A role for Notch signaling in trophoblast endovascular invasion and in the pathogenesis of pre-eclampsia

Nathan M. Hunkapiller1,2, Malgorzata Gasperowicz3, Mirhan Kapidzic1,2, Vicki Plaks4, Emin Maltepe1,5,6,7, Jan Kitajewski8,9,10, Jay C. Cross3 and Susan J. Fisher1,2,4,5,11,12,*

SUMMARY
Placental trophoblasts (TBs) invade and remodel uterine vessels with an arterial bias. This process, which involves vascular mimicry, re-routes maternal blood to the placenta, but fails in pre-eclampsia. We investigated Notch family members in both contexts, as they play important roles in arterial differentiation/function. Immunohistological analyses showed step-wise modulation of Notch receptors/ligands during human TB invasion. Inhibition of Notch signaling reduced invasion of cultured human TBs and expression of the arterial marker EFN B2. In mouse placentas, Notch activity was highest in endovascular TBs. Conditional deletion of Notch2, the only receptor upregulated during mouse TB invasion, reduced arterial invasion, the size of maternal blood canals by 30-40% and placental perfusion by 23%. By E11.5, there was litter-wide lethality in proportion to the number of mutant offspring. In pre-eclampsia, expression of the Notch ligand JAG1 was absent in perivascular and endovascular TBs. We conclude that Notch signaling is crucial for TB vascular invasion.

KEY WORDS: Endovascular Invasion, Notch, Pre-eclampsia, Trophoblast

INTRODUCTION
Human placentation involves unique interactions between embryonic/fetal cytotrophoblasts (CTBs) and maternal cells. CTBs that arise from the placental surface colonize the uterine wall and resident maternal vessels (Fig. 1A), a process that requires aggressive invasion of maternal tissues (Fisher et al., 1989; Librach et al., 1991). During interstitial invasion, CTBs commingle with decidual, myometrial and immune cells. During endovascular invasion, CTBs breach maternal spiral arterioles that supply blood to the placenta. Subsequently, they replace the maternal endothelium and regions of the smooth muscle wall, creating a novel chimeric vasculature composed of maternal and fetal cells, a process that greatly increases arteriolar diameter. By contrast, CTBs form only superficial connections with uterine veins. These remarkable cell-cell interactions are accompanied by changes in fundamental aspects of the phenotype of the cells with the net effect of mimicking many aspects of endothelial cells (ECs). They switch on the expression of vascular-type adhesion molecules (Zhou et al., 1992; Zhou et al., 1997), vasculogenic/angiogenic factors (Zhou et al., 2003; Zhou et al., 2002) and Ephrin family members that play a role in arterial function and identity (Red-Horse et al., 2005). The significance of CTB endovascular invasion is illustrated by the fact that this process largely fails in pre-eclampsia (PE).

PE is a serious complication that affects ~7% of first-time pregnancies (Levine et al., 1997; Redman and Sargent, 2005). The mother shows signs of widespread alterations in EC function such as hypertension, proteinuria and edema (Roberts and Lain, 2002). Sometimes fetal growth is impaired. In PE, the extent of CTB interstitial invasion is variable, but frequently shallow; endovascular invasion is consistently rudimentary (Brosens et al., 1972; Naicker et al., 2003; Zhou et al., 2007), resulting in increased vascular resistance and decreased placental perfusion (Mattijevic and Johnston, 1999). The PE syndrome reveals the significance of CTB differentiation/invasion. Biopsies of the uterine wall of individuals with PE show that invasive CTBs fail to upregulate receptors that promote invasion and/or assumption of an EC-like phenotype (Zhou et al., 2003; Zhou et al., 2002). The upstream regulatory mechanisms that are responsible for these defects remain enigmatic. Thus, ligands/receptors that are involved in vascular patterning are of interest.

The Notch signaling pathway governs differentiation and function during cell-cell contact in many tissues (Bianchi et al., 2006; Miele, 2006) with particularly important roles in vascular patterning (Roca and Adams, 2007; Swift and Weinstein, 2009). Mechanistically, Notch receptors operate both on the cell surface to receive activating signals and within the nucleus as transcriptional modulators (Alva and Iruela-Arispe, 2004; Kopan and Iliagan, 2009). The core mammalian pathway is a conserved family of four transmembrane receptors (NOTCH1-4) and five ligands (DLL1/3/4 and JAG1/2). Binding of receptors and ligands on adjacent cells triggers serial proteolytic cleavages of the receptor, releasing the Notch intracellular domain (NICD) via γ-secretase-mediated processing (Schroeter et al., 1998). Subsequently, cleaved NICD translocates to the nucleus, binds to CBFI/Su(H)/Lag2 family transcription factors, and induces downstream targets such as Hes and Hey (Weinmaster, 1998).
Notch signaling regulates the differentiation of primitive ECs into hierarchical networks by specifying arterial identity. By embryonic days (E) 9.5-11.5 of mouse development, when the primary vascular plexus begins to remodel into arterial and venous networks, Notch1/Notch3/Notch4, Jag1 and Dll4 mRNAs are expressed within the developing vasculature. However, by E13.5, their expression is largely confined to arterial endothelium (Villa et al., 2001). Concurrently, Efnb2 and Ephb4 emerge as markers of arterial and venous identity, respectively (Wang et al., 1998). In zebrafish, loss of Notch signaling reduced EC efnb2 expression while simultaneously enhancing ephb4 expression (lower inset) as they invaded the uterine wall and blood vessels. CTBs expressed NOTCH3 at all stages of differentiation/invasion (insets). (E) CTB progenitors and cell columns (upper inset) expressed NOTCH4, which was downregulated in proximity to maternal blood vessels (lower inset). (F) Dll1 was expressed by maternal cells in the uterus that associated with CTBs (inset). (G) DLL4 immunoreactivity, which was absent in progenitors (upper inset), increased as CTBs entered the cell columns (lower inset) and declined with deeper invasion. (H,I) CTBs expressed JAG1 only in proximity to maternal spiral arterioles. Scale bars: 100 μm. The direction of invasion is indicated by arrows.

In culture, undifferentiated mouse trophoblast stem (TS) cells express all members of the Notch family (Cormier et al., 2004). In vivo, differentiated trophoblasts at the junctional zone (the maternal-fetal interface) express only Notch2, which is restricted to a narrow temporal window at midgestation (Nakayama et al., 1997), suggesting that trophoblast differentiation in vivo probably involves significant modulation of Notch expression. As to function, Notch2, Hes2 and Hes3 are co-expressed in trophoblast giant cells (TGCs), which, along with glycogen trophoblast cells (GlyTCs), carry out interstitial and endovascular invasion. Tetraploid rescue experiments in Notch2-deficient mice revealed that placental defects involving the labyrinthian circulatory system are associated with the lethality in Notch2-deficient animals (Hamada et al., 2007). However, the mechanisms involved remained elusive. We theorized that defects in trophoblast endovascular invasion contributed to the observed phenotype. Here, we used a combination of mouse and human models to test this theory and to identify PE-associated aberrations in CTB expression of Notch family members.
MATERIALS AND METHODS

Human tissue collection

This study was approved by the UCSF Institutional Review Board. Written informed consent was obtained from donors. Biopsies of normal placentas were either fresh-frozen for immunolocalization, paraffin-embedded fixed for in situ hybridization or immediately processed for CTB isolation. Equivalent specimens were obtained from women who experienced either pre-term labor, severe pre-eclampsia, or hemolysis, elevated liver enzymes and low platelets syndrome using clinical criteria and methods we have published (Zhou et al., 2007).

Immunofluorescence

Fresh-frozen basal plate biopsies were embedded and sectioned as described previously (Zhou et al., 2007). Following fixation in cold methanol:acetone (1:6) for 5 minutes, sections were washed in PBS. Non-specific reactivity was blocked by incubation in 0.3% bovine serum albumin/PBS. They were incubated (overnight at 4°C) with anti-KRT7 (diluted 1:50; produced by the Fisher group), which reacts with all trophoblast populations, and an anti-Notch family member diluted 1:200 [NOTCH1 (Santa Cruz Biotechnology, sc-9011), NOTCH2 (R&D Systems, AF3735), NOTCH3 (Santa Cruz Biotechnology, sc-5593), NOTCH4 (Santa Cruz Biotechnology, sc-8643), JAG1 (Santa Cruz Biotechnology, sc-8303), JAG2 (Santa Cruz Biotechnology, sc-5604), DLL1 (Santa Cruz Biotechnology, sc-9102) and DLL4 (Santa Cruz Biotechnology, sc-18640)]. Subsequently, the sections were rinsed in PBS, incubated with the appropriate species-specific secondary antibody conjugated to either fluorescein (Notch family members) or rhodamine (KRT7), washed in PBS and mounted under Vectashield containing DAPI. Controls were incubated with the primary or secondary antibody alone. Sections were photographed using a Leica DM5000B microscope. Immunostaining with the Tromo-1 antibody (anti-mouse cytokeratin; Developmental Studies Hybridoma Bank) was performed as described above on tissue sections of E12.5 Notch2flox/flox; Tpbpa-Cre and Notch2flox/+; Tpbpa-Cre placentas.

Immunoblotting

Immunoblotting was performed as previously described (Zhou et al., 2002). Primary antibodies that reacted with NOTCH2 (Developmental Studies Hybridoma Bank; C651.6DbHN), NOTCH3 (Santa Cruz Biotechnology, sc-9011) and NOTCH4 (Santa Cruz Biotechnology, sc-8643), JAG1 (Santa Cruz Biotechnology, sc-8303), JAG2 (Santa Cruz Biotechnology, sc-5604), DLL1 (Santa Cruz Biotechnology, sc-9102) and DLL4 (Santa Cruz Biotechnology, sc-18640). Subsequently, the sections were rinsed in PBS, incubated with the appropriate species-specific secondary antibody conjugated to either fluorescein (Notch family members) or rhodamine (KRT7), washed in PBS and mounted under Vectashield containing DAPI. Controls were incubated with the primary or secondary antibody alone. Sections were photographed using a Leica DM5000B microscope. Immunostaining with the Tromo-1 antibody (anti-mouse cytokeratin; Developmental Studies Hybridoma Bank) was performed as described above on tissue sections of E12.5 Notch2flox/flox; Tpbpa-Cre and Notch2flox/+; Tpbpa-Cre placentas.

Cell isolation and culture

Primary human CTBs were isolated and cultured up to 36 hours as described (Hunkapiller and Fisher, 2008). For functional analyses, CTBs were cultured in medium containing either 10 μM L-685,458 (Bachem), or DMSO alone. After 36 hours, the percentage of cells undergoing programmed cell death was estimated using an In Situ Cell Death Detection kit (Roche).

RNA in situ hybridization and KRT7 immunohistochemistry

Single probe RNA in situ hybridization was performed as previously described (Simmons et al., 2007). Notch family cDNAs were amplified from CTB RNA by using the RT-qPCR primers described in Table 1, which included either a 5′ T7 promoter sequence (TAATACGACTCACTATAGGG) or a 5′ T3 promoter sequence (AATTAACCTCTACTAAAGGG). Mouse cDNAs for Prl2c2, Pcdh12 (Simmons et al., 2008), Gcm1 (Basyuk et al., 1999), Hand1 (Cross et al., 1995) and Tpbpa (Lescisin et al., 1988) have been described previously. The Prl3b1 cDNA was provided by J. Rossant (The Hospital for Sick Children, Toronto, Canada) and the Prl3d1 cDNA was provided by D. Linzer (Northwestern University, IL, USA). cDNAs were used for digoxigenin probe synthesis according to the manufacturer’s protocol.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTCH1</td>
<td>CTCACCTGGTGCCAGACCCACAG</td>
<td>GACACCTGTAGCTGTGGCTG</td>
<td>383</td>
</tr>
<tr>
<td>NOTCH2</td>
<td>CAGTTGACCACCTCTGCTGTTACTCTG</td>
<td>CATGATACGAGGAAACCATTTAC</td>
<td>456</td>
</tr>
<tr>
<td>NOTCH3</td>
<td>CGCTCTGAGAATGATCTAGCTTCTTC</td>
<td>TACACCTGGGCGATGTCTTCTTC</td>
<td>352</td>
</tr>
<tr>
<td>NOTCH4</td>
<td>ATGACCTGTGCAACAGGCTTC</td>
<td>GAAGATCAAGAGGCGAGTGGCTC</td>
<td>237</td>
</tr>
<tr>
<td>JAG1</td>
<td>GCTTGGAGTCTGTGGTGGAC</td>
<td>ACCTTTACGAACCTTGTTGGC</td>
<td>386</td>
</tr>
<tr>
<td>JAG2</td>
<td>GCTTTCTTCAGAGGTCCTGGAG</td>
<td>GCACGGAGATAGAGGAGTTG</td>
<td>236</td>
</tr>
<tr>
<td>DLL1</td>
<td>CTACAGGCGGGAGAATGTGAG</td>
<td>GCCTCTTTTGTTGTGGTCTTG</td>
<td>441</td>
</tr>
<tr>
<td>DLL4</td>
<td>CGGGTACATCTGAGCTGACAC</td>
<td>AGTTGAGATCTTGGTACCAAACACG</td>
<td>348</td>
</tr>
<tr>
<td>RN18S1</td>
<td>CGGCTGCTGAGGAGAATGCTTCT</td>
<td>CGAACCTCCGACTTGTCTTC</td>
<td>101</td>
</tr>
<tr>
<td>EFNB2</td>
<td>Taqman Assay: Hs00187950_m1</td>
<td>Taqman Assay: Hs00187950_m1</td>
<td>63</td>
</tr>
<tr>
<td>EPHB4</td>
<td>Taqman Assay: Hs00174752_m1</td>
<td>Taqman Assay: Hs00174752_m1</td>
<td>82</td>
</tr>
<tr>
<td>MMP9</td>
<td>Taqman Assay: Hs00234579_m1</td>
<td>Taqman Assay: Hs00234579_m1</td>
<td>54</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Taqman Assay: Hs02758991_g1</td>
<td>Taqman Assay: Hs02758991_g1</td>
<td>93</td>
</tr>
</tbody>
</table>

Genotyping primers

<table>
<thead>
<tr>
<th>Target allele</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnr</td>
<td>CACATGAAGCAGCAGCAGCTT</td>
<td>ACTGGGTTGCTGAGTGG</td>
<td>385</td>
</tr>
<tr>
<td>Notch2flox/+</td>
<td>TAGGAAAGCAGCTGAGCTGAGAGG</td>
<td>ATAAGGCTAAAGGAGCTGAGGAG</td>
<td>200/161</td>
</tr>
<tr>
<td>Gt(Rosa)26SoFm15or</td>
<td>CGGCCCTGGGACACCACAGAGG</td>
<td>CCAGTGGGTAATAGGAGAG</td>
<td>370</td>
</tr>
</tbody>
</table>
Development 138 (14)

Glycine (pH 2.2) for 30 minutes. Non-specific reactivity was blocked in acid buffer/0.1% Tween-20. Then the slides were incubated in 0.1 M maleic acid buffer/0.1% Tween-20. Bound antibody was heat inactivated (65°C; 30 minutes) in maleic acid buffer/0.1% Tween-20. Then the slides were incubated in 0.1 M maleic acid buffer/0.1% Tween-20. Bound antibody was heat inactivated (65°C; 30 minutes) in maleic acid buffer/0.1% Tween-20. Staining for NOTCH2 (Fig. 1B) was either absent or weak and sporadic in CTB progenitors that attached to the trophoblast basement membrane. However, NOTCH2 expression was dramatically upregulated in the CTB cell compartment as invasion began. CTBs at all stages of differentiation stained for NOTCH3 (Fig. 1C,D). NOTCH4 immunoactivity (Fig. 1E) was high in CTB progenitors and cell columns, and abruptly declined with deeper invasion, particularly as the cells approached spiral arterioles. Maternal and fetal cells stained for several Notch ligands. DLL1 was expressed (Fig. 1F) in maternal cells adjacent to CTBs in the spiral arterioles and endovascular compartments. DLL4 staining (Fig. 1G) was not detected in CTB progenitors but increased as the cells entered the columns. However, DLL4 immunoactivity was rapidly lost as CTBs invaded the uterine wall, although small fields of immunopositive cells were occasionally observed in the deeper regions. JAG1 expression (Fig. 1H,I), which was absent in early stages of CTB differentiation/invasion, was significantly upregulated in CTBs that associated with the maternal spiral arterioles. CTBs did not express JAG2 (not shown). The staining patterns for individual family members and a diagrammatic summary of the results are presented in Fig. S2 in the supplementary material. Together, these data show that CTBs dramatically altered their expression of Notch receptors and ligands as they differentiated/invaded.

Next, we used a RT-qPCR approach to assess Notch family mRNA expression patterns in an in vitro system (Fig. 2A). CTBs grown on Matrigel model interstitial differentiation/invasion in vivo (Librach et al., 1991; Zhou et al., 1997). In general, the results recapitulated the immunoanalyses. NOTCH2 mRNA levels peaked at 3 hours, NOTCH3 mRNA levels remained constant and NOTCH4 expression quickly declined with differentiation. DLL4 mRNA levels increased with differentiation. However, we repeatedly failed to detect JAG1 expression at either mRNA or protein levels under a variety of culture conditions we used to simulate the uterine vascular environment, e.g. EC, decidual cell or placental spiral arteriole co-culture, as well as physiological hypoxia, shear stress or high serum concentrations (data not shown). This was consistent with our conclusion that this culture model does not replicate the terminal stages of CTB endovascular invasion, e.g. upregulation of NCAM1 (Blankenship and King, 1996). Thus, the results of these experiments showed that this culture model is useful for studying Notch functions during the interstitial phases of differentiation/invasion but not during the final stages of endovascular remodeling.

Statistical analyses

Differences between means were assessed using either a paired two-tailed Student’s t-test (α=0.05) for RT-qPCR and CTB invasion experiments or assuming equal variance for the perfusion and casting experiments.
Therefore, this inhibitor concentration was subsequently employed. Next, we assessed the effects of inhibiting Notch activation on CTB invasion. L-685,458 significantly decreased invasion (Fig. 2D), evidence that Notch family members play a role in this process. Finally, we asked whether Notch inhibition affected the ability of the cells to upregulate the expression of markers of arterial ECs as they differentiated/invaded in culture. These experiments exploited our observation that, during this process, CTBs downregulate EPHB4 and upregulate EFNB2, markers of venous and arterial ECs, respectively (Red-Horse et al., 2005). Under control conditions, we observed robust upregulation of EFNB2 mRNA after 36 hours (Fig. 2E). In the presence of L-685,458, EFNB2 expression was blunted by 27% ($P=0.025$), as measured by Taqman RT-qPCR. By contrast, L-685,458 had no effect on EPHB4 or MMP9, which is required for CTB invasion (Librach et al., 1991). Together, these results supported a model in which Notch activity promoted CTB differentiation/invasion and acquisition of an arterial EC-like phenotype. Because cultured CTBs failed to replicate the terminal stages of CTB endothelial differentiation, we used mouse models to study the role of Notch in this process.

**Placental Notch activity was largely confined to the mouse ectoplacental cone and endovascular trophoblasts**

As the co-expression of Notch ligands and receptors does not necessarily correlate with function, independent methods are required to estimate Notch activity. However, few tools have been developed for use in human cells. Accordingly, we used the mouse transgenic Notch reporter (Tnr) line that expresses Gfp under the control of a Notch responsive promoter (Duncan et al., 2005) to assess Notch activity during development of the placenta. Tnr offspring and placentas were generated from timed matings between wild-type females and males harboring the Tnr transgene. Notch activity was first observed at E7.5 in a few rare cells at the leading edge of the ectoplacental cone (EPC), the primary source of invasive trophoblasts (data not shown). By E8.5, Notch activity was detected in association with many more EPC cells, particularly in the outer layers that were in contact with the uterus (Fig. 3A). Between E8.5 and E10.5, a subset of TGCs at the periphery of the implantation site acquired Notch activity, which was also evident in a growing population of trophoblast cells within the junctional zone (see Fig. S3A-C in the supplementary material), a region analogous to the maternal-fetal interface in humans. By E14.5 (Fig. 3B,C), the highest Notch activity was restricted to trophoblasts that were associated with maternal spiral arterioles with lower levels observed in the spongiotrophoblast region and in association with other cells that were sparsely distributed throughout the labyrinth. Placentas without the Tnr allele lacked Gfp signal (see Fig. S3D in the supplementary material). In mice, spiral artery-associated TGCs (SpA-TGCs) and GlyTCs carry out endovascular invasion, suggesting that Notch activity was dramatically increased in these cells. To confirm this theory, we quantified Tnr and trophoblast lineage marker co-expression by RNA in situ hybridization. Because of differences in probe strength, we performed double hybridizations for Tnr (Notch activity) and the TGC marker Prl2c2.
(Fig. 3D), and analyzed serial sections for Tnr (Fig. 3E) and the GlyTC marker Pcdh12 (Fig. 3F) \((n=13)\). Prl2c2-positive SpA-TGCs (48±8%) and approximately half of Pcdh12-positive endovascular GlyTCs had Notch activity. Accordingly, we profiled mouse TB expression of Notch family members with the goal of focusing subsequent studies on receptors that could mediate differentiation/invasion. We compared RNA levels in TS cells, which are known to express all components of the Notch pathway (Cormier et al., 2004), to their differentiated progeny, grown under normoxic conditions that produce primarily TGCs (Fig. 3G). Very similar to the patterns observed in human CTBs in vivo, only Notch2, Jag1 and Dll4 expression increased with differentiation, whereas Notch1, Notch3, Notch4, Jag2 and Dll1 expression declined. Taken together, these findings bolstered the conclusions of the in vitro human experiments that implicated Notch activity as a driver of CTB endovascular invasion.

**Notch2 deletion in invasive trophoblast lineages led to litter-wide lethality in proportion to the number of mutant offspring**

To investigate Notch function with regard to trophoblast invasion of spiral arterioles, we designed a strategy for conditional deletion of Notch2 in the invasive trophoblast lineages, which includes all SpA-TGCs and GlyTCS. Briefly, we bred mice harboring floxed alleles of *Notch2* (*Notch2fl*) (McCright et al., 2006) to Tp翰pa-Cre mice (Simmons et al., 2007) in which Cre recombinase is specifically expressed by EPC cells that later give rise to all GlyTCs, SpA-TGCs and spongiotrophoblasts. Following their differentiation from EPC precursors, spongiotrophoblasts normally maintain Tp翰pa expression, which GlyTCs and SpA-TGCs lose. To confirm the expected expression pattern, we performed serial RNA in situ hybridization for both Tp翰pa and Gfp, which is also a component of the Tp翰pa-Cre construct. Gfp expression by
spongiotrophoblasts typically occurred in nearly all cells at E9.5 (see Fig. S4A,B in the supplementary material) and in the majority of cells at E12.5 (see Fig. S4C,D in the supplementary material), evidence that the construct was expressed in the desired location.

Accordingly, we generated Notch2floxl+;Tpbpa-Cre (heterozygote) and Notch2floxl+;Tpbpa-Cre (mutant) embryos and offspring by breeding Notch2floxl+ and Tpbpa-Cre founders. In preliminary crosses, Notch2floxl+;Tpbpa-Cre offspring were born that were used for breeding purposes. Given that complete embryonic loss of Notch2 results in lethality by E11.5 (Hamada et al., 1999), we focused our analysis on this developmental window. First, we surveyed resorption rates in litters generated from different combinations of Notch2floxl+;Tpbpa-Cre or Notch2floxl+;Tpbpa-Cre parents, designed to produce 25%, 50% or 100% Notch2floxl+;Tpbpa-Cre progeny (see Table S1 in the supplementary material). In litters bred from Notch2floxl+;Tpbpa-Cre animals, only one resorption was observed among the 39 implantation sites (2.6%) examined between E10.5-E12.5. Breeding of Notch2floxl+;Tpbpa-Cre and Notch2floxl+;Tpbpa-Cre animals produced litters with many more resorptions. Loses, which were observed as early as E9.5, increased to a maximal frequency of 29% by E11.5. A similar temporal pattern was observed in Notch2floxl+;Tpbpa-Cre matings, but the resorption rate increased to 54%. Maternal factors did not contribute to increases in embryonic lethality, as crosses of Notch2floxl+;Tpbpa-Cre females to Notch2floxl+;Tpbpa-Cre males and reciprocal crosses of Notch2floxl+;Tpbpa-Cre females to Notch2floxl+;Tpbpa-Cre males exhibited similar rates of resorption (see Table S2 in the supplementary material). Next, we correlated embryonic genotype with resorption/survival (see Table S3 in the supplementary material). Surprisingly, in Notch2cloxl+;Tpbpa-Cre × Notch2floxl+;Tpbpa-Cre matings, resorptions involved equal numbers of Notch2cloxl+;Tpbpa-Cre and Notch2floxl+;Tpbpa-Cre embryos. This conclusion was substantiated by the fact that the surviving offspring had the same genetic distribution. Consistent with this observation, we did not note any differences in embryonic or placental weight between Notch2cloxl+;Tpbpa-Cre and Notch2floxl+;Tpbpa-Cre offspring (see Table S4 in the supplementary material). Taken together, these results suggest that embryonic lethality correlated with the presence of Notch2floxl+;Tpbpa-Cre embryos rather than the genotype of individual fetuses.

In initial breeding experiments, we noted that Notch2floxl+;Tpbpa-Cre mothers had significantly fewer implantation sites than Notch2cloxl+;Tpbpa-Cre mothers (5.7±2.8 versus 7.5±2.8, P=0.03). This result was independent of embryonic genotype, as the implantation rate was identical in Notch2cloxl+;Tpbpa-Cre females bred to Notch2cloxl+;Tpbpa-Cre or Notch2cloxl+;Tpbpa-Cre males. In searching for a possible explanation, we discovered that the Notch2floxl+;Tpbpa-Cre females exhibited severe ovarian defects, including hemorrhagic and cystic follicles, which were not observed in Notch2cloxl+;Tpbpa-Cre females (see Fig. S5A in the supplementary material). These observations suggested that the Tpbpa-Cre transgene might also have ovarian expression. To address this possibility, we bred Tpbpa-Cre females to males of the Cre reporter strain Gt(Rosa26)26Soim1locZ, which has a ROSA26-lox-stop-lox-lacZ sequence, and examined Gt(Rosa26)26Soim1locZ;Tpbpa-Cre embryos/offspring for β-galactosidase activity (i.e. evidence of Tpbpa-Cre expression). In placenta, we observed β-galactosidase activity in spongiotrophoblasts, TGC, and GlyTCs as previously reported (Simmons et al., 2007). In the adult ovaries, we observed β-galactosidase activity in the granulosa cells (see Fig. S5B in the supplementary material). Accordingly, we generated Tpbpa-Cre females to address this possibility, we bred Tpbpa-Cre females to males of the Cre reporter strain Gt(Rosa26)26Soim1locZ, which has a ROSA26-lox-stop-lox-lacZ sequence, and examined Gt(Rosa26)26Soim1locZ;Tpbpa-Cre embryos/offspring for β-galactosidase activity (i.e. evidence of Tpbpa-Cre expression). In placenta, we observed β-galactosidase activity in spongiotrophoblasts, TGC, and GlyTCs as previously reported (Simmons et al., 2007). In the adult ovaries, we observed β-galactosidase activity in the granulosa cells (see Fig. S5B in the supplementary material). We also noted infrequent Rosa26-lacZ recombination events in a small percentage of cells (~1-5%) in other organs, including the uterus (see Fig. S5C in the supplementary material). We performed RNA in situ hybridization for Tpbpa to assess possible ovarian expression. In the absence of signal (see Fig. S5D in the supplementary material), the ovarian phenotype was attributable to nonspecific promoter activity. Thus, to avoid possible confounding ovarian effects in Notch2floxl+;Tpbpa-Cre mothers, all the data used to compare Notch2floxl+;Tpbpa-Cre and Notch2floxl+;Tpbpa-Cre offspring were generated from experiments using Notch2cloxl+;Tpbpa-Cre mothers, and comparisons were made between offspring of the same litter. Additionally, to ensure that Notch2floxl+;Tpbpa-Cre

Fig. 4. Conditional deletion of Notch2 in invasive trophoblasts reduced TGC and GlyTC invasion of spiral arterioles. (A-H) In situ hybridization was performed on tissue sections of E12.5 Notch2floxl+;Tpbpa-Cre and Notch2floxl+;Tpbpa-Cre placentas for the TGC marker PrI2c2 (A,B, magnified in E,F, respectively) and the GlyTC marker Pcdh12 (C,D, magnified in G,H, respectively). Hybridization was visualized by NBT/BCIP staining (purple). Nuclei were stained with nuclear Fast Red. The decidua (dec), junctional zone (jz) and labyrinth (lab) are shown. In Notch2floxl+;Tpbpa-Cre mice, very few TGCs and GlyTCs invaded spiral arterioles (arrowheads). Scale bars: 400 μm in A-D; 100 μm in E-H.
In the absence of Notch2, TGCs and GlyTCs failed to invade maternal spiral arterioles, which was associated with reduced canal size and placental perfusion

To assess whether trophoblast differentiation/invasion failed in Notch2<sup>floxlflox</sup>;Tpbpa-Cre placentas, we used RNA in situ hybridization to survey lineage-specific markers. No overt differences were observed at E9.5 or E12.5 between Notch2<sup>flxflo</sup>;Notch2<sup>flox+</sup>;Tpbpa-Cre and Notch2<sup>flxflo</sup>;Tpbpa-Cre placentas with regard to the presence/absence of markers of labyrinthian trophoblast cell types (Gem1, Hand1), spongiotrophoblasts (Tpbpa), TGCs (Prl3d1, Prl3b1 and Prl2c2) or GlyTCs (Pcdh12) (see Fig. S6 in the supplementary material and data not shown). However, although TGCs and GlyTCs in Notch2<sup>flx+</sup>;Tpbpa-Cre placentas robustly invaded maternal spiral arterioles at E12.5 (10/11 placentas examined) (Fig. 4A,C; enlarged in Fig. 4E,G, respectively), this process failed in the large majority of the Notch2<sup>flxflo</sup>;Tpbpa-Cre placentas (7/9 placentas examined) (Fig. 4B,D; enlarged in Fig. 4F,H, respectively). To rule out possible differences in marker expression levels in Notch2<sup>flxflo</sup>;Tpbpa-Cre placentas, we confirmed these results by immunostaining with the Tromo-1 antibody (see Fig. S7 in the supplementary material), which reacts with a cytokeratin that is expressed by all trophoblast populations (Amarante-Paffaro et al., 2011).

Accordingly, we explored the functional consequences of these observations. Specifically, we looked for abnormalities in the structure of the trophoblast-lined canals that transport maternal blood to the placenta. To obtain an integrated view of the anatomical basis of placental perfusion, we prepared vascular corrosion casts of the maternal circulation at E10.5 and E14.5 (Fig. 5). Overall, the branching patterns in Notch2<sup>flxflo</sup>;Tpbpa-Cre and Notch2<sup>flx+</sup>;Tpbpa-Cre placentas were similar; maternal blood entered the decidua through a tortuous network of ~8-12 interconnected spiral arterioles that converged into ~1-4 canals at the junctural zone boundary before spreading into a sinusoidal network at the base of the labyrinth. Given the complexity of the spiral artery network, we focused our analysis on the anatomical regions of the canals. At E10.5, the canals in Notch2<sup>flx+</sup>;Tpbpa-Cre placentas (Fig. 5A; magnified in Fig. 5C) were larger than those in Notch2<sup>flxflo</sup>;Tpbpa-Cre placentas (Fig. 5B; magnified in Fig. 5D). We noted similar differences between Notch2<sup>flx+</sup>;Tpbpa-Cre (Fig. 5E; magnified in Fig. 5G) and Notch2<sup>flxflo</sup>;Tpbpa-Cre animals (Fig. 5F; magnified in Fig. 5H) at E14.5. The results were quantified by translating the estimated vessel diameters into cross-sectional areas (Fig. 5I). The results showed significant decreases in Notch2<sup>flx+</sup>;Tpbpa-Cre placentas at E10.5 (40%) and E14.5 (34%). No significant differences were observed between Notch2<sup>flx+</sup>;Tpbpa-Cre (n=14) and Notch2<sup>flxflo</sup>;Tpbpa-Cre (n=11) animals in the average number of canals per placenta (3.2±1.2 versus 2.9±1.0; P=0.51).

The reduced canal size in Notch2<sup>flx+</sup>;Tpbpa-Cre placentas suggested that placental perfusion might be compromised. To test this theory, we estimated possible differences by injecting a 70 kDa fluorescein-conjugated dextran via the tail vein and measuring its short-term accumulation within the labyrinth at E12.5 (Plaks et al., 2010). Analysis of placental tissue sections from Notch2<sup>flx+</sup>;Tpbpa-Cre animals (Fig. 6A,B) when compared with their Notch2<sup>flx+</sup>;Tpbpa-Cre counterparts (Fig. 6C,D) revealed increased fluorescence intensity. Although we did not observe differences between the two groups in the relative size of the placental labyrinth or its vascular spaces, quantification of the total labyrinthian-FITC signal showed that Notch2<sup>flx+</sup>;Tpbpa-Cre placentas accumulated 23% less fluorescein-conjugated dextran (Fig. 6E). Together, the
results of the marker analyses, vascular corrosion casting and placental perfusion experiments supported a possible causal relationship between Notch2-directed trophoblast invasion of spiral arteries and corresponding increases in placental perfusion.

**In PE, CTBs that associated with spiral arterioles failed to express JAG1**

Based on the mouse data, we tested the hypothesis that the faulty CTB differentiation/invasion observed in PE is associated with alterations in the expression of Notch molecules. As we previously described, pre-term labor (PTL) cases, which we used as controls, were associated with normal levels of CTB interstitial, perivascular and endovascular invasion (Zhou et al., 2007) (Fig. 7A-C). In addition, CTB differentiation in PTL is normal, as are gene expression patterns in the basal plate (Winn et al., 2009).

By contrast, CTB invasion was variably reduced in severe pre-eclampsia (SPE), and in the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. In some instances, CTB invasion failed altogether. In others, CTBs were either confined to perivascular spaces (Fig. 7D), exhibited limited endovascular invasion (Fig. 7E) or transformed maternal arteries as in normal pregnancy (Fig. 7F). To assess the expression of Notch family members, we used immunolocalization and RNA in situ hybridization approaches. We failed to detect any differences between cases and controls in CTB NOTCH2-4 and DLL4 expression patterns and staining intensities (data not shown).

Similar to our observations in second trimester biopsies, the majority of perivascular and endovascular CTBs exhibited robust JAG1 mRNA expression in PTL samples (Fig. 7A-C). However, a CTB JAG1 signal was absent in many vessels from PE cases (Fig. 7D-F) regardless of the extent of vascular remodeling. We confirmed these findings at the protein level (Fig. 7G,H). We calculated the percentage of JAG1-positive CTB-modified vessels in individual PTL and PE cases (Fig. 7I and see Table S5 in the supplementary material). All the modified vessels in each of six PTL control cases contained JAG1-positive CTBs. This fraction was reduced to 53% in ten SPE cases and to 26% in three HELLP cases. Taken together, the correlation between reduced JAG1 expression and failed vascular remodeling in PE suggests that Notch signaling plays an important functional role in remodeling of human spiral arterioles.

**DISCUSSION**

Through their aggressive invasion/remodeling of uterine vessels, CTBs incorporate the placenta into the maternal circulation. As this component of placentation fails in PE, the mechanisms involved are of great interest. The first evidence that Notch might regulate CTB differentiation/invasion came from experiments in which we profiled Notch expression in tissue sections of the human placenta and in CTBs that were differentiating in vitro. Notably, multiple Notch pathway components were similarly expressed by CTBs in both contexts and were precisely modulated during specific stages of the invasion process. Functional inhibition in cultured CTBs confirmed that Notch signaling influenced interstitial CTB invasion and EFNB2 expression. To understand Notch functions during endovascular remodeling, we used mouse models to demonstrate that the endovascular trophoblast populations possessed the highest levels of Notch activity and that Notch expression patterns were similar in both species. As Notch2 was the only receptor upregulated during mouse trophoblast invasion, we targeted this molecule for deletion in the invasive trophoblast lineages. Unexpectedly, Notch2floxflox; Tpbpa-Cre and Notch2floxflox; Tpbpa-Cre embryos were lost in proportion to the number of Notch2floxflox; Tpbpa-Cre offspring, suggesting that failures of individual feto-placental units had litter-wide effects. Notch2 proved to be a crucial determinant of endovascular invasion; TGCs and GlyTCs from Notch2floxflox; Tpbpa-Cre placenta failed to invade maternal spiral arterioles. Furthermore, failed endovascular invasion was associated with decreased canal size in Notch2floxflox; Tpbpa-Cre animals, a likely cause of the observed decline in placental perfusion. As Notch signaling was required for endovascular invasion in the mouse, we investigated the expression of family members in PE, which is associated with failures in this process. The results showed that artery-associated CTBs often lacked JAG1 expression. To our knowledge, JAG1 is the only marker that distinguishes between endovascular CTBs in normal pregnancies and those complicated by PE.

Notch functions in other cellular contexts offer unique insights into how these signaling pathways specify trophoblast identity and regulate endovascular invasion. Notch2 is the only receptor that has not been associated with arterial identity (Villa et al., 2001). In developing vascular beds, Notch signaling in EC progenitors promotes their assumption of arterial identity, which is
characterized by \textit{Efnb2} expression in the absence \textit{Ephb4}. The converse is observed in venous endothelium, helping to organize arteriovenous boundaries through repulsive interactions generated through bi-directional signaling between adjacent cells. We have previously demonstrated that \textit{EFNB2}/\textit{EPHB4} play similar roles in directing CTB invasion away from the placenta and toward uterine spiral arterioles (Red-Horse et al., 2005). Here, we showed that Notch plays an upstream role in regulating CTB \textit{EFNB2} expression.

The striking upregulation of \textit{JAG1} expression and Notch activity in the endovascular trophoblast populations suggests that CTBs experience physiological cues that promote Notch signaling and subsequent \textit{EFNB2}-dependent endovascular invasion. Shear stress and cyclic strain, which are high in uterine spiral arterioles and low in uterine veins during pregnancy, offer possible explanations of how Notch signaling is initiated in the arteries. In ECs, these forces rapidly enhance Notch receptor cleavage/activation, increase expression of Notch receptors and ligands, and promote Notch-dependent \textit{EFNB2} upregulation (Masumura et al., 2009; Morrow et al., 2007; Wang et al., 2007). Interestingly, ligand endocytosis potentiates cleavage and activation of Notch receptors, leading several groups to propose that mechanical forces expose the S2 cleavage site within the Notch receptor (Kohan and Ilagan, 2009). Thus, it is possible that cyclic strain and shear stress experienced by CTBs near spiral arterioles provides a mechanical force that potentiates Notch activation, \textit{JAG1} upregulation and \textit{EFNB2}-dependent signaling. However, our attempts to modulate CTB expression of Notch family members with shear stress were unsuccessful. Likewise, CTB co-culture with ECs or decidual cells, and in a low oxygen tension environment failed to upregulate \textit{JAG1} expression. Thus, it is likely that a complex interplay among cell types and physiological factors is required.

\textit{Notch2} function is required for mouse placental development (Hamada et al., 2007). Tetraploid complementation, in which \textit{Notch2}-deficient embryos were provided with wild-type placentas, revealed placental insufficiency as the primary cause of lethality. \textit{Notch2}–/– animals exhibited dramatic reductions in maternal blood spaces within the placental labyrinth without obvious defects in the differentiation of labyrinthian trophoblasts, spongiotrophoblasts or TGCs. However, the mechanism of placental insufficiency remained to be elucidated. Our observation that Notch activity was highest in the endovascular trophoblast populations suggested that this remodeling process played an important role in determining the \textit{Notch2}–/– placental phenotype. We addressed this possibility by specifically eliminating \textit{Notch2} in this population. These experiments revealed similar phenotypes in terms of the timing of embryo loss and reduced accumulation of maternal blood in the labyrinth, but differed in that we did not observe changes in the size of the maternal sinusoidal spaces. Rather, our study uniquely identified deficits in endovascular invasion and decreases in the
size of canals that supply blood to the labyrinth. The disparate phenotypic outcomes of the two gene deletion strategies may highlight unique functions of endovascular and labyrinthian Notch2 activity; labyrinthian cells appear to play a distinct role in organizing the sinusoidal blood spaces, whereas endovascular trophoblasts coordinate maternal increases in blood supply.

Interestingly, disrupting perfusion of individual placentas had litter-wide effects on pregnancy outcome. The frequency of fetal resorption correlated with the percentage of Notch2flox/flox;Tpbpa-Cre offspring in the litter. Unexpectedly, fetal loss was equally distributed among littersmates regardless of genotype. As mice with Notch2 haploinsufficiency are viable (Witt et al., 2003), these results suggested that reducing perfusion in Notch2flox/flox;Tpbpa-Cre placentas had negative effects on the survival of both Notch2flox/flox;Tpbpa-Cre and Notch2flox/flox;Tpbpa-Cre animals. Although the mechanism(s) is unknown, it is well established that endovascular invasion produces local decreases in vascular resistance of the arteries that supply each fetus. Conversely, failed vascular transformation is associated with local increases in vascular tone that drive parallel systemic changes. These effects could account for our finding. This theory is supported by observations made in the reduced utero-placental perfusion model (RUPP) in which the uterine blood flow of pregnant rats is surgically reduced. RUPP increases sensitivity to vasoactive factors at both local and systemic levels, which results in hypertension (Anderson et al., 2005; Crews et al., 2000). These findings were further substantiated by a recent study of placental gene expression in the Norway Brown rat (Goyal et al., 2010), a strain that exhibits significantly decreased trophoblast vascular invasion. Compared with control strains, the placentas of these animals secrete increased levels of vasoconstrictive factors and decreased amounts of vasodilators. Additionally, they have a much higher rate of fetal loss. Taken together, these studies may explain how local reductions in utero-placental perfusion can produce systemic changes in vascular contractility with litter-wide effects.

Our findings that Notch2flox/flox;Tpbpa-Cre placentas failed to remodel maternal blood vessels adequately, which resulted in reduced placental perfusion, suggested that aberrations in the expression of Notch family members might be associated with PE. Another study that addressed this possibility reported expression changes in vascular contractility with litter-wide effects. The distinctive expression pattern of JAG1, combined with the failure of endovascular remodeling in PE, suggested that Notch signaling is a crucial component of this unusual process. Although it is difficult to determine whether altered JAG1 expression in PE is a prerequisite or a by-product of failed endovascular invasion, the phenotype of the Notch2flox/flox;Tpbpa-Cre placenta supports a causal role.

In summary, we provide evidence that Notch signaling is a crucial component of the process whereby fetal trophoblast cells invade and remodel maternal blood vessels. Failure of this physiological transformation in the absence of Notch2 is associated with reduced vessel diameter and placental perfusion, findings that support the conclusion that trophoblasts coordinate increases in maternal vascular supply through the progressive invasion and the resultant dilation of maternal vessels. The finding that peri- and endovascular CTBs often fail to express JAG1 in PE provides further evidence that defects in Notch signaling are an important part of the pathogenesis of this pregnancy complication.

Acknowledgements
We thank H. Stolp, M. J. Scott and G. Goldfien for assisting with human tissue collection, and acknowledge the experimental assistance of Y. Zhou, K. Red-Horse, P. A. Murphy, C. J. Shawber and D. Miniati. The Notch2flox/flox mice were a generous gift from T. Gridley. Work was supported by NIH grant HD055764. V.P. was supported by Machiah and Bikura foundation fellowships from the Israel Science Foundation. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl;doi:10.1242/dev.066589/-/DC1

References


