Hohn et al., 2000), the percentage of Jeg-3 spheroids attaching was 75% compared with 45% observed with BeWo and JAr spheroids. These data indicate that in studies of interventions aimed at increasing embryo attachment rates, the BeWo and JAr trophoblast spheroids may be the models of choice. A key unanswered question is the extent to which adhesion by these cells to endometrial epithelial cell utilizes similar mechanisms to those employed by the trophectoderm cells of the blastocyst.

A fourth trophoblast cell line that has been used to create spheroids is the Sw.71 (first-trimester) trophoblast cell line. This cell line has stem cell characteristics, including the capacity to form spheroids (Straszewski-Chavez et al., 2005). The use of the Sw.71 cell line has been reported in combination with the ECC-1 cell line (Holmberg et al., 2012). An advantage of this combination is that the receptivity of the ECC-1 cell line for Sw.71 trophoblast spheroids can be modulated through addition of inflammatory cytokines to the culture medium. This model may therefore be particularly suitable for the evaluation of putative factors influencing endometrial epithelial cell receptiveness. However the analysis by Korch et al. (2012) also suggested that the integrity of the ECC-1 cell line may have been compromised. The key points relating to early-phase implantation models are summarized in **Table 2**.

B. Later-phase implantation models

Using embryos

After the short initial apposition and attachment phases, the embryo breaches the luminal epithelial and starts to invade the underlying decidua, consisting of endometrial stromal cells, various types of immune cells, including uterine natural killer cells, macrophages, dendritic cells and T cells, and vascular (endothelial) cells (Erlebacher, 2013). During this invasion-phase, the embryo primarily encounters decidual stroma. Therefore, in addition to models with endometrial epithelial cell-like cells and embryos or embryo surrogates, endometrial stromal cells may also be used as a monolayer in a co-culture model for the study of embryo–endometrium interactions in the later phase of embryo implantation (**Figure 1** and **Table 1**).

Primary human endometrial stromal cells are generally obtained from luteal-phase endometrial biopsies taken from fertile patients of reproductive age undergoing sterilization or hysterectomy for benign conditions (Carver et al., 2003; Evron et al., 2011; Weimar et al., 2012). Ideally, donors should have regular menstrual cycles and should not be receiving hormonal medication. The cells can be isolated by finely mincing the endometrial tissue into small pieces (mechanical digestion) followed by enzymic digestion in collagenase type I in a standard cell culture incubator. The resulting cell suspension is filtered through a 40-μm cell strainer and allowed to adhere to a culture flask with standard medium supplemented with 10% fetal calf serum. Following cell attachment, medium is replaced to select out non-adherent cells (intact glands, the majority of immune cells and epithelial cells). Alternative strategies are gradient centrifugation or magnetic bead isolation. The purity of the isolated cells should be verified by using immunohistochemistry with specific antibodies to vimentin (positive) and cytokeratin-7 (negative). Cells can then be used for experiments for up to 6–10 passages. Various protocols exist to decidualize endometrial stromal cells, including the administration of 8-Br-cAMP, medroxyprogesterone acetate or both for 3–5 days. To be able to use the cells for more passages with a stable phenotype, primary endometrial stromal cells have recently been immortalized by the transduction of telomerase. The resulting St-T1b cell line displays many features of endometrial stromal cells, such as decidualization after cAMP treatment, and forms a good alternative when primary cells cannot be obtained (Samalecos et al., 2009).

In an early co-culture model with endometrial stromal cells described by Mardon and colleagues (Carver et al., 2003), human primary undifferentiated endometrial stromal cells were grown to confluent monolayers. Subsequently, a hatched human blastocyst was placed on top of the monolayer for co-culture over 3 days. This model of implantation showed that blastocysts were able to attach to endometrial stromal cells and that trophoblast cells penetrate the in-vitro endometrial stromal cell compartment completely (Carver et al., 2003). The human endometrial stromal cell–embryo co-culture model was further employed to study embryonic trophoblast invasion into the stroma, the extent of which was shown to be dependent on both embryonic and stromal factors.

A key group of invasion modulators was shown to be Rho GTPases (Rac1 and RhoA), which belong to a family of proteins that have been shown of particular importance for cell migration (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). In co-culture experiments, it was demonstrated that Rac1 silencing in endometrial stromal cells inhibited

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Table 2 Key points for early implantation models.

In the assisted reproduction setting, co-culture of embryos with endometrial epithelial cells contributes to their development and implantation rate
Human embryos and primary uterine epithelial cells have been successfully used for the study of early embryo implantation, for instance in the study of endometrial receptivity markers
Epithelial cell lines (e.g. ECC-1, RL-95) are available as surrogates for primary epithelial cells; regular validation of cell line stocks by DNA microsatellite typing is essential to ensure cell line integrity
Cell-line-derived trophoblast spheroids can be employed successfully as an embryo surrogate

human embryo invasion (quantified by trophoblast spreading) into endometrial stromal cell monolayers, while RhoA silencing promoted embryo invasion (Grewal et al., 2008). A further key observation in this study was that the inhibition of ROCK (a RhoA inhibitor) promoted endometrial stromal cell migration, but that inhibition of Rac1 reduced cell migration and increased cell motility at the site of implantation.

In recent years, the human endometrial stromal cell–embryo co-culture model has been further developed to study the endometrial stromal cell response upon co-culture with human embryos from day 5 to day 8 of development. Validation studies have shown that embryos demonstrate normal embryonic lineages with segregated hypoblast and epiblast lineages (Teklenburg et al., 2012). In the same study, analysis of embryos indicated that the mechanisms contributing to X chromosome inactivation were functioning. The ability to maintain human embryos in culture to this later stage allows the in-vitro study of human embryo development beyond the stage at which they would normally initiate implantation *in vivo* or indeed be transferred to the uterus after IVF. Using this model, the incidence of chromosomal mosaicism exhibited by embryos was shown to fall from 83% on day 4 to 42% on day 8 (Santos et al., 2010).

This model also enables interrogation of the response of both undifferentiated and decidualized endometrial stromal cells to the presence of embryos of varying quality. After endometrial stromal cell co-culture with embryos for 3 days (from day 5 to day 8), supernatants were collected and analysed for a panel of cytokines and growth factors such as IL-1β, HB-EGF, IL-6, IL-10, IL-17, IL-18 and eotaxin known to play key roles during implantation (Teklenburg et al., 2010b). The endometrial stromal cell cytokine profile was measured in co-cultures with arresting and developing embryos, as well as in controls with no embryo. At the level of cytokine secretion, normal endometrial stromal cells showed little change in their cytokine secretion in the presence of a developing embryo. However, in the presence of an arresting embryo, the decidualized cells (not undifferentiated cells) showed decreased expression of a panel of implantation modulators. These findings provided the first indication that decidualizing endometrial stromal cells have the potential to function as a biosensor for the detection of poor embryo quality. This study was limited by the use of primary cell cultures from just one normal fertile individual and these preliminary findings require further confirmation.

To gain more insight into the dynamics of endometrial stromal cell migration during the implantation phase and to further investigate the biosensor concept in terms of selective migration towards high-quality embryos, the current study group employed a modified version of this model in which human decidualizing endometrial stromal cells migrate towards a human day-5 embryo. The model was expanded by using both high-quality embryos and chromosomally abnormal (3 pronuclei) embryos in combination with endometrial stromal cells from women with recurrent miscarriage. Analogous to the response to poor- and high-quality embryos reported by Teklenburg et al. (2010b), migration of cells from normal fertile women was inhibited in the presence of chromosomally abnormal embryos. This inhibition was not observed in decidualized endometrial stromal cells obtained from women with recurrent miscarriage (Weimar et al., 2012).

Thus, embryo selection may also be exerted via selective endometrial stromal cell migration. Women with recurrent miscarriage may be less selective for embryo quality and thus more receptive for embryo implantation. This reduced selectivity may increase the risk of poorly viable embryos implanting, resulting in a clinically revealed miscarriage. This mechanism, first proposed as the 'selection failure hypothesis' (Quenby et al., 2002) is more fully described elsewhere (Brosens and Gellersen ,2010; Lucas et al., 2013, this volume; Teklenburg et al., 2010a).

Despite its incomplete representation of the in-vivo components of the endometrial stroma, the human endometrial stromal cell–embryo co-culture model appears to provide an environment that is suitable for the developing embryo. This conclusion is supported by the observation of high concentrations of HCG derived from trophoblast cells measured in co-culture supernatants and by normal embryonic lineage development in the embryo (Teklenburg et al., 2010b, 2012; Carver et al., 2003). Similarly to the endometrial epithelial cell–embryo model, the endometrial stromal cell–embryo model forms a robust and relatively simple system in which embryo–endometrium interactions may be studied. Selective gene inhibition studies have also been reported using such a model (Jones et al., 2006). The primary disadvantage of this model, however, is that only one single endometrial cell type is studied, ignoring the in-vivo endometrial stromal cell–endometrial epithelial cell interactions and involvement of the abundant uterine leukocytes and vascular cells. Multilayer (3D) co-culture models form an attractive alternative, as will be discussed. Furthermore, it should be noted that variations in the use of undifferentiated or decidualizing endometrial stromal cells differentiated by varying protocols make any direct comparisons between studies difficult.

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Using embryo surrogates

Knowledge of endometrial stromal cell–embryo interactions is mostly derived from in-vitro models using surrogate human embryos (**Figure 1B** and **C**). These models use cell cultures of decidualizing endometrial stromal cells on which either a mouse embryo or a trophoblast spheroid derived from a human cell line is placed. Even simpler models using decidualizing endometrial stromal cells in co-culture with trophoblast cells or trophoblast-cell-conditioned medium have also been reported (Gellersen et al., 2013; Hess et al., 2007). Studies employing a co-culture model with mouse embryos and decidualizing human endometrial stromal cells to study embryo–endometrium interactions revealed the importance of endometrial stromal cell integrins in blastocyst development (outgrowth) and differentiation following attachment (Shiokawa et al., 1996) and showed that endometrial stromal cell histone deacetylase may promote trophoblast spreading of a day-4 hatched mouse embryo (as well as endometrial stromal cell migration and Jeg-3 trophoblast invasion) (Estella et al., 2012). In another heterologous in-vitro co-culture model, it was shown that inhibition of endometrial stromal cell motility suppressed mouse blastocyst invasion into the monolayer (Grewal et al., 2008).

An alternative to the endometrial stromal cell–embryo model is to co-culture human primary endometrial stromal cells with human trophoblast spheroids derived from cell lines. Trophoblast spheroids have been employed as an embryo surrogate in a co-culture model on top of an endometrial stromal cell monolayer (Gellersen et al., 2013; Gonzalez et al., 2011; Holmberg et al., 2012). An attractive alternative source of trophoblast cells for the preparation of spheroids are cytotrophoblast stem cells derived from human embryonic stem cells. When co-cultured for up to 6 days on a confluent monolayer of endometrial stromal cells, collected in the luteal phase of the menstrual cycle and primed with progesterone, these human embryonic stem cell–derived trophoblast spheroids closely imitate the early invasive stages of the implanting embryo (Harun et al., 2006). In an implantation model using AC-1M88 trophoblast spheroids, trophoblast expansion was enhanced in the presence of decidualized endometrial stromal cells compared with cultures of undifferentiated endometrial stromal cells (Gonzalez et al., 2011).

Since migration of endometrial stromal cells was suggested to be an important step in embryo implantation in the early-phase co-culture model (Grewal et al., 2008), the current study group investigated AC-1M88 trophoblast spheroids in a cell migration assay. A cell-free 'scratch' (migration zone) was generated in a monolayer of decidualized cells (Liang et al., 2007). Decidualized cells derived from normal fertile women showed no difference in migration into the scratch in the presence or absence of a trophoblast spheroid, placed in the migration zone. However, decidualized cells derived from women with recurrent miscarriage showed enhanced migration in the presence of a trophoblast spheroid compared with the migration in the absence of a trophoblast spheroid (Weimar et al., 2012). Interestingly, decidualized cells derived from women with recurrent miscarriage also showed increased migration in the presence of a low-quality embryo compared with the migration in the absence of an embryo. This result suggests that trophoblast spheroids serve as a valid embryo surrogate in this assay.

As a resource for trophoblast cells, Holmberg et al. (2012) used the first-trimester trophoblast cell line Sw.71. After 2 h of co-culture with the telomerase-immortalized H-ESC cell line (Krikun et al., 2004), the trophoblast cells of the spheroid started to migrate towards the endometrial stromal cells in a polar manner. In addition, the trophoblast cells were capable of penetrating the monolayer, which was nicely visualized by two-colour fluorescence (GFP-labelled spheroids and tdTomado-labelled endometrial stromal cells).

A third model is the use of primary human trophoblast cells in co-culture with human endometrial stromal cells. This model has been employed to study endometrium–trophoblast interactions and the impact on the endometrial stromal cell gene expression profile by genome-wide microarray technology. In co-culture with trophoblast cells, 171 genes were found to be up-regulated and 119 genes were found to be down-regulated when compared with growth in the absence of trophoblast cells. Many genes were identified that were previously not known to play a role in endometrium–trophoblast interactions. Most of the up-regulated genes were involved in inflammatory responses and chemotaxis (e.g. *IL-8*, *CXCL2*, *CXCL1*, *PTX-3*), lipid and steroid metabolism, proteolysis (*MMP12*, *TIMP3*), development (e.g. *DKK1*), cell growth (e.g. *IGFBP1*) and oxidative stress responses (e.g. *SOD2*). Most of the genes down-regulated were related to cell motility, apoptosis, proteolysis (e.g. *MMP11*) and growth hormones (e.g. FGF) (Popovici et al., 2006).

A fourth variant of the endometrial stromal cell–embryo model is one in which human endometrial stromal cells are cultured in human trophoblast-conditioned culture media. By genome-wide gene expression profiling, the paracrine effect of trophoblast-derived factors (in supernatants derived from invasive first- or second-trimester human cytotrophoblast cultures) on human primary endometrial stromal cells was explored. After 3 and 12 h in culture, total RNA was isolated and processed for microarray analysis on 54,600 transcripts. There were 1374 genes significantly up-regulated and 3443 genes significantly down-regulated after 12 h of co-incubation of endometrial stromal cells with trophoblast-conditioned media, compared with incubation with control media. Among the most up-regulated genes were those involved in chemotaxis, cytokine production (e.g. *CXCL1*, *IL-8*) and proteolysis (*MMP1*, *MMP10* and *MMP14*). A cluster of growth factors (e.g. FGF1, TGFB1, angiopoietin-1) was identified from the down-regulated genes (Hess et al., 2007). The data from Popovici et al. (2006) and Hess et al. (2007) demonstrate a significant induction of proinflammatory cytokines and chemokines as well as factors associated with cell motility in endometrial stromal cells in response to trophoblast-secreted products. However, while comparing the two studies it must be noted that Popovici et al. (2006) used undifferentiated cells while Hess et al. (2007) used decidualized cells. This model system can evaluate the paracrine effects of trophoblast cells co-cultured with decidualized endometrial stromal cells in both directions. However, this approach ignores the fact that, *in vivo*, it is the extravillous trophoblast that interacts

with endometrial stromal cell while migrating through the decidualizing endometrium, whereas many studies employ medium conditioned by placental villi comprised of the villous trophoblast subsets, which have different secretory profiles (Apps et al., 2011).

To investigate the effect of extravillous trophoblast-derived products on endometrial stromal cell motility, multilayered trans-well migration and invasion assays are used under co-culture conditions (**Figure 1C**). Gellersen et al. (2010) adopted this model and showed that decidualizing cells migrate significantly more than undifferentiated cells in the presence of extravillous trophoblast (AC-1M88) secretory products. In addition, decidualizing endometrial stromal cells showed invasive behaviour in the Matrigel invasion assay, which was increased significantly in co-culture with extravillous trophoblast cells (Gellersen et al., 2010). The paracrine effect of endometrial stromal cell-secreted factors, derived from human in-vitro decidualized cells, on the invasive capability of trophoblast cells can also be studied in a similar fashion (Estella et al., 2012). The key points relating to later-phase implantation models are summarized in **Table 3**.

C. Complex in-vitro implantation models

The in-vivo implantation environment involves synchronous paracrine signals, cell–cell communication and cell–extracellular matrix interactions between multiple cell types of the developing embryo and the decidualizing endometrium. This complexity is illustrated by the endometrial stromal cell response to paracrine signals from trophoblast cells (Hess et al., 2007) and endometrial stromal cells influencing the development of epithelial cells (Mahfoudi et al., 1992). More extensive models have been designed to try and imitate the in-vivo implantation milieu more closely. These complex implantation models include multilayer co-culture models, created by using whole endometrial explants or stacking multiple single-cell monolayers, or multilayer invasion assays (**Figure 1C**).

More than 50 years ago, the in-vitro co-culture of rabbit blastocysts on a rabbit uterus organ culture was reported (Glenister, 1961). The key advantage of this system was the close simulation of the in-vivo situation. However, the model was limited by the difficulty of keeping the explant cells alive; necrotic cells would appear in the uterine tissue within hours. Similar problems were encountered in other organ explant culture systems using mouse and human blastocysts (Grant et al., 1975; Landgren et al., 1996). In addition, explant cultures did not induce endometrial epithelial cell polarization, which is suggested to be an important step in embryo implantation (Bentin-Ley et al., 1994; Birkenfeld et al., 1988).

This led to the development of alternative multilayer co-culture models utilizing multiple cell layers seeded on top of each other, resulting in improved viability in culture (Bentin-Ley et al., 1994; Evron et al., 2011; Park et al., 2003; Wang et al., 2012). These models generally consist of a layer of endometrial stromal cells, which in some set ups reside within a matrix scaffold, imitating the extracellular matrix by using fibrin-agarose or collagen. A layer of endometrial epithelial cells is grown on top of this stromal layer, resembling the uterine epithelium, and can be separated by an artificial basement membrane of Matrigel (an extracellular matrix preparation rich in laminin and collagen). This type of model system more closely mimics the complex 3D tissue architecture during tissue regeneration than the simplified monolayer co-culture models on plastic or glass (Schindler *et al.*, 2006). The multilayered approach, with collagen as an extracellular matrix, was used to study embryo attachment. It effectively induces endometrial epithelial cell polarization, resulting in embryo attachment rates of 80% within 48 h. Moreover, electron scanning micrographs can be made of the in-vitro implantation site, enabling the visualization of the intimate interaction between the embryo and endometrial epithelial cell luminal surface (Bentin-Ley et al., 2000). This model provided novel insights into the process of embryo implantation from early trophoblast cell attachment to subsequent penetration of the endometrial epithelial cell and basement membrane layers followed by invasion of the endometrial stromal cell compartment.

An attachment assay between trophoblast spheroids and a multilayered endometrium model was subsequently developed by Wang et al., (2012), who constructed a multilayered co-culture model with both primary endometrial cell types (epithelial and stromal) and established cell lines (H-ESC cell line for stromal cells and the JAr cell line for trophoblasts). The endometrial epithelial cells grew in an organized polarized fashion on top of the stromal cell layer and even epithelial cell glands appeared to form

Table 3 Key points for later-phase implantation models.

Primary endometrial stromal cells can be isolated and maintained in culture relatively easily for multiple passages
There is no consensus on how to 'correctly' decidualize endometrial stromal cells for in-vitro experiments, making comparisons between laboratories difficult
Embryos or trophoblast spheroids in co-culture with endometrial stromal cells are valuable models for the study of the later phase of implantation when trophoblast cells start to invade the decidualizing endometrium
Co-culture with endometrial stromal cells modulates in-vitro embryo development
Migration of endometrial stromal cells is a hallmark of embryo implantation and can be studied in a 'scratch' assay in combination with an embryo or trophoblast spheroid
Genome-wide gene expression profiling allowed for the discovery of new genes involved in the complex interaction between trophoblast and endometrial stromal cells
Multilayer trans-well systems have been employed to study the influence of trophoblast secretory products on endometrial stromal cell migration and invasion

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spontaneously. The JAr spheroid attachment rate was reported to be highest in the presence of the Ishikawa cell line (Heneweer et al., 2005), followed by the model using primary endometrial epithelial cells. Poorer JAr spheroid adhesion rates were observed in the presence of the H-ESC cell line. In the multilayered in-vitro model by Evron et al. (2011), JAr spheroid attachment was highest in the presence of receptive-phase primary endometrial stromal cells, compared with the model in which non-receptive-phase endometrial stromal cells were used. Interestingly, progesterone treatment elevated spheroid attachment in the presence of endometrial epithelial and non-receptive-phase endometrial stromal cells (Evron et al., 2011).

The combination of established trans-well model systems for trophoblast invasion on the one hand and endometrial epithelial cell–endometrial stromal cell interaction on the other, could lead to even more representative in-vitro embryo implantation models. Invasion assays are dual-chambered (Boyden chamber) systems widely used for the study of trophoblast invasion (Aplin, 2006; Lash et al., 2007). In short, a trophoblast cell suspension is allowed to adhere to a Matrigel-coated filter insert with 8-μm pores. After attachment to the Matrigel, trophoblast cells start to invade this extracellular matrix surrogate and filter. The amount of trophoblast cells that reaches the lower surface of the filter insert can be than quantified over time. Substances can be added to the insert allowing the study of undirectional motility (chemokinesis) and to the culture well for the study of directed motility (chemotaxis). This assay can also be adapted to more closely mimic trophoblast invasion during early implantation when integrated in a co-culture model that has been established to study paracrine interactions between endometrial epithelial and stromal cells (Arnold et al., 2001; Blauer et al., 2005; Classen-Linke et al., 1998; Pierro et al., 2001). Endometrial epithelial cells should then be seeded on the Matrigel prior to addition of a trophoblast surrogate and endometrial stromal cells can be cultured on the bottom of the culture plate (**Figure 1C**). Although cell–cell contact between the epithelial and stromal cell layers is lost, this model would provide some additional major advantages. It would be possible to easily quantify trophoblast invasion through the endometrial epithelial cell and Matrigel layer, during both chemokinesis and chemotaxis experiments. Furthermore, trophoblast invasion can be compared with migration when paralleled by the use of non-Matrigel coated inserts. This hypothetical model would also allow the study of phenotypic changes of trophoblast cells along the invasive pathway by comparing trophoblasts on the top and bottom of the filter insert. Changes in the endometrial stromal cell compartment may also be monitored throughout the experiment (using time-lapse microscopy). Addition of other endometrial cell subsets (e.g. uterine natural killer cells) in combination with endometrial stromal cells or alone would allow for even more experimental options. A practical issue could be migration or invasion of both endometrial epithelial cells and trophoblast cells through the insert membrane. This potential issue could be addressed by prelabelling or transfecting one of these cell types with a fluorescent marker (Holmberg et al., 2012) or evaluating the experiment afterwards by staining with a trophoblast- or endometrial-epithelial-cell-specific antibody. The key points relating to complex implantation models are summarized in **Table 4**.

Conclusions and future perspectives

Human embryo implantation is the rate-limiting step in both unassisted and assisted reproduction, and implantation failure is a major cause of infertility. To acquire better understanding of the human implantation environment, increasing attention has focused on studying human embryo–endometrium interactions. However, due to the inaccessibility of the human in-vivo implantation environment, several in-vitro models have been developed to study human embryo–endometrium interactions. No model fully imitates the normal human endometrium *in vivo*. The most 'complete' implantation models, such as that reported by Bentin-Ley et al. (2000), use human embryos in combination with multilayer endometrial cultures, consisting of both endometrial epithelial cells and endometrial stromal cells. However, even these more sophisticated approaches do not contain the vascular or immune cellular components that constitute the in-vivo decidualizing endometrium. In recent years, appreciation has grown of the important role played by maternal immune cells such as uterine natural killer cells at the implantation site. These cell types should also be considered for inclusion in future studies of embryo–endometrium interactions.

The in-vitro implantation models established thus far provide the means of analysing discrete parts of the embryo–endometrium interaction. They have added valuable insights to the understanding of the complex process of human embryo implantation. The simpler implantation models with fewer cell types have been shown to be valid approaches to study the crucial expression and interactions between the adhesion and chemokine molecules of both embryonic trophoblast cells and maternal endometrium (Dominguez et al., 2003; Liu et al., 2011). The development of a model that include receptive and non-receptive endometrial epithelial cells may prove to be a promising tool

Table 4 Key points for complex implantation models.

Multi-layer co-cultures more closely mimic the complex three-dimensional tissue architecture of the implantation site during embryo implantation
Besides endometrial epithelial cell–embryo and endometrial stromal cells–embryo interactions, such models enable interrogation of the dialogue between endometrial epithelial cells and endometrial stromal cells
New complex implantation models can be developed in a trans-well system with multiple cell types, allowing the study of endometrial stromal cells, endometrial epithelial cells and trophoblast cells in one set up

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Table 5 Practical considerations when setting up an in-vitro model of embryo implantation.

Obtain written informed consent after ethical approval and conform to the Declaration of Helsinki before collection of human tissues
Specifically define and describe characteristics of the patient/donor population
Confirm phenotype (e.g. endometrial stromal cells are vimentin positive and cytokeratin negative), purity and viability after primary cell isolation by immunohistochemistry (the same holds for cell lines)
Decidualize endometrial stromal cells before utilization in in-vitro experiments
Use a standardized protocol for decidualization of endometrial stromal cells and confirm the resulting decidualized endometrial stromal cells morphologically (e.g. shape, size), phenotypically and functionally (e.g. prolactin production)
Allow embryos to hatch before using them in co-culture and describe their specific stage of development (morula, blastocyst) and viability at the start and end of the experiment
(Co-)culture under standardized (5% CO_2, 37°C) and sterile conditions
Critically select endometrial cell types which are representative of those interacting with the embryo in the phase of implantation that is studied
Ideally, the model should be reproducible within the same set up as well as between different laboratories

to further explore the aetiology behind implantation failure, which is still poorly understood (Holmberg et al., 2012). The processes and interactions that occur later during implantation, in the period that the embryo is completely surrounded by endometrial stromal cells, have been well studied using trophoblast co-cultures with endometrial stromal cells. A key finding here is the observations of endometrial stromal cell motility that may promote implantation and of the role of endometrial stromal cell Rho GTPases in this process (Grewal et al., 2008). A modified endometrial stromal cell–embryo model in which endometrial stromal cells were left to migrate towards an embryo or trophoblast spheroid added to the understanding of the pathophysiology of recurrent miscarriage (Weimar et al., 2012).

In practice, the model of choice should represent the key cellular players pertinent to the specific phase of implantation under study. As there are many limitations inherent to the use of cell lines compared with primary cell cultures, the preferential approach is to include the use of embryos or primary trophoblast cells in combination with primary endometrial stromal cells/endometrial epithelial cells instead of cell-line-derived trophoblast (spheroid) or endometrial cells. However, the use of cell lines has certain benefits. Cell lines are readily accessible, more easily handled, are cost effective and form reasonable surrogates for endometrial stromal cells, endometrial epithelial cells and trophoblast cells. These aspects represent major advantages when combining multiple cell types in complex models.

Some studies have reported the use of non-decidualized endometrial stromal cells. Because decidualizing endometrial stromal cells are the primary endometrial stromal cells present in the implantation environment and these cells are critical in allowing correct trophoblast invasion to occur, undifferentiated endometrial stromal cells should only be used as a model for non-receptive endometrium (Gellersen et al., 2010). In-vitro decidualization of endometrial stromal cells is therefore a key step when modelling later phases of implantation. Unfortunately, while in-vitro decidualization will normally involve incubating endometrial stromal cells with cAMP and progesterone, procedures are not uniform, making comparisons between studies more difficult.

Looking to the future, recent findings and new techniques may be incorporated into model systems to study embryo–endometrium interactions in a clinical context, as bioassays guiding individual patient management. For instance, the use of implantation models which include endometrial epithelial and stromal cells obtained from women with recurrent miscarriage, unexplained implantation failure and endometriosis provides exciting opportunities to advance the understanding of these poorly understood yet common and distressing conditions. Studies with endometrial stromal cells from women diagnosed with recurrent miscarriage strongly suggest an epigenetic component, since the endometrial stromal cells from such patients respond differently to embryos compared with those from fertile women, even after multiple passages in culture. Although this raises major questions about the validity of models employing transformed endometrial epithelial and stromal cell lines, it clearly demonstrates how embryo–endometrium co-culture models can reveal novel mechanisms underlying reproductive failure. The development and clinical introduction of time-lapse imaging (Aparicio et al., 2013, this volume; Futures in Reproduction Symposium, in press) to monitor early embryogenesis offers the prospect of developing this technology into a robust and clinically feasible means to monitor embryo–endometrium interactions in individual patients (see also Brison et al., 2013, this volume; Futures in Reproduction Symposium, in press). In-vitro models of human embryo–endometrium interactions are now establishing themselves as very valuable research tools for studying a complex and fragile in-vivo process. Their potential as diagnostic and therapeutic tools is yet to be exploited. The practical issues that should be considered when setting up an in-vitro model for embryo implantation are summarized in **Table 5**.

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