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Molecular mechanisms of membrane interaction at implantation

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ABSTRACT

Successful pregnancy is dependent upon the implantation of a competent embryo into a receptive endometrium. Despite major advancement in our understanding of reproductive medicine over the last few decades, implantation failure still occurs in both normal pregnancies and those created artificially by assisted reproductive technology (ART). Consequently, there is significant interest in elucidating the aetiology of implantation failure. The complex multi-step process of implantation begins when the developing embryo first makes contact with the plasma membrane of epithelial cells within the uterine environment. However, although this biological interaction marks the beginning of a fundamental developmental process, our knowledge of the intricate physiological and molecular processes involved, remains sparse. In this synopsis, we aim to provide an overview of our current understanding of the morphological changes which occur to the plasma membrane of the uterine endothelium, and the molecular mechanisms that control communication between the early embryo and the endometrium during implantation. A multitude of molecular factors have been implicated in this complex process, including endometrial integrins, extra cellular matrix molecules, adhesion molecules, growth factors, and ion channels. We also explore the development of *in vitro* models for embryo implantation to help researchers investigate mechanisms which may underlie implantation failure. Understanding the precise molecular pathways associated with implantation failure could help us to generate new prognostic/diagnostic biomarkers, and may identify novel therapeutic targets.

Keywords: implantation failure, embryo, endometrium, receptivity

INTRODUCTION

The generation of a successful pregnancy requires the implantation of a competent blastocyst into a receptive endometrium. Despite advances in our understanding of fertility, implantation failure still remains a significant problem for both spontaneous and assisted pregnancies (Margalioth et al., 2006; Cha et al., 2012). While 25% of embryos implant successfully following *in vitro* fertilization (IVF), it is not yet possible to accurately determine the rate of implantation failure in humans because we have yet to develop a reproducible method with which to evaluate the pre-implantation period (Diedrich et al., 2007; de Mouzon et al., 2010; Cha et al., 2012). Successful implantation of the embryo represents a fundamental biological interaction involving intricate molecular signalling between the implanting embryo and the host endometrium (Dey et al., 2004; Nimbkar-Joshi et al., 2009). Acquiring an enhanced understanding of the molecular signalling networks that coordinate strategies for successful implantation is critical to our basic knowledge of human reproduction and may lead to novel approaches to improve the outcome of both natural pregnancies and pregnancy via assisted reproductive technology (ART). However, our understanding of the cellular and molecular pathways underlying implantation remain disappointingly limited (Singh et al., 2011).

The physiological and molecular processes initiated during implantation are complex and highly organized. The uterus becomes receptive to an embryo only during a discrete temporal phase known as the 'window of implantation'. During this phase, ovarian steroids induce a number of physiological changes in the endometrium to create a receptive and appropriate environment (Dey et al., 2004). The window of implantation is a limited period of time when blastocyst competency coincides with a

receptive uterus. If this coordination is out of phase, implantation becomes defective and fails (Cha et al., 2012).

The first physiological contact between an implantation-competent blastocyst and the receptive uterus involves sequential stages of apposition, adhesion, and invasion, which together, steer the process of successful implantation (Norwitz et al., 2001; Dey et al., 2004). During apposition, a multitude of small protrusions, referred to as uterodomes (or pinopodes) develop on the apical surface of the luminal uterine epithelium and subsequently inter-digitate with the blastocyst via surface microvilli on the apical cytotrophoblast (Figure 1; Bentin-Ley et al., 1999; Lopata et al., 2002). Once the embryo has settled upon the endometrium, a series of interactions take place to provide more stable adhesion with the uterine epithelium. Blastocyst attachment with the luminal epithelium overlaps with decidualization of the endometrial stroma, which is required for successful implantation. Once adhesion is complete, the trophoblast differentiates into the syncytiotrophoblast and cytotrophoblast. Located on the endometrial side of the embryo is the inner cell mass which penetrates the syncytiotrophoblast via the uterine epithelium and the underlying basement membrane. Eventually, the inner cell mass invades the endometrial stroma to interact with the maternal vasculature (Bentin-Ley and Lopata, 2000; Carson et al., 2000).

The events that orchestrate the crucial cross-talk between the embryo and the uterine epithelium are primarily coordinated by estrogen and progesterone from the ovaries, however, the molecular dialogue that originates locally from the mother and embryo which governs the orderly stages of these events is poorly understood (Dey et al., 2004; Cha et al., 2012). Unravelling the molecular foundation of implantation and implantation failure has significant implications for both understanding the underlying cause of infertility as well as determining patient prognosis.

MORPHOLOGICAL CHANGES TO THE PLASMA MEMBRANE

Contact between the plasma membrane of uterine epithelial cells and that of the developing trophoblast represents a common beginning to implantation. The luminal epithelium is perceived as a major mediator of uterine receptivity, transmitting signals to other compartments, since this is destined to be the site of blastocyst attachment (Murphy, 2004). There are a series of important events involved in preparing the luminal epithelium for implantation, as described below.

APICAL MEMBRANE CHANGES

The apical epithelial surface, in addition to providing an immune barrier (innate and acquired via immunoglobulin A), is the first site of interaction with the embryo, and thus, features of the apical human plasma membrane are of particular clinical interest. Uterodomes, originally known as ‘pinopodes’, are large, rounded, smooth-surfaced projections of the apical plasma membrane and have been identified in the uterine epithelium of a range of species during early pregnancy (Daikoku et al., 2004; Nallasamy et al., 2012). These smooth-surfaced projections appear during the receptive phase for blastocyst implantation in humans, and represent important indicators of normal endocrine progression, as well as uterine receptivity for blastocyst implantation (Murphy, 2004). The formation of uterodomes demonstrates the ability of the luminal epithelium plasma membrane to transform from a microvillous plasma membrane into a smooth and flattened plasma membrane (Murphy, 2004).

Interestingly, transmission electron microscopy has revealed that disruption of the apical junction complex creates additional space between the lateral borders of

epithelial cells for trophectoderm intrusion. Direct membrane-to-membrane interactions are then able to occur between the trophectoderm and the lateral (not apical) surfaces of endometrial cells. These findings indicate that interaction between the embryo and apical luminal epithelium surface is transient, and suggest that interactions with the lateral borders play a key role in implantation (Murphy, 2004).

BASAL AND LATERAL MEMBRANE CHANGES

In several species, the basolateral plasma membrane of luminal epithelial cells undergoes significant transformation at the time of implantation. Noteworthy changes occur in various junctions involving the lateral plasma membrane of uterine epithelial cells, which give epithelial cells their unique character (Murphy, 2004). During the mid-secretory phase in humans, tight junctions move to a deeper level in the luminal epithelium and tight junction claudin 1, 4, and 5 proteins begin to concentrate in the lower parts of lateral cell borders (Murphy, 2004). In both mice and humans, desmosomes, another junction of the lateral plasma membrane, show both morphological changes and down-regulation of certain desmosomal proteins by the time of uterine receptivity (Illingworth et al., 2000). It has also been shown that adheren junctions, along with their associated terminal web, are lost completely from the lateral plasma membrane by day 6 of pregnancy in rats, although this has not been demonstrated in other species (Murphy, 2000). Another common change during early pregnancy is an increase in the thickness of the basal lamina (Murphy, 2004). Components of the lateral plasma membrane, in particular junction complexes, are transformed during the period leading up to uterine receptivity in preparation for implantation.

MOLECULAR CHANGES TO THE PLASMA MEMBRANE

Under the influence of ovarian hormones, and in the period preceding implantation, the uterine epithelial layer acquires a receptive state (Carson et al., 2000; Lessey, 2011). Implantation is a complicated bi-directional process between the embryo and the endometrium and is regulated by complex signalling pathways. This process relies upon the fact that the embryo and receptive endometrium develop in a synchronous fashion (Simon et al., 2000ab; Garrido-Gomez et al., 2010). Presently, little is known about the specific molecular mechanisms that control communication between the early embryo and the endometrium during the window of implantation, and a variety of molecular factors including endometrial integrins, extra cellular matrix molecules, adhesion molecules, growth factors, and ion channels have been implicated (Calmak and Taylor, 2011).

ENDOMETRIAL INTEGRINS

Endometrial cells are host to a wide range of integrins, which are known to mediate a number of cellular processes, including adhesion, migration, and invasion in response to both intracellular and extracellular signals (Lessey et al., 1992; Singh and Aplin, 2009). Some integrins are constitutively expressed on the luminal epithelium, while others, located in the uterus, are regulated during the menstrual cycle in a spatial and temporal fashion (Bowen and Hunt, 2000; Singh & Aplin, 2009). Integrins exhibiting increased levels of expression during the mid-luteal phase of cycle are believed to represent useful markers for uterine receptivity, and integrins of the $\beta 1$ and αv subfamilies have been widely investigated for their relative roles in regulating implantation (Aplin and Kimber, 2004). Embryos arising from integrin $\beta 1$

knockout mice undergo implantation failure, largely because they are unable to adhere to, or invade, the basement membrane of the endometrium (Brakebusch et al., 1997). Furthermore, mouse embryos lacking the integrin αv gene die owing to defective vasculogenesis and angiogenesis (McCarty et al., 2002).

$\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 6$ are expressed by the trophectoderm during early apposition and adhesion (Sutherland et al., 1993; Bloor et al., 2002) while $\alpha 1\beta 1$, $\alpha 6\beta 1$, and $\alpha 7\beta 1$ tend to dominate during embryonic invasion (Bowen and Hunt, 2000). In early blastocysts, $\alpha 5\beta 1$ is initially expressed by cells located between the inner cell mass and the blastocoel cavity. However, as the trophectoderm differentiates, $\alpha 5\beta 1$ undergoes translocation to the apical surface of trophoblast cells, which are the first cells to come into contact with the uterus (Clark et al., 2005). Two lines of evidence indicate that integrin $\alpha 4\beta 1$ is associated with successful implantation. Firstly, reduced expression of $\alpha 4\beta 1$ in the endometrial stroma have been associated with recurrent miscarriage (Skrzypczak et al., 2001). Secondly, intrauterine blocking using a specific antibody raised against $\alpha 4\beta 1$ led directly to a failure to implant or delayed implantation (Basak et al., 2002).

Integrin $\alpha v\beta 3$ is of particular interest in terms of implantation due to the presence of $\alpha v\beta 3$ ligands on both the embryo and endometrial epithelium in humans (Lessey, 1994, 1995; Casals et al., 2012), and in other mammals (Aplin et al., 1996; Johnson et al., 2001; Kaneko et al., 2011). Moreover, defects in the expression of $\alpha v\beta 3$ has been associated with infertility, endometriosis (Lessey et al., 1994) and polycystic ovarian syndrome (Apparao et al., 2002). Moreover, an absence or delayed expression of integrin $\beta 3$ has been reported in patients with unexplained infertility and menopausal endometrium (Lessey et al., 1995).

Integrin $\alpha\text{v}\beta 3$ has been shown to localise in the luminal and glandular epithelium of both mice and humans during the window of implantation (Lessey et al., 1992; Lessey et al., 1994; Merviel et al., 2001; Srinivasan et al., 2009). It has also been shown to be expressed in the mouse and rat blastocyst (Sutherland et al., 1993; Kaneko et al., 2011). Using an *in vitro* rat model, Kaneko, et al. (2011) showed that integrin $\beta 3$ moves from the cytoplasm of trophoblast cells to the apical membrane in response to embryo attachment. However, this phenomenon failed to occur when rat embryos adhered onto glass coverslips, suggesting that the apical re-location of $\beta 3$ in trophoblast cells may be induced by specific patterns of crosstalk between the embryo and the luminal epithelium and that $\beta 3$ might be important during initial interactions between the embryo and endometrium (Kaneko et al., 2011).

In the human endometrium, integrin $\alpha\text{v}\beta 3$ exhibits a mid-secretory phase increase of expression influenced by ovarian hormone signalling (Daftary et al., 2002; Lessey, 2002). Indeed, $\alpha\text{v}\beta 3$ is up-regulated in immature ovariectomised rats in response to exogenous progesterone, or a combination of estrogen and progesterone, indicating that the epithelial expression of $\alpha\text{v}\beta 3$ is regulated, at least in part, by maternal hormones, and progesterone in particular (Srinivasan et al., 2009). Moreover, embryo-derived interleukin-1 (IL-1) can initiate the up-regulation of epithelial $\alpha\text{v}\beta 3$ at the specific site of embryo attachment. *In vitro* experiments showed that IL-1 was secreted only when embryos were co-cultured with endometrial epithelial cells, and that once secreted, IL-1 bound and activated endometrial IL-1 receptor type I (De los Santos et al., 1996; Simon et al., 1997, Van Sinderen et al., 2013). The consensus of opinion, therefore, is that IL-1 may function as a paracrine factor in order to regulate $\alpha\text{v}\beta 3$ in the endometrial epithelium during implantation.

A number of studies have reported that the expression of epithelial $\alpha\beta3$ is associated with the window of implantation and that the endometrium is devoid of this particular integrin in certain cases of infertility (Lessey et al., 1992; Castelbaum et al., 1994), endometriosis (Lessey et al., 1994; Lessey, 2002; Wei et al., 2009; Casals et al., 2012), and idiopathic infertility (Lessey et al., 1995). The level of integrin $\beta3$ mRNA on day 21 has been suggested as a possible indicator with which to predict success rates following ART, while $\alpha\beta3$ has been considered as a clinical biomarker for human implantation (Elnashar and Aboul-Enein, 2004). Data suggests that maternal cycle-dependent $\alpha\beta3$ expression in the epithelium may be a prerequisite for endometrial receptivity and that embryo-derived signals could be essential factors for the mediation of local cell-cell interaction between the implanting embryo and the host epithelium.

OSTEOPONTIN (OPN)

By binding to integrins, osteopontin (OPN), an extra-cellular matrix (ECM) protein, is able to mediate a range of biological events, including the maintenance of tissue integrity, bone mineralisation, and angiogenesis (Johnson et al., 2003a; Standal et al., 2004). During the early secretory phase, OPN is expressed weakly inside the cells of the human endometrial epithelium adjacent to the cell surface membrane, and its expression increases gradually in response to progesterone (Apparao et al., 2001; Carson et al., 2002; Quenby et al., 2007). Comparative global gene expression studies of the human endometrium have illustrated this increase in OPN following the luteinizing hormone (LH) surge (Kao et al., 2002). During the mid- to late-secretory phase of the menstrual cycle, OPN has been identified on the apical surface of uterine luminal and glandular epithelial cells, particularly in the vicinity of pinopodes

(Quenby et al., 2007). The expression of OPN subsequently extends into the decidualising stroma and embryonic trophectoderm (Omigbodun et al., 1997; Quenby et al., 2007), a distinctive pattern of events that has also been identified in other mammals, including pigs, rabbits and sheep (Johnson et al., 1999, 2003b; Garlow et al., 2002; Apparao et al., 2003; White et al., 2005). Moreover, while knockout OPN mice are fertile, they exhibit reduced pregnancy rates during mid-gestation, suggesting peri-implantation pregnancy loss (Weintraub et al., 2004), highlighting a functional role for OPN during pregnancy.

Interaction between OPN and $\alpha v \beta 3$ also appears to play a role in regulating embryo implantation (Casals et al., 2008; Altmae et al., 2012; Kang et al., 2014). Levels of both proteins are increased in the epithelium of the human endometrium during the mid- to late-secretory phase, and have thus been proposed as clinical markers for discriminating receptive from non-receptive endometrium (Apparao et al., 2001; Lessey, 2002; Lessey and Castelbaum, 2002; Quenby et al., 2007; Erikson et al., 2009). Binding of OPN to integrins activates integrin receptors and cytoskeletal proteins and subsequently promotes focal adhesions in the trophectoderm of embryos. Polystyrene beads coated with OPN were shown to activate $\alpha v \beta 3$ at focal adhesion sites, indicating that successful binding of these two proteins could enhance the attachment of trophoblast cells to the epithelium (Johnson et al., 2001). It has also been suggested that both integrin $\alpha v \beta 3$ and OPN may be related to endometrial receptivity for implantation and that OPN binds to two populations of $\alpha v \beta 3$, on the embryo and endometrium respectively, in order to facilitate connections between these surfaces for implantation (Figure 2; Apparao et al., 2001).

The functional significance of increased OPN expression in the secretory-phase endometrium requires further investigation, however, its temporal and spatial

patterns of expression suggest an important role during implantation and placentation. Moreover, the aberrant expression of OPN has been linked with both idiopathic infertility and polycystic ovarian syndrome (PCOS)(Quenby et al., 2007; DuQuesnay et al., 2009).

CELL SURFACE ASSOCIATED MUCIN 1 (MUC1)

The apical surface of the uterine epithelial layer is coated by glycocalyx, a biological barrier which prevents microbial and proteolytic attack. This glycocalyx allows the uterus to become accessible to a prospective embryo (Thathiah and Carson, 2002). The epithelial glycocalyx contains higher molecular mass mucin glycoproteins, which are key attributes of the apical epithelial surface. The absence of such mucins from the basolateral cell surface in normal luminal endometrial epithelium defines these cells as showing a primary polarity and an apical barrier. The anti-cellular adhesion molecule mucin 1 (MUC1), an integral membrane glycoprotein expressed on the apical surface of secretory epithelial cells, is a major component of interest in the glycocalyx (Meseguer et al., 1998). MUC1 is capable of sterically hindering interactions with other cell surfaces that are mediated by adhesion molecules with smaller conformations, due to its high expression, density of glycosylation, and extended conformation (Hilkens et al., 1992). MUC1 is thought to be one of the first molecules on the uterine surface to encounter the implanting embryo, and is thus of significant interest to scientists investigating the process of implantation.

However, the potential role of MUC1 in regulating endometrial receptivity appears to vary across species. In mice and rabbits, MUC1 levels are reduced during implantation (Aplin and Hey, 1995; Surveyor et al., 1995; Hoffman et al., 1998; Meseguer et al., 1998,2001; Dharmaraj et al., 2010). Conversely, in humans, MUC1

levels increase during the mid-secretory phase and peak 7 days after the LH surge luteal hormone surge to coincide with embryo implantation (Meseguer et al., 1998). Multiple studies have reported that patients suffering from recurrent implantation failure have significantly lower levels of MUC1 in the endometrium during the window of implantation compared to healthy fertile women (Aplin et al., 1996; Xu et al., 2012; Bastu et al., 2015). Moreover, patients experiencing infertility due to hydrosalpinx, a condition in which the distal end of a fallopian tube is blocked with serous fluid, have exhibited lower MUC1 expression in the endometrium (Li et al., 2010; Song et al., 2012). Human MUC1 is only moderately expressed upon the apical surface of the glandular and luminal epithelium during proliferation and is under the control of progesterone. Despite differences between species, consensus of opinion appears to suggest that MUC1 plays a key role in the acquisition of endometrial receptivity. It is possible that the intrinsic heterogeneity of MUC1 glycosylation may allow receptive sites upon the endometrium to be recognised, and that embryos may recognise MUC1-associated glycans, or its protein backbone (Meseguer et al., 2001; Horne et al., 2006).

In vitro studies on implantation have shown that MUC1 is down-regulated specifically in the region of the implanting blastocyst in both humans and mice (Meseguer et al., 2001; Singh et al., 2010). Low levels of MUC1 in the region of the implanting blastocyst during the window of implantation is hypothesized to be an important factor for successful embryo-endometrium interactions during implantation, since MUC1 otherwise conceals the expression of cell adhesion molecules that are important for blastocyst attachment (Figure 3; Horne et al., 2005; Singh et al., 2010, Bastu et al., 2015). Moreover, paracrine signals from embryos appear to regulate MUC1 to alter local epithelial phenotypes, possibly via growth factors including

insulin-like growth factor (IGF)(Fluhr et al., 2008) and heparin-binding epidermal growth factor (HB-EGF)(Paria et al., 2001; Pochampalli et al., 2007; Lim and Dey, 2009). MUC1 therefore appears to be a key player in regulating endometrial receptivity for implantation and should be the focus of continued research.

NOTCH SIGNALLING

A diverse range of cellular processes are regulated via Notch signalling pathways, including cell invasion, apoptosis, adhesion, and differentiation (Bray, 2006; Leong & Karsan, 2006). Humans express four notch receptors (1-4), which are transmembrane proteins with an extracellular and intracellular domain, and all four of these are present in the endometrium (Mitsunashi et al., 2012; Mori et al., 2012). Notch1-3 are additionally expressed by the trophectoderm of human blastocysts (Aghajanova et al., 2012). Notch ligands and receptors are localized to the apical surface of the luminal epithelium during the mid-secretory phase of the menstrual cycle where they are accessible to ligands and receptors on the trophectoderm in order to facilitate endometrial-embryo attachment (Cuman et al., 2014).

GROWTH FACTORS

During implantation, several growth factors bind to specific cell surface receptors and initiate downstream phosphorylation signalling cascades which, in turn, regulate the actions of the functional blastocyst and receptive uterus (Dey et al., 2004; Guzeloglu-Kayisli et al., 2009; Singh et al., 2011).

Heparin-binding epidermal growth factor (HB-EGF)

348 Heparin-binding epidermal growth factor (HB-EGF) is a juxtacrine, paracrine,
349 and autocrine signalling mediator. During the mid-secretory phase of the menstrual
350 cycle, HB-EGF is expressed in abundance on the apical surface of the receptive
351 luminal epithelium. HB-EGF is also expressed from 6 – 8 weeks of human pregnancy
352 in both the cytotrophoblast and syncytiotrophoblasts of the chorionic villi (Leach et
353 al., 1999; Hamatani et al., 2004a). HB-EGF is thought to be important during the
354 early dialogue between the embryo and endometrium since its expression has been
355 associated with increased rates of embryo hatching and development, and since it can
356 promote trophoblast outgrowth *in vitro* (Das et al., 1994; Martin et al., 1998).

357 HB-EGF, and its ErbB receptors, are expressed by the preimplantation embryo
358 as well as the luminal epithelium, indicating that HB-EGF-ErbB binding may serve as
359 a bi-directional signalling system between the embryo and endometrium to mediate
360 early attachment (Hamatani et al., 2004b; Nishi and Klagsbrun, 2004). Two types of
361 ErbB receptors exist on the embryo, ErbB1 which is localised to the inner cell mass,
362 and ErbB4 which is expressed on the surface of trophoblast cells, and in particular,
363 close to the inner cell mass (Paria et al., 1999). The differential localisation of ErbB
364 and ErbB4 receptors may suggest that these receptors adopt distinct roles during
365 implantation and that while juxtacrine and paracrine interactions are both required for
366 implantation, such interactions may occur in a differential manner (Chobotova et al.,
367 2002).

368 In mice, distinctive indicators of implantation, such as increased endometrial
369 vascular permeability at the site of apposition site, appear to coincide with the
370 temporal and spatial expression of HB-EGF, which appears to suggest a role in the
371 molecular pathways underlying implantation (Psychoyos, 1973; Das et al., 1994).
372 Paria et al. (2001) suggested a more defined role for HB-EGF during implantation

based upon the fact that when transferred to pseudo-pregnant mice, HB-EGF-soaked beads induced implantation-like responses, including the expression of HB-EGF on the luminal epithelium, increased vascular permeability, decidualisation, and the expression of a variety of implantation markers. However, blastocysts that were devoid of the HB-EGF gene successfully implanted on the uteri of wild-type mice, calling into question the necessity for HB-EGF expression on the blastocyst surface (Xie et al., 2007). Further investigations are required to elucidate the specific role of HB-EGF expression and binding with regards to implantation.

Insulin-like growth factor 1 (IGF-1)

Mitogenic insulin-like growth factors (IGFs) are thought to be involved in the proliferation and differentiation of several cell types during reproduction (Giudice et al., 1998). For example, insulin-like growth factor 1 (IGF-I) appears to guide a range of events, including endometrial proliferation (Rutanen, 1998), placental function (Murata et al., 1994), and preimplantation embryo development (Humbel, 1990). Under the influence of estrogenic stimulation, IGFs are expressed abundantly in endometrial stromal cells, particularly during the early-secretory phase (Fluhr et al., 2008). It has been hypothesised that in response to estrogen stimulation, IGF-I production mediates biological mitogenesis via paracrine mechanisms and binding to IGF-IR on the epithelium (Oner and Oner, 2007).

By increasing the number of cells in the trophoctoderm and inner cell mass, IGF-I can also enhance both embryo development and quality (Kim et al., 2005). By causing cytotrophoblast cells to proliferate and by enhancing syncytial formation via the MAP kinase pathway, IGFs appear to play an important role in both implantation and placentation (Forbes et al., 2008). While current literature suggests that IGF-I and

IGF-II facilitate the early stages of implantation by acting on both the endometrium and blastocyst, the precise role that IGF-I plays in the mechanism of embryo implantation remains to be elucidated (Van Sinderen et al., 2013).

GLYCODELIN A

Glycodelin A (GdA) is reportedly one of the most abundant glycoproteins in the secretory and decidualized endometrium and is thought to play a vital role in preparing the endometrium for implantation (Seppala et al., 2002; Uchida et al., 2007). During pregnancy, levels of GdA production in the decidua increases. It is theorized that since GdA exhibits immunosuppressive activity, it may facilitate embryo implantation and the maintenance of pregnancy by preventing the rejection of the foreign fetal allograft (Brown et al., 2000). Low levels of GdA are associated with recurrent miscarriage (Tulppala et al., 1995; Dalton et al., 1998) and idiopathic infertility (Mackenna et al., 1993). A recent study by Bastu et al., (2015) reported that women with repeated implantation failure exhibited significantly lower levels of GdA in primary endometrial cells and peripheral blood during the window of implantation, compared to healthy fertile females (Bastu et al., 2015). GdA is thus emerging as a candidate biomarker for assessing recurrent implantation failure and idiopathic infertility.

ION CHANNELS

Numerous different ion channels have been identified on the endometrium of different species, including humans and rodents. The specific functional roles of such ion channels are not yet fully understood, however evidence is emerging that some of these ion channels may be key players in the processes occurring during embryo

implantation (Ruan et al., 2014; Liu et al., 2014). The majority of ion channels in the endometrium are tightly regulated by ovarian hormones, or factors from the implanting embryo. Ion channel proteins exhibit their classical role in establishing a resting membrane potential, are regulated by steroid hormones (such as estrogen and progesterone), and function as a signal cascade initiator via stimulation from their ligand (Liu et al., 2014). For example, interplay between the cystic fibrosis transmembrane conductance regulator (CFTR) and epithelial sodium channel (ENaC), as well as potassium (K^+) channels, are vital in regulating uterine electrolyte and fluid transport. This is very important in terms of reducing the volume of uterine luminal fluid in order to lock the embryo in place prior to implantation (Nobuzane et al., 2008). ENaC also senses mechanical and chemical signals from the embryo and transduces downstream cellular responses to promote stromal decidualization (Enuka et al., 2012; Ruan et al., 2014). It has also been suggested that calcium (Ca^{2+}) channels play a role in regulating the adhesion of blastocysts to the endometrium, although the precise mechanism involved remains unknown and requires further investigation (Thie & Denker, 2002). The importance of ion channels in implantation is highlighted by implantation failure observed in animals or humans with aberrant expression or function of certain ion channels (Ruan et al., 2014).

THE NEED TO DEVELOP NOVEL RESEARCH METHODOLOGY

It is clearly evident from a mounting body of literature that numerous cell-cell and cell-matrix interactions are vital in modulating signal transduction to mediate embryo-endometrial interactions, uterine receptivity, and implantation. Such mechanisms are of huge importance as deficiencies are likely to manifest in

implantation failure or infertility. However, specific molecular cross-talk between these pathways remains poorly understood, and much work is needed to elucidate and manipulate these transitions. Such research may identify important prognostic or diagnostic biomarkers, or indeed novel therapeutic strategies.

Due to the multitude of technical and ethical restrictions involved in studying the precise site of human implantation *in vivo*, the majority of existing data regarding implantation are derived from animal studies (Mardon et al., 2007). Transgenic mouse models have helped to improve our understanding of the molecular basis of uterine receptivity and implantation in humans (Cha et al., 2012). While animal studies have been extremely helpful in providing clues to human implantation, there are many differences between species that need to be taken into account (Banerjee and Fazleabas, 2010). For example, murine rodents undergo eccentric implantation, where the luminal epithelium forms implantation chambers by invagination surrounding the trophoblast. (Dey et al., 2004; Reese et al., 2008). Conversely, primates undergo interstitial implantation, where the syncytial trophoblast formed near the inner cell mass adheres to the uterine epithelium and penetrates into the stroma by intruding between endometrial epithelial cells (Nimbkar-Joshi et al., 2009). In humans, specific interactions such as apposition and early embryo attachment to the uterine epithelium are difficult to observe due to the tenuous and transient nature of these mechanisms, and investigations rely on biopsies obtained during the estimated time of embryo attachment, or at predicted times in the menstrual cycle (Klemmt et al., 2009; Sela et al., 2013). There is a distinct lack of knowledge regarding the specific mechanisms underlying the early stages of implantation, and as the human implantation site is inaccessible *in vivo*, *in vitro* models are increasingly being developed and relied upon (Mardon et al., 2007).

473

474 *IN VITRO MODELS*

475 A variety of *in vitro* models have been developed in order to investigate
476 interactions between the embryo and endometrium. The primary driver for the
477 development of such models is the fact that it is almost impossible to study human
478 implantation *in vivo* (Mardon et al., 2007; Kang et al., 2014). *In vitro* models of
479 human implantation have been developed using tissues acquired by hysterectomy or
480 endometrial biopsy. These tissues can be co-cultured with trophoblast cells from
481 embryos to investigate embryo positioning and invasion (Kliman et al., 1990;
482 Landgren et al., 1996; Klemmt et al., 2009; Chillakuri et al., 2010). While
483 endometrial tissue explants can be cultured in appropriate media to mimic the *in vivo*
484 endometrial environment, tissue explant models are associated with the major
485 problem of extensive tissue necrosis, thus prompting the use of cell lines as a more
486 promising avenue.

487 Various cell lines have been investigated as a suitable *in vitro* representation
488 of the receptive endometrium. To examine later stages of implantation such as
489 invasion *in vitro*, human trophoblast cells and embryos have been cultured with
490 human endometrial stromal cells derived from patients with benign disease or primary
491 endothelial cells which have been decidualised by cyclic adenosine monophosphate
492 (cAMP)(Popovici et al., 2000; Carver et al., 2003). Models such as these have
493 allowed the observation of blastocyst outgrowth and invasion into decidualised
494 stromal cells, and potential mediators of invasion can be elucidated (Grewal et al.,
495 2008). While *in vitro* models demonstrating interaction between the embryo and
496 stromal cells have helped us to investigate the invasion phase of implantation, this
497 type of *in vitro* model is unable to explore the key initial events of implantation, such

as apposition, attachment, and adhesion. Since it has been shown that *in vitro* blastocysts attach non-selectively to endometrial stromal cells, as well as to tissue culture dishes, it is likely that it is the luminal epithelium that confers unique properties of implantation resistance in phases other than the mid-secretory phase (Carver et al., 2003; Aplin, 2007).

There is a clear need to develop improved *in vitro* models of luminal epithelium to investigate embryo attachment and early implantation. Current developments involve immortalized endometrial cell lines are derived from endometrial adenocarcinomas such as RL95-2, HEC-1, ECC1, and Ishikawa cell lines (Table 1; Hannan et al., 2010). These cells can be cultured in matrices such as collagen to create a three-dimensional model with different layers (Bentin-Ley and Lopata, 2000). Trophoblast spheroids and embryos can then be used with these luminal epithelium models to investigate early attachment (Ho et al., 2012; Uchida et al., 2012).

While multiple cells lines have been investigated, many have limitations such as lack of apical polarity, surface receptors, tight junctions, or adhesiveness to trophoblast cells. However, Ishikawa cells appear to be a promising model with which to investigate the normal endometrial epithelium and study potential endometrial crosstalk with the embryo. The Ishikawa cell line, originally derived from endometrial adenocarcinoma cells harvested from an Asian woman (39 years of age), expresses progesterone and estrogen receptors which are able to respond to steroid stimulation (Nishida et al., 1985). Moreover, this cell line exhibits a range of enzymes and proteins that are commonly found in the normal endometrium (Castelbaum et al., 1997; Mo et al., 2006). Ishikawa cells also express $\alpha 1\beta 1$ and $\alpha v\beta 3$ integrins, which are also present in the normal endometrium, and appear to play key

roles in endometrial receptivity and cell adhesion (Castelbaum et al., 1997). Investigations have shown that Ishikawa cells are an exciting model for investigating endometrial integrins (Lessey et al., 1992; Castelbaum et al., 1997). They grow stably, form a monolayer, and maintain epithelial polarity, however can exhibit some morphological changes following long periods of culture (Singh et al., 2010).

The development of an *in vitro* model which completely mimics the complex *in vivo* endometrial environment has yet to be developed. Nonetheless, *in vitro* models using epithelial cells provide a useful means with which to investigate potential interactions that might occur *in vivo* and allow workers to systematically evaluate the complex network of biological pathways involved in implantation (Teklenburg and Macklon, 2009; Weimar et al., 2013; Berger et al., 2015).

FUTURE DIRECTIONS

In the future, new techniques may be incorporated into model systems to study embryo-endometrium interactions in a clinical context, such as bioassays to guide individual patient therapies (Weimar et al., 2013). Markers such as periostin are already being investigated as clinical biomarkers for poor implantation (Morelli et al., 2014). The use of implantation models incorporating epithelial and stromal cells from women with endometriosis, recurrent implantation failure and idiopathic infertility would be an exciting venture to explore and advance our comprehension of these poorly understood conditions. Moreover, the development of time-lapse imaging to monitor early embryo development could be developed as a means to monitor embryo-endometrium interactions during attachment in research settings and perhaps one day in individual patients (Brison et al., 2013).

In vitro models of embryo-endometrium interactions have great potential for expansion in the coming years. An in-depth spatial and temporal analysis of key molecular stages in the fragile and complex process of implantation would be very useful in order to determine the molecular mechanisms involved in specific stages and disease. Robust *in vitro* models of embryo-endometrium implantation could represent a significant milestone in reproductive research, allowing the exploration of unseen mechanisms of implantation to suggest key clues to solve problems of infertility and pregnancy failure, and perhaps generate new prognostic/diagnostic biomarkers and/or therapeutic targets.

Figure legends

Figure 1. A schematic representation of early signalling between the embryo and endometrium preceding implantation. The blastocyst approaches the receptive endometrium, defined by the appearance of uterodomes and integrin profiles. (Adapted from Norwitz et al., 2001).

Figure 2. (A) Initial attachment of the embryo to the glycocalyx. (B) Second phase of attachment after clearance of apical glycocalyx MUC1 in response to embryonic signals. The lateral luminal epithelium borders open and trophoctoderm inserts in between the luminal epithelium cells. MUC1 = mucin 1; OPN = osteopontin; $\alpha\beta3$ = integrin $\alpha\beta3$. (Adapted from Aplin et al., 2007).

Figure 3. MUC1 as an anti-adhesion molecule acting as a barrier to embryo attachment to the apical surface of the uterine epithelium. MUC1 clearance allows access to the apical cell surface receptors for implantation.

Table legends

Table 1. Epithelial cell characteristics and hormone receptors expressed by different endometrial cell lines with respect to implantation

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