Fetal–maternal communication: the role of Notch signalling in embryo implantation

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Abstract

The establishment of a successful pregnancy requires the implantation of a competent blastocyst into a 'receptive' endometrium, facilitating the formation of a functional placenta. Inadequate or inappropriate implantation and placentation is a major reason for infertility and is thought to lead to first-trimester miscarriage, placental insufficiency and other obstetric complications. Blastocyst–endometrial interactions are critical for implantation and placental formation. The Notch signalling family is a receptor–ligand family that regulates cellular processes as diverse as proliferation, apoptosis, differentiation, invasion and adhesion. Notch signalling is achieved via cell–cell interaction; thus, via Notch, cells can have direct effects on the fate of their neighbours. Recently, a number of studies have identified Notch receptors and ligands in the endometrium, blastocyst and placenta. This review collates current knowledge of this large receptor–ligand family and explores the role of Notch signalling during implantation and placentation, drawing on information from both human and animal studies. Overall, the evidence suggests that Notch signalling is a critical component of fetal–maternal communication during implantation and placentation and that abnormal Notch expression is associated with impaired placentation and pre-eclampsia.

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Introduction

Establishment of a successful pregnancy requires implantation of a competent blastocyst into a 'receptive' endometrium, facilitating the formation of a functional placenta (Fig. 1). Inadequate or inappropriate implantation and placentation is a major reason for infertility (Dimitriadis *et al.* 2005, Koot *et al.* 2011) and is thought to lead to first-trimester miscarriage, placental insufficiency and other obstetric complications (Aplin 2010, Knofler 2010). 'Unexplained' infertility accounts for 25% of couples unable to conceive (Cahill & Wardle 2002). Additionally, even if embryos undergo chromosome screening and fresh embryo transfer, 33% will still fail to implant during IVF (Scott *et al.* 2013). Implantation failure in these cases is predominantly attributed to a non-receptive endometrium (Norwitz *et al.* 2001). Blastocyst–endometrial interactions are critical for implantation and placental formation; however, very little is known about human blastocyst–endometrial interactions, predominantly due to the difficulty in studying implantation in humans.

Recently, a number of studies have identified endometrial, blastocyst and placental expression of many members of the Notch signalling family. This review will assemble current knowledge of this large receptor–ligand family and explore the role of the Notch signalling family during implantation and placentation. While the expression of Notch in the placenta has been recently reviewed (Zhao & Lin 2012), this review will focus on the role of the Notch signalling throughout the entire implantation process, highlighting the importance of signalling pathways in establishing implantation and placentation.

Implantation and placentation

To facilitate successful implantation and placentation, the blastocyst and endometrium must be synchronized – the endometrium is receptive to blastocyst implantation for a short time period (4–5 days) during the mid-secretory phase (6–9 days following the luteinizing hormone peak) of the menstrual cycle (Paiva *et al.* 2009). This is

Figure 1 Protein production of Notch receptors and ligands during the establishment of pregnancy. (A) Mid-late secretory phase endometrium showing blastocyst apposition to a suitable region of the luminal epithelium. Trophoblast expression is from mRNA only. Stromal fibroblasts differentiate (decidualize) into decidual cells during the mid-secretory phase of the menstrual cycle regardless of conception/pregnancy. (B) Invasion of extravillous trophoblast (EVT) into the decidua and spiral artery (SA) remodelling during the first trimester. Cytotrophoblast in the cell column differentiate into EVTs, which invade into the decidua (red arrows). SA remodelling is initiated when uterine natural killer cells disrupt SA integrity via interactions with vascular smooth muscle cells. Subsequently, EVTs can invade and replace the SA endothelial cells creating spiral arteries lined by endovascular EVTs – remodelled SA (RSA). (C) Placental villous structure.

referred to as the ‘window of implantation’. For the remainder of the menstrual cycle, the endometrium is ‘refractory’ to implantation.

The uterine endometrium undergoes significant remodelling in preparation for pregnancy during every menstrual cycle (Paiva *et al.* 2009). Uterine glands differentiate into a highly secretory state, with altered expression of cell surface proteins and adhesion molecules on the apical and basolateral surfaces of the luminal epithelium. Stromal fibroblasts surrounding the spiral arterioles differentiate (decidualize) into morphologically and functionally distinct decidual cells (Fig. 1A; Popovici *et al.* 2000, Lunghi *et al.* 2007, Paiva *et al.* 2009). Immune cells are present in the uterus at all stages of the menstrual cycle, but during the mid-secretory phase associated with the onset of decidualization, there is an influx of macrophages and proliferation of uterine natural killer (uNK) cells (van Mourik *et al.* 2009, Salamonsen *et al.* 2009) such that during the first trimester, around 30–40% of the cells in the decidua are leukocytes (Bulmer *et al.* 2010). Leukocytes within the decidua are highly specialized compared with peripheral leukocytes (Erlebacher 2013).

The conceptus enters the uterine cavity up to 72 h prior to implantation (Norwitz *et al.* 2001) and it is likely

that during this time the blastocyst releases soluble factors that interact with the endometrial epithelium to induce or facilitate endometrial receptivity (Fig. 1A; Cuman *et al.* 2013).

Implantation and subsequent placentation is a continuum involving initially the apposition (Fig. 1A) and subsequent adherence of the blastocyst trophoblast to the endometrial luminal epithelium. Firm adhesion is the initiating event of implantation – failure of adhesion results in implantation failure or infertility. Following attachment, trophoblast cell proliferation, migration and invasion into the endometrium are critical events that ensure placental development. Cytotrophoblast cells within placenta differentiate into two main cell types: syncytiotrophoblasts and extravillous trophoblasts (EVTs). EVTs within the cell columns proliferate before subpopulations migrate and invade into the decidua. EVT cell migration and invasion are tightly regulated by numerous factors produced within the trophoblast–endometrial microenvironment (Salamonsen *et al.* 2009, Burton *et al.* 2010).

There are two subpopulations of invasive EVT (Fig. 1B): endovascular EVTs, which initially plug, then remodel maternal spiral arterioles into low resistance, high flow vessels creating the placental blood supply

at the end of the first trimester (Fig. 1B; Burton *et al.* 2010), and interstitial EVT (Fig. 1B), the role of which is unclear. Spiral artery remodelling begins in the absence of any invasive EVTs whereby uNK cells disrupt the vascular smooth muscle surrounding the arteries priming the arteries for EVT colonization (Harris 2011).

The placenta forms the interface between the maternal and fetal circulation. The fetal component of the placenta undergoes extensive morphogenesis to develop villous projections (Fig. 1C). The structure of the chorionic villous increases the available surface area, maximizing fetal–maternal exchange of nutrients, gases and wastes. Villi are lined by trophoblasts and contain a network of fetal capillaries that lead to the umbilical cord, circulating to the developing embryo (Fig. 1C). These villous projections are bathed in maternal blood as a result of EVT arterial remodelling that occurs early in placental development, as aforementioned (Enders & Carter 2012). During the formation of the placental villi (5–12 weeks gestation), post-proliferative cytotrophoblasts fuse with the overlying syncytium, into which the underlying mesenchyme invades to form the early villi (Kaufmann *et al.* 1985).

Vasculogenesis describes the formation of new vessels from mesenchymal progenitor cells within these early villi that eventually form the fetal capillaries. Although limited data are available on early villous vascularization in humans, reviewed by Castellucci *et al.* (2000), this is thought to be the underlying mechanism of placental villous vessel formation. From the 23rd week of gestation in women, mesenchymal villi transform into mature intermediate villi characterized by reduced cytotrophoblast density and increased capillary density and diameter to ensure efficient exchange (Kaufmann *et al.* 1985).

Notch signalling

The Notch signalling pathway regulates diverse cellular processes such as cell invasion, adhesion, survival, apoptosis and differentiation (Artavanis-Tsakonas *et al.* 1999, Bray 2006, Leong & Karsan 2006). In humans, there are four Notch receptors (1–4; Chu *et al.* 2011). Notch receptors are single pass transmembrane proteins composed of a functional extracellular domain (NECD), transmembrane and intracellular domain (NICD) (Hurlbut *et al.* 2007).

Canonical signalling

Canonical Notch signalling is achieved via cell–cell interaction, in which Notch receptors expressed on the surface of one cell interact with ligands present on the surface of a neighbouring cell (D'Souza *et al.* 2010). Signal-sending and signal-receiving cells can be of equivalent or differential cellular type (*trans*-activation) or alternatively intracellular auto-inhibition can occur

within the same cell (*cis*-inhibition). In humans, there are five membrane-bound ligands: the Delta-like (DLL) 1, 3 and 4 and Jagged1 and 2 (Kopan & Ilagan 2009). Canonical Notch ligands are transmembrane proteins containing EGF (Epidermal growth factor)-like repeats in their extracellular domain, as well as a single Delta or serrate & Lag-2 (DSL) domain necessary for the interaction with Notch receptors (Artavanis-Tsakonas & Muskavitch 2010, D'Souza *et al.* 2010). Notch ligand–receptor binding leads to receptor cleavage by proteases, including ADAM (A Disintegrin And Metalloprotease) and γ -secretase, and release of the NCID that translocates to the nucleus, where it binds to Recombining Binding Protein, Suppressor of Hairless (RBPSUH), and induces gene transcription of the Notch target genes (Ilagan & Kopan 2007, Kopan & Ilagan 2009), including members of the basic helix–loop–helix and hairy/enhancer of split/-related (e.g. Hes and Hey) families (Mikhailik *et al.* 2009, Mitsuhashi *et al.* 2012) and myc, cyclin D and p21 (Bray & Bernard 2010).

Non-canonical signalling

The non-canonical signalling pathway is not fully understood. Two signalling pathways have been identified as non-canonical: ligand-dependent and ligand-independent. Non-canonical ligand-dependent signalling refers to membrane-bound and secreted ligands that do not contain the DSL domain (D'Souza *et al.* 2010, Wang 2011). These proteins act as both ligands and modulators of Notch signalling and are comprehensively described in Wang (2011). Much less is known about non-canonical ligand-independent signalling (Andersen *et al.* 2012). Recent studies suggest that the normally membrane-bound Notch receptors localize in the cytoplasm and post-translationally target Wnt/ β -catenin signalling, via binding and titration of active β -catenin (Andersen *et al.* 2012). To date, ligand-independent Notch signalling in humans has only been investigated in human embryonic stem cells; however, the available information suggests that ligand-independent signalling is found mainly in stem or progenitor cells across species (Andersen *et al.* 2012). A comprehensive review of Notch non-canonical signalling has recently been published; therefore, it is not reviewed in the current paper (Andersen *et al.* 2012).

Notch signalling at the human blastocyst–maternal interface

Notch signalling in the endometrium

Much research into the Notch family in the endometrium has focused on its role in disease states such as cancer and hyperplasia due to its roles in proliferation, differentiation and angiogenesis. All four Notch receptors are present in the endometrium (Table 1 and Fig. 1A),

Table 1 Notch ligand localization in cycling endometrium and blastocyst trophectoderm.

	Endometrium				Blastocyst
	Luminal epithelium	Glandular epithelium	Stroma	Endothelial cells	Trophectoderm
Notch1		++ ^{a,b}	+ ^{a,b}	— ^a	α^c
Notch2		α^d	α^d		$\alpha^{c,e}$
Notch3		++ ^b	+ ^b		α^e
Notch4		++ ^a	+ ^a	+ ^a	
DLL1					
DLL3					α^c
DLL4	++ ^f	++ ^{b,f}	+ ^{b,f}	+ ^f	α^e
Jagged1	+ ^g	+ ^{a,b}	+ ^{a,b}	— ^a	
Jagged2					
Hes1		α^d	α^d		
Hey1		α^d	α^d		
Hey2					α^e

α , mRNA expression; +, present low levels; ++, present in high levels; — absent; blank, unknown.

^aCobellis *et al.* (2008). ^bMitsuhashi *et al.* (2012). ^cAdjaye *et al.* (2005). ^dMikhailik *et al.* (2009). ^eAghajanova *et al.* (2012). ^fMazella *et al.* (2008).

^gCuman *et al.* (2013).

although Notch2 has only been identified at the mRNA level (Cobellis *et al.* 2008, Mikhailik *et al.* 2009, Mitsuhashi *et al.* 2012, Mori *et al.* 2012).

Notch1 and 3 protein primarily localize to the glandular epithelium (Cobellis *et al.* 2008, Mitsuhashi *et al.* 2012), whereas Notch4 localizes primarily to the stroma (Cobellis *et al.* 2008). In the only study to examine transcript, Notch1 and 3 expressions are higher in endometrial stromal cells compared with epithelium (Mikhailik *et al.* 2009); however, this analysis was performed following isolation and culture and therefore may not represent *in vivo* expression.

Two studies have previously investigated Notch family protein expression throughout the menstrual cycle. Cobellis *et al.* (2008) identified changes in the expression of Notch1, which was strongest during the mid-secretory phase, and Notch4, which was stronger during the proliferative phase. Conversely, Mitsuhashi *et al.* (2012) more recently identified no differences in Notch1 and 3 across the cycle. This is despite using the same antibodies for Notch1, albeit at different concentrations. In support of cyclical variation in Notch expression, the expression of Notch1 and 4 correlates with the endometrial expression of downstream Notch target genes: *P21* (CDKN1A), an epithelial cell proliferation inhibitor, and cyclin D (Cobellis *et al.* 2008) a promoter of cell proliferation.

Presently, Jagged1 and DLL4 are the only Notch ligands known to localize to the endometrium (Table 1; Mazella *et al.* 2008, Mikhailik *et al.* 2009, Mitsuhashi *et al.* 2012). Both ligands strongly localize to the endometrial glandular and luminal epithelium, with very little to no expression in the stroma. Jagged1 mRNA and protein are highest during the mid-secretory phase compared with the proliferative phase (Cobellis *et al.* 2008, Cuman *et al.* 2013). Whilst *DLL4* mRNA expression is highest during the early secretory phase (Mazella *et al.* 2008), *DLL4* protein shows no significant changes across the cycle (Mitsuhashi *et al.* 2012). Taken

together, the up-regulated expression of these ligands during the mid-secretory phase suggests a role in receptivity. There is very little information regarding the endometrial expression of downstream signalling targets Hes and Hey1. Their mRNA expression is higher in epithelial cells compared with the stromal cells, which correlates with high Jagged1 and *DLL4* protein in the epithelium (Mikhailik *et al.* 2009), suggesting that Notch signalling is active in these cells.

Despite the identification of the presence of both Notch receptor and ligand within the endometrium, there is no evidence that investigates whether juxtacrine activities maybe occurring between the same or different cell types.

Notch signalling in the blastocyst

Notch1, 2, 3, *DLL4*, Jagged1 and Hey2 have been identified by microarray studies as present in human blastocyst trophectoderm (Fig. 1A and Table 1; Adjaye *et al.* 2005, Aghajanova *et al.* 2012). However, the ethical difficulties in obtaining human blastocysts have limited the functional studies examining blastocyst–endometrial interactions during the early stages of implantation.

Function of Notch signalling in blastocyst–maternal interactions

The expression of the Notch receptors and ligands by the trophectoderm and immunolocalization of Notch1, *DLL4* and Jagged1 to the apical surface of the luminal epithelium during the mid-secretory phase of the menstrual cycle (Fig. 1A; Mazella *et al.* 2008, Cuman *et al.* 2013) highlights the accessibility of Notch ligands and receptors for interactions during endometrial epithelial–trophectoderm attachment (Fig. 1A). In mice, *DLL1* is shown to act as an adhesion molecule directly

regulating mast cell–stromal cell adhesion via binding to Notch2 (Murata *et al.* 2010). To date, however, no studies have investigated the role of Notch in endometrial receptivity or blastocyst attachment in humans. However, there is reduced or absent immunostaining of Jagged1 in the mid-secretory luminal epithelium of women with primary infertility (Cuman *et al.* 2013), suggesting that this ligand may be important for blastocyst–epithelial attachment. Furthermore, microarray analysis demonstrates that blastocyst-conditioned media regulates the endometrial epithelial expression of Notch1 and Jagged1 *in vitro* (Cuman *et al.* 2013); thus, blastocysts may facilitate endometrial Notch expression, and possibly endometrial receptivity, via soluble mediators.

Notch signalling in implantation and placentation

Tissue localization of Notch family in human placenta and decidua

To date, only one study has investigated the expression of Notch family members in implantation sites during early implantation and placentation (Table 2 and Fig. 1B; Hunkapiller *et al.* 2011). The individual expression pattern observed for each protein (Table 2) highlights the possible role that each Notch family member plays in regulating trophoblast differentiation and invasion.

Invasive trophoblast

No information is available for the expression of Notch family members during the first trimester. During the second trimester (Table 2 and Fig. 1B), Notch1, DLL1 and Jagged2 are absent in all trophoblast lineages. Notch3 is present in all trophoblast lineages. The expression of Notch2 and 4, DLL4 and Jagged1 is associated with the differentiation of cytotrophoblast to invasive EVT lineages: as cytotrophoblast differentiate towards invasive EVT, Notch2 and Jagged1 expression is up-regulated, whereas DLL4 is expressed only by

trophoblasts residing in the cell column and Notch4 is down-regulated in EVTs in close proximity to spiral arterioles (Hunkapiller *et al.* 2011). Interestingly, despite not being identified in EVTs in the second trimester, Notch1, along with Notch4 and Jagged1, is localized to EVTs in third-trimester decidua (De Falco *et al.* 2007).

Decidua

Little is known about decidual Notch expression (Table 2 and Fig. 1B). uNK cells express Notch1 and 2 (Manaster *et al.* 2010). To our knowledge, no study has localized Notch signalling family members in human decidualized stromal cells, except Notch1 *ex vivo* in decidual cells isolated from term placentas (Afshar *et al.* 2012b). Immunofluorescence figures highlighting trophoblast expression of Notch family members in Hunkapiller *et al.* (2011) provide some clues as to decidual Notch expression. Unfortunately, this study does not investigate Notch1 expression. Intriguingly, these data suggest that Notch4, Jagged1 and DLL4 are likely absent from maternal cells in the decidua during the second trimester (Hunkapiller *et al.* 2011). Notch3 and DLL1 are clearly visible in maternal cells of the decidua; however, from the figures provided, it is not possible to identify the cell types. Strong expression of DLL1 is observed in maternal cells associating with EVTs remodelling spiral arterioles during the second trimester (Hunkapiller *et al.* 2011); however, the identity of these cells is not clear. These cells are likely either decidual cells or uNK cells; certainly, DLL1 is expressed by uNK cells in mice (Degaki *et al.* 2012).

Placenta

Studies examining Notch expression in the placenta are conflicting, with localization studies not showing consistent expression. One study investigating transcript levels of Notch3, 4, Jagged1, 2 and DLL4 found that expression increased from the first to the third trimester

Table 2 Notch ligand localization in the decidua during pregnancy.

Notch members	Decidua			Cytotrophoblast cell column			EVTS			Endothelial cells			Immune cells		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Notch1			+					– ^b	++ ^c				+ ^{uNKd}		
Notch2								+					+ ^{uNKd}		
Notch3					+			+							
Notch4		– ^b			+			+	+						
DLL1					– ^b			– ^b							
DLL3															
DLL4		– ^b			+			– ^b							
Jagged1		– ^b			– ^b			+	+						
Jagged2					– ^b			– ^b							
Hes1															
Hey1															

α, mRNA expression; +, present low levels; ++, present in high levels; –, absent; blank, unknown.

^aAfshar *et al.* (2012b). ^bHunkapiller *et al.* (2011). ^cDe Falco *et al.* (2007). ^dManaster *et al.* (2010).

(Herr *et al.* 2011); however, many studies, investigating a variety of genes, also identify increased gene expression in third-trimester placentas compared with first-trimester placentas, suggesting that this may just reflect a change in global gene expression.

The limited number of studies to date makes it difficult to determine whether there are differences in Notch receptor or ligand protein localization and levels between the first and third trimesters, although the only study to directly compare the two trimesters found no differences (Herr *et al.* 2011). Further, immunolocalization of Notch receptors and ligands is variable between studies, despite likely using the same antibodies. Generally, however, most proteins are present in both first- and third-trimester tissue with very few factors showing differential staining between the two gestation times (Table 3).

Overall, the more recent studies have less background, suggesting that these studies reflect true positive staining. Therefore, in the text, we refer only to the most recent studies, although all studies are represented in Table 3.

Notch1 is expressed by trophoblasts, certainly syncytiotrophoblasts (Cobellis *et al.* 2007, De Falco *et al.* 2007, Herr *et al.* 2011, Sahin *et al.* 2011), and possibly cytotrophoblast (Cobellis *et al.* 2007, De Falco *et al.* 2007, Herr *et al.* 2011), but staining is absent in the EVT of the cell column (Table 2; Hunkapiller *et al.* 2011). Notch2 (Sahin *et al.* 2011), Notch3 (Herr *et al.* 2011) and Notch4 (Sahin *et al.* 2011) are also localized to the syncytiotrophoblasts and Notch3 localizes to the cytotrophoblasts (Herr *et al.* 2011). DLL1 (Herr *et al.* 2011) and Jagged1 and 2 (Sahin *et al.* 2011) are expressed by syncytiotrophoblast and DLL1 also by cytotrophoblast (Herr *et al.* 2011). In non-trophoblast lineages, Notch1, Jagged1, DLL1 and DLL4 are expressed by endothelial cells (Herr *et al.* 2011, Sahin *et al.* 2011), while Hofbauer cells express Notch2, 3 and 4 (Herr *et al.* 2011) and Notch3 and Jagged2 localize to the villous stroma (Herr *et al.* 2011; Table 3).

Function of Notch signalling during implantation and placentalization

Decidualization

Decidualization describes the terminal differentiation of uterine endometrial stromal cells such that their morphology and function change. Notch1 signalling has been shown *ex vivo* to be critical for the progression of decidualization; silencing Notch1 in stromal cells isolated from decidua parietalis (decidua of term pregnancy, from a site not associated with the placenta) impaired decidualization *in vitro* (Afshar *et al.* 2012b). Furthermore, this study showed that Notch1 is induced by human chorionic gonadotrophin (hCG) and the authors suggest that Notch1 responds to hCG to mediate a survival signal in the uterine endometrium so that menstrual sloughing is avoided. Certainly, Notch1 is a known mediator of survival signals and in mice abrogation of Notch1 signalling during decidualization decreased proliferation and up-regulates apoptosis-associated genes (Afshar *et al.* 2012b). Intriguingly, a global gene array study identified up-regulation of the Notch signalling pathways in decidual cells exposed to trophoblast conditioned media, suggesting that Notch family members other than Notch1 may regulate decidualization (Hess *et al.* 2007).

EVT invasion

While the precise role of Notch signalling in EVT invasion remains unclear, the pattern of Notch family expression during early placentalization strongly suggests a role in EVT differentiation and invasion into the decidua (Table 2). Some functional data to strengthen this notion demonstrate that invasion through Matrigel of cytotrophoblasts isolated from term placentas was impaired in the presence of a γ -secretase inhibitor, which acts to inhibit canonical Notch signalling (Hunkapiller *et al.* 2011). In the same study, DLL4 was found to be transiently up-regulated during cytotrophoblast invasion *in vitro*. Meanwhile, the *in vivo* localization of DLL4

Table 3 Localization of Notch-ligand in placenta.

Notch members	Cytotrophoblast		Syncytiotrophoblast		Stroma		Immune cells		Endothelial cells	
	1st	3rd	1st	3rd	1st	3rd	1st	3rd	1st	3rd
Notch1	+ ^{a,b}		+ ^{a,b}	+ ^{a,b,c,d}		— ^d , + ^a			+ ^a	+ ^{a,b,d}
Notch2	— ^b		— ^b	+ ^d	— ^b	— ^d	+ ^{HCB}	+ ^{HCB}		— ^d
Notch3	+ ^b		— ^b		— ^b	+ ^b	+ ^{HCB}	+ ^{HCB}		
Notch4	— ^b , + ^a	+ ^c	— ^b	+ ^{a,d}	— ^b , + ^a	— ^{b,d}	+ ^{HCB}	+ ^{HCB}		+ ^{a,c,d}
DLL1	+ ^b	+ ^b		+ ^b		+ ^b			+ ^b	+ ^b
DLL3										
DLL4	— ^b		— ^b	— ^b					+ ^b	+ ^b
Jagged1	+ ^a	+ ^{a,c}		+ ^{a,c}	— ^b	— ^b , + ^{a,c}			+ ^b	+ ^{a,b,c}
Jagged2				+ ^d	+ ^b	+ ^b				
Hes1										
Hey1										
Hey2										

^a, mRNA expression; +, present low levels; ++, present in high levels; —, absent; blank, unknown; HC, Hofbauer cell.

^aDe Falco *et al.* (2007). ^bHerr *et al.* (2011). ^cCobellis *et al.* (2007). ^dSahin *et al.* (2011).

is restricted to cytotrophoblasts in the cell columns (Hunkapiller *et al.* 2011), suggesting that DLL4 could be involved in the differentiation of cytotrophoblasts towards invasive EVT, an important step in invasion.

Spiral artery remodelling

Notch signalling may be critical for spiral artery remodelling. Prior to EVT invasion of spiral arteries, uNK cells act to disrupt the integrity of the arteries, particularly the vascular smooth muscle (Harris 2011). Recently, uNK-conditioned media and interferon- γ alone were shown to disrupt vascular smooth muscle cell integrity (Robson *et al.* 2012), a critical step in spiral artery remodelling. Furthermore, both DLL1 and 4 interact with Notch on uNK cells to induce interferon- γ secretion (Manaster *et al.* 2010). As uNK cells express Notch1 and 2 and DLL1 is expressed by maternal cells surrounding spiral arteries (Hunkapiller *et al.* 2011) and by uNK cells in mice (Degaki *et al.* 2012), it is tempting to speculate that Notch activation in uNK cells is regulated by DLL1, leading to interferon- γ secretion and vascular smooth muscle disruption, prior to EVT invasion.

Placentation

There are few functional studies examining the role of Notch signalling in human placental vasculogenesis. The localization of Notch family members DLL1, 4, Jagged1 and Notch1 to the endothelial cells in tertiary villi implies a role in placental vasculogenesis, as recently reviewed (Zhao & Lin 2012). In addition, Hofbauer cells and cytotrophoblast induce angiogenesis during placental vasculogenesis via the secretion of growth factors (Demir *et al.* 2007). The localization of Notch receptors 2, 3 and 4 to Hofbauer cells (Herr *et al.* 2011) in the villous stroma and the ligands to the endothelial cells suggests a role for Notch signalling in angiogenesis, although this remains to be experimentally proven. Certainly, Hofbauer cells are in close contact with endothelial progenitor cells (angiogenic cell cords), during the initiation of vascularization (Seval *et al.* 2007), potentially allowing direct cell communication via Notch signalling.

Meanwhile, the differential expression of Notch receptors and ligands by the cytotrophoblast compared with syncytiotrophoblasts (Table 3) suggests that Notch signalling may play a role in differentiation from cytotrophoblasts to syncytiotrophoblasts or possibly that Notch signalling is involved in interactions between syncytiotrophoblasts and cells in the intervillous space. Differentiation of human embryonic stem cells towards a trophoblast lineage is shown to require inhibition of Notch activation (Yu *et al.* 2008); however, no studies have investigated differentiation towards the cytotrophoblast or syncytiotrophoblast lineages. Further studies are

clearly needed to confirm the function and regulation of Notch signalling in human placentation.

Clues from animal studies

Pre-implantation development and Notch signalling in mice

Mice studies have provided clearer identification of Notch signalling in the pre-implanted embryos, with mRNA expression of the different genes identified throughout the stages of embryo pre-implantation development. Notch1, 2, Jagged1, 2, DLL3 and RBPSUH are expressed in oocytes and by the conceptus at all stages of pre-implantation development, while expression of Notch4 and DLL4 was observed only from the two cell stage through to the hatched blastocyst stage. Interestingly, the mRNA expression of *NOTCH3*, DLL1 and Deltex homologue 1 is stage dependent. Their expression is detected at the two-cell embryo and hatched blastocyst stage, while low or absent at the morula stage (Cormier *et al.* 2004). Furthermore, *in vitro* studies demonstrate a role for Notch signalling in mouse blastocyst spreading (Chu *et al.* 2011) while *in vivo* studies showed that administration of a γ -secretase inhibitor disrupts endometrial stromal cell decidualization (occurs post-implantation in mice), leading to pregnancy loss (Afshar *et al.* 2012a). Thus, in mice, these studies suggest that Notch signalling is important in endometrial epithelial cell function, highlighting the importance of examining blastocyst–endometrial epithelial interactions in humans.

Notch signalling in the mouse placenta

Mouse placental development involves a number of processes, including trophoblast differentiation, chorioallantoic fusion and branching, fetal vascular morphogenesis and the establishment of a maternal blood supply. Both the human and mouse placenta are haemochorial, where trophoblasts are directly bathed in maternal blood (Watson & Cross 2005), and both share a high degree of structural, proteomic and molecular genetic homology (Cox *et al.* 2009, Dilworth & Sibley 2013).

Several Notch signalling family members are expressed in the mouse placenta. Targeted single-gene mutations in *Notch1*, 2, 4 and *DLL1*, 4 result in mid-gestation embryonic lethality, indicating a pivotal role for this pathway in the formation of a functional placenta, as previously reviewed by Gasperowicz & Otto (2008).

However, of these family members, only a clearly defined role for Notch2 has been elucidated. Of the Notch receptors, Notch2 is the only receptor up-regulated during trophoblast invasion in mice. Notch2 deletion results in embryonic lethality at E11.5 (Hamada

et al. 1999). Hamada *et al.* 2007 demonstrated that whole embryo culture evaded lethality in mutant embryos, suggesting an extraembryonic cause. Notch2 localizes to maternal sinusoidal trophoblasts in the mouse placental labyrinth, the site of fetal–maternal exchange, and from the histology of early Notch2-deficient placentas, poor maternal blood sinus formation was evident (Hamada *et al.* 2007). More recently, conditional deletion under the *Tpba* promoter (Simmons *et al.* 2007) specific for invasive spiral arteriole giant trophoblast Cell (TGC) and glycogen trophoblast cell lineages has shown impaired trophoblast invasion into the maternal arteries and reduced maternal canal size and placental perfusion (Hunkapiller *et al.* 2011). Taken together, these findings imply a fundamental role for Notch2 signalling in endovascular trophoblast invasion in the mouse placenta.

While previously, the Notch2 ligands, Jagged1, 2 and DLL1, 4, were thought not to be expressed in trophoblast cell subsets of the developing mouse placenta during this time between E9.5 and E12.5, the latest investigation by Gasperowicz *et al.* (2013) suggests otherwise. In this study, the expression pattern of all Notch receptors and ligands were investigated from E7.5 to E12.5, during the time of mouse placental development. Notch2 and Jagged1 and 2 were complementarily expressed in the labyrinth layer. Meanwhile, Notch2 and DLL4 expression were shared at the decidual interface, suggesting that DLL4 could be the ‘missing’ ligand involved in endovascular trophoblast cell invasion mediated by Notch2 receptor signalling.

Conversely, the remaining Notch family members, including the Notch1 and 4, appear to be highly involved and crucial for fetal angiogenesis and maternal vascular formation as previously reviewed (Gasperowicz & Otto 2008). The Notch signalling effectors, Hey1/2, are also highly expressed in the endothelial cells associated with fetal vasculature in the chorioallantoic layer that gives rise to the placental labyrinth (Fischer *et al.* 2004). Furthermore, double-knockout Hes/Hey mutant mice fail to undergo chorioallantoic branching during the formation of this layer (Fischer *et al.* 2004).

Overall, these insights from the roles of the Notch signalling family members in the formation of the functional mouse placenta could provide insight as to the potential functional roles for distinct Notch family members in various aspects of human placental development. Meanwhile, further mouse studies could elucidate the precise regulatory mechanisms of the Notch pathway during the development of the placenta.

Clinical implications of inadequate implantation/placentation

Implantation failure results in infertility and is also a major cause of unsuccessful assisted reproduction

technologies such as IVF. Meanwhile, placental dysfunction is widely accepted as the underlying cause of two of the most common and severe complications of pregnancy, pre-eclampsia (PE) and intrauterine growth restriction (IUGR). These disorders contribute to significant maternal and fetal mortality and morbidity.

A role for Notch in PE and IUGR

PE is the single most important cause of maternal morbidity in antenatal care. PE only presents after 20 weeks of gestation (more commonly in the third trimester), although it is thought to initiate during the first trimester (Red-Horse *et al.* 2004, Redman & Sargent 2005). Women who develop PE are at an increased risk of cardiovascular complications later in life (Smith *et al.* 2001). Some women with PE also develop IUGR (Fisher 2004). IUGR is associated with poor transformation with the maternal spiral arteries, and importantly, IUGR is the most important trigger of perinatal mortality. Moreover, low-birth-weight babies have an increased risk of developing heart disease, hypertension and diabetes in adult life (Barker & Clark 1997).

Many studies have identified dysregulated expression of Notch family members in pre-eclamptic placentas (Cobellis *et al.* 2007, Sitrass *et al.* 2009, Hunkapiller *et al.* 2011, Løset *et al.* 2011, Sahin *et al.* 2011, Meng *et al.* 2012, Taki *et al.* 2012). Unfortunately, in many studies, the gestational age of the samples is not matched between pre-eclamptic and control (Sitrass *et al.* 2009, Løset *et al.* 2011, Meng *et al.* 2012). In the most recent study (Taki *et al.* 2012), gestational age was matched; however, the PE fetus likely also had IUGR. To date, only one study has utilized chorionic villus samples (CVS) to prospectively compare gene expression by microarray, in placentas that go on to develop PE and those that do not. No differences in expression of Notch family members were identified (Founds *et al.* 2009); however, the indication for the majority of women undergoing CVS was advanced maternal age, thus these tissues may be biased.

However, in placentae collected at delivery, differences have been identified in both gene (microarray) and protein (immunohistochemistry and western blotting) expression. *NOTCH3* gene expression is up-regulated in pre-eclamptic placentas (Sitrass *et al.* 2009), while Deltex 3 Homolog, *HES1*, *NOTCH3* and *NOTCH4* gene expression are down-regulated in the decidua of pre-eclamptic pregnancies (Johansson *et al.* 2011, Løset *et al.* 2011).

Immunolocalization of proteins suggests that there is down-regulation of many Notch family proteins in pre-eclamptic placentas, including Notch1 (Cobellis *et al.* 2007, Sahin *et al.* 2011), Notch2 (Sahin *et al.* 2011) and Notch4 (Cobellis *et al.* 2007, Sahin *et al.* 2011), Jagged1 (Cobellis *et al.* 2007) and Jagged2 (Sahin *et al.* 2011). Interestingly, this down-regulation is associated with

a shift in protein localization from membrane-bound protein to cytoplasmic in Notch1, 2, 4 and Jagged1 (Sahin *et al.* 2011), possibly indicating a shift from canonical to ligand-independent Notch signalling. Notch expression in the placenta from IUGR is very similar to PE; immunostaining of Notch1, 2 and 4 and Jagged2 is reduced in the syncytiotrophoblast and the cellular localization of these receptors and ligand changes from being membrane-bound to cytoplasmic (Sahin *et al.* 2011).

A few studies have also identified altered expression of non-canonical Notch ligands in PE. EGF-like domain, multiple 7 (EGFL7) is down-regulated in the placenta of pre-eclamptic pregnancies (Johansson *et al.* 2011, Junus *et al.* 2012) and is further down-regulated in early-onset compared with late-onset PE (Junus *et al.* 2012). Nephroblastoma overexpressed (NOV) is down-regulated in the placenta from early-onset PE (Gellhaus *et al.* 2006, 2007). Serum periostin is elevated in women with PE compared with normotensive pregnant women and is localized by *in situ* hybridization to stromal cells in the placenta (Sasaki *et al.* 2002). It should be noted that none of these studies have determined whether these proteins are signalling via Notch in these cells; however, the prevalence of Notch receptors and both canonical and non-canonical Notch ligands being dysregulated during PE does strongly suggest a role for Notch signalling in PE.

Overall, it is interesting to note that the majority of studies investigating a role for Notch signalling in PE or IUGR have focused on Notch members expression in trophoblasts. Formation and maintenance of the placental vasculature is of critical importance for normal placental formation. Many Notch proteins identified also have roles in vascularization. In the deciduas, Jagged1 immunostaining is reduced in EVT, which have invaded maternal spiral arteries of pre-eclamptic and HELLP (haemolysis, elevated liver enzymes and low platelet count) pregnancies, regardless of the extent of remodelling (Hunkapiller *et al.* 2011). It should be noted that this study also shows that Jagged1 is strictly up-regulated as EVTs come into proximity of blood vessels. Thus, in a pre-eclamptic pregnancy where there is limited invasion, Jagged1 may not be up-regulated. Nevertheless, a functional role for Jagged1 in spiral artery remodelling should be investigated, particularly as the Jagged1-null mouse shows defects in angiogenesis, although this has not been investigated in the context of placental angiogenesis (Gasperowicz & Otto 2008).

In pre-eclamptic placentas with IUGR, DLL4 is up-regulated in the syncytiotrophoblasts and the vascular smooth muscle of the umbilical artery (Taki *et al.* 2012). DLL4 reduces vessel sprout length and inhibits angiogenesis and may be up-regulated in response to hypoxia (Taki *et al.* 2012). It is interesting that both DLL4 and Jagged1 have been attributed to PE and poor vessel remodelling, as they are thought to antagonize one

another during angiogenesis (Benedito *et al.* 2009). These changes in DLL4 expression are likely to represent direct effects on angiogenesis. However, these differences may reflect global changes in expression, and future studies should investigate expression in villous endothelial and associated cells, preferably from CVS sampling to allow prospective identification of protein alterations in PE and IUGR.

Conclusions

This review has summarized the currently available knowledge regarding Notch signalling during the establishment of pregnancy and highlights the role of Notch signalling during implantation and placentation. Overall, the evidence supports an important role for Notch signalling in implantation/placentation. Current data show that Notch signalling is involved in endometrial-trophoblast interactions during the initiation of attachment, regulation of EVT invasion and spiral artery remodelling in the decidua and angiogenesis in the placenta. Furthermore, aberrant Notch signalling is found in the diseases of gestation, such as PE and IUGR.

However, our current knowledge of Notch receptor and ligand expression and functional studies investigating their role in implantation and placentation is limited. Further studies are required, particularly functional studies. The current paradigm suggests that Notch signalling is important for normal placentation and that Notch signalling may be a clinical target to improve IVF outcomes and to prevent or treat placental insufficiency.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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Author contribution statement

C Cuman and E Dimitriadis conceived the study and wrote the manuscript. E Menkhurst wrote the manuscript and designed the figure. A Winship and M Van Sinderen wrote the manuscript. T Osianlis and L J Rombauts did critical analysis of the manuscript.

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