Notch signalling in placental development and gestational diseases

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Abstract

Activation of Notch signalling upon cell-cell contact of neighbouring cells controls a plethora of cellular processes such as stem cell maintenance, cell lineage determination, cell proliferation, and survival. Accumulating evidence suggests that the pathway also critically regulates these events during placental development and differentiation. Herein, we summarize our present knowledge about Notch signalling in murine and human placentation and discuss its potential role in the pathophysiology of gestational disorders. Studies in mice suggest that Notch controls trophoderm formation, decidualization, placental branching morphogenesis and endovascular trophoblast invasion. In humans, the particular signalling cascade promotes formation of the extravillous trophoblast lineage and regulates trophoblast proliferation, survival and differentiation. Expression patterns as well as functional analyses indicate distinct roles of Notch receptors in different trophoblast subtypes. Altered effects of Notch signalling have been detected in choriocarcinoma cells, consistent with its role in cancer development and progression. Moreover, deregulation of Notch signalling components were observed in pregnancy disorders such as preeclampsia and fetal growth restriction. In summary, Notch plays fundamental roles in different developmental processes of the placenta. Abnormal signalling through this pathway could contribute to the pathogenesis of gestational diseases with aberrant placentation and trophoblast function.

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1. General role of Notch signalling

Notch represents an evolutionarily conserved signalling pathway critically involved in embryonic development and tissue homeostasis of adult organs. It controls a vast range of cellular processes including proliferation, survival, differentiation and motility. Signalling through Notch requires direct cell-cell contact allowing for short-range communication between neighbouring cells [1]. Hence, the pathway is ideally suited to control self-renewal and differentiation in stem cell niches [2,3]. In the latter, Notch was shown to mediate binary cell fate decisions: different levels of Notch activation are created in two initially equipotent, adjacent precursor cells thereby promoting adoption of distinct cell fates. For example, in the developing mouse brain Notch inhibited neuronal differentiation, but induced a glial cell fate and promoted astrocyte differentiation [2]. Likewise, Notch signalling is crucial for epithelial stemness and cell fate specification. It maintains murine intestinal stem cells and balances the cell fate of terminally differentiating gut cells [3]. Interestingly, Notch was shown to have an opposed role in intestinal stem cells of Drosophila, impairing their self-renewal. Hence, Notch signalling is highly complex and depends on the cellular context as well as on its cross-talk with other developmental signalling cascades mediated through members of the Wingless (Wnt), epidermal growth factor (EGF) or transforming growth factor-β (TGF-β) family [4,5]. Conditions of the particular microenvironment such as composition of the extracellular matrix, shear stress or hypoxia also determine the various effects of Notch in cells and tissues [4]. Accordingly, Notch signalling components may act as tumour suppressors or oncogenes in different organs and differentially influence cancer development and progression [6–8]. Considering its regulatory role in de novo vessel formation, angiogenesis and branching morphogenesis [9–11], it may not be surprising that Notch also controls these processes during placental development [12]. The present review summarises our current knowledge about Notch signalling in murine and human placentation and discusses its role in the context of gestational diseases and choriocarcinoma cell function.

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1.1. Canonical Notch signalling

Canonical Notch signalling is activated upon interaction of a membrane-anchored ligand on the signal-sending cell with a Notch receptor on the adjacent signal-receiving cell [1,13]. In contrast, binding of the ligand to a receptor on the same cell was shown to inhibit the pathway [14]. In mice and humans four different Notch receptors (Notch1-4) and five distinct Notch ligands, the Serrate-like ligands (Jagged1 and 2) and the Delta-like ligands (DLL1, 3 and 4) have been identified [1]. Notch receptors are single transmembrane proteins. After translation the Notch polypeptide chain is cleaved by furin-like convertases generating a heterodimer of the Notch transmembrane domain (TMCD) non-covalently linked to the Notch extracellular domain (NECD) (Fig. 1). NECD harbours epidermal growth factor (EGF)-like repeats necessary for ligand interaction. These repeats can be modified by different types of glycosylation thereby governing differential ligand binding and receptor activation [15].

NTM-ICD contains a trans-membrane domain (TMD) and different intracellular protein motifs required for the nuclear function of the Notch intracellular domain (NICD). The latter arises after two sequential cleavages from the receptor [1]. Upon interaction of Notch with its ligand, members of the ADAM family, such as ADAM10 or ADAM17, cleave NTM-ICD 12 amino acids before the trans-membrane domain thereby generating the intermediary membrane protein Notch extracellular truncation (NEXT). Subsequently, NEXT is recognized by γ-secretase [16], cutting the protein at two different cleavage sites in the TMD. These proteolytic steps release NICD from the membrane allowing it to translocate into the nucleus where it acts as a co-activator of transcription. Besides a transactivation domain, NICD comprises a RAM (RBPJκ-associated module) domain for high affinity binding of the transcription factor recombination signal binding protein for immunoglobulin kappa J (RBPJκ) as well as ankyrin repeats for RBPJκ activation through binding of co-activators of the mastermind-like (MAML) family. In the absence of NICD RBPJκ operates as a repressor recruiting histone deacetylases and other co-repressors such as CtBP (C-terminal-binding protein 1), CIR (CBF1 interacting co-repressor) or SHARP (SMRT and HDAC associated repressor protein) [5]. Upon Notch activation these repressors are displaced by NICD thereby activating Notch target genes after binding of MAML proteins and further co-activators [17]. Activity of canonical Notch signalling is regulated by multiple mechanisms controlling post-translational Notch modification, receptor availability at the membrane, and expression of soluble Notch ligands, discussed elsewhere [1,13].

The expression of Notch targets strictly depends on the cell- and tissue-specific role of the pathway. Whereas NICD induces proliferation by directly activating cyclin D1 and CDK5, it promotes growth arrest and differentiation by inducing kinase inhibitors such as p21 [18–20]. However, the prime targets of canonical Notch signalling are members of the Hairy/Enhancer of Split (HES) family, HES1, HES5, HES7 and Hairy/Enhancer-of-split related with YRPW motif (HEY) proteins, HEY1, HEY2 and HEYL [21]. HES and HEY proteins are basic helix loop helix proteins (bHLH) acting as transcriptional activators. 

![Fig. 1. The canonical Notch pathway.](image-url)

Membrane-anchored Notch ligands contain a transmembrane domain (TMD), a cysteine (C)-rich domain and EGF-like repeats for binding to Notch receptors. Moreover, the N-terminal DSL (Delta/Serrate/LAG-2 proteins) domain and the DOS (Delta and OSM-11-like proteins) motif contribute to receptor binding. In contrast to Jagged1 and Jagged 2, the C-rich region is absent from DLL1, whereas DLL3 and DLL4 additionally lack the DOS domain [1]. The co-ligands Delta-like homologue 1 and 2 (DLK1 and 2) harbour a DOS domain but do not contain a DSL motif. Depending on the receptor subtype, the Notch extracellular domain (NECD) contains differential numbers of EGF-like repeats. In addition, NECD comprises cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD) for binding to the Notch transmembrane and intracellular domain (NTM-ICD). Upon Notch activation, cleavage by ADAM proteases generates the Notch extracellular truncation (NEXT) which is further processed by γ-secretase yielding Notch intracellular domain (NICD). The latter harbours a nuclear localisation sequence (NLS), the RBPJκ-associated module (RAM) for binding to RBPJκ and ankyrin (ANK) repeats for interaction with mastermind-like (MAML) proteins. Nuclear translocation of NICD displaces co-repressors (Co-R) from RBPJκ and recruits co-activators (Co-A) thereby promoting transcription of canonical Notch target genes such as repressors of the HES and HEY family. Stability of NICD is regulated via ubiquitination of proline/glutamic acid/serine/threonine-rich (PEST) motifs and proteosomal degradation.
transcriptional repressors, particularly upon binding to co-repressors of the Groucho/Transducin-like enhancer of split (TLE) family [22,23]. These protein complexes are critically involved in the maintenance of stem and progenitor cells by repressing key regulators of cell fate determination.

Along those lines, the role of canonical Notch signalling in stemness and cell line lineage decisions is highly complex and explained by different regulatory mechanisms [2,3,13]. In lateral inhibition equivalent precursors undergo different cell fates since high expression of a Notch ligand in a given cell activates the pathway in a neighbouring cell. In the latter, NICD-RBPJκ prevents Notch ligand expression through the induction of HES. As a consequence of subsequent feedback loops, the ligand-expressing cell is maintained in a Notch-inactive state whereas the activated cell adopts a different cell fate. Alternatively, Notch-dependent cell fate decisions are regulated by inhibitors of the NUMB family which have been described as multifunctional adaptor proteins involved in endocytosis and intracellular trafficking of membrane-bound molecules [24]. NUMBs interact with proteins of the endocytic machinery, such as α-adaptin and Eps15, thereby promoting internalisation and sequestration of Notch receptors. Moreover, NUMB was shown to antagonise canonical Notch signalling by binding to NTM-ICD and membrane-tethered NICD, thus recruiting E3-ubiquitin ligases for proteasomal degradation. The adoption of distinct cell fates is achieved by asymmetrical segregation of NUMB during cell division of a progenitor, thereby creating two different daughter cells with active and inactive Notch signalling, respectively [13].

1.2. Non-canonical Notch signalling

Besides the canonical activation of RBPJκ, full-length Notch receptors as well as NICD exert non-canonical effects in cells and tissues in a ligand-dependent or -independent manner [25]. Notch has been initially identified as a cell adhesion molecule in Drosophila and was also shown to promote cell-cell interactions in mammals independently of NICD generation [26]. Moreover, NTM-ICD and/or membrane-tethered NICD bind signalling components, such as phosphatidylinositol-3-kinase (PI3K) and mammalian target of rapamycin C2 (mTORC2) [27], promoting cell survival via AKT (Fig. 2). Notch-mediated activation of these kinases suppresses pro-apoptotic p53 or stabilizes X-linked inhibitor of apoptosis (XIAP) [28,29]. Moreover, nuclear NICD interacts with a variety of non-canonical transcription factors, for example nuclear κB (NFκB), hypoxia-inducible factor 1α (HIF1α) or Ying Yang1 (YY1) [30–32], promoting their nuclear retention and/or activation of Notch targets upon binding to RBPJκ (Fig. 2). Notch enhances NFκB signalling at various levels [33]. In addition to direct binding of the NFκB p52 subunit, NICD interacts with κB kinase α (IKKα) promoting phosphorylation of inhibitor of κB (IkB). Degradation of the latter allows for nuclear translocation of the active transcription factor and expression of its target genes. Notch’s cross-talk to Wnt signalling is mediated through binding of its central player, active β-catenin, to the ankyrin repeats of the full-length receptor. Subsequently, Notch promotes lysosomal degradation of active β-catenin involving NUMB-mediated endocytosis thereby antagonizing canonical Wnt signalling [34].

2. Notch signalling in reproductive tissues

Besides other pathways Notch-mediated cell-cell communication plays a crucial role in the development of reproductive organs and their homeostasis in adults. In females, the particular signalling cascade controls formation of the reproductive tract, folliculogenesis, proliferation of granulosa cells during oogenesis, as well as oocyte survival and maturation [35–37]. Likewise, Notch is also involved in spermatogenesis [38]. Abnormal Notch activation has been noticed in ovarian and testicular cancer [39,40]. Functions of individual Notch proteins and their cross-talk with other signalling pathways in germ cells are above the scope of this review and discussed elsewhere [41].

2.1. Notch signalling in blastocyst formation, implantation and decidualization

Expression of Notch signalling components has been observed in embryos commencing with the 4 cell-stage suggesting that the pathway could be required for pre-implantation development [42]. Moreover, Notch expression in murine and human trophoectoderm (TE) suggested that it could be involved in early lineage decisions [43,44]. However, homozygous mutation of RBPJκ in mice did not affect progression of zygotes to the blastocyst stage indicating that Notch signalling might not be necessary for early embryonic development [45]. On the other hand, RBPJκ was shown to also have Notch-independent roles, largely by acting as a transcriptional repressor [46]. Indeed, a more recent study suggested that Notch1 ICD promotes expression of the key regulator of TE formation, caudal-related homeobox transcription factor 2 (Cdx2), in conjunction with the Hippo signalling-dependent activator TEAD4 and converts outer blastomere cells to a TE cell fate [47].

Notch signalling also critically regulates implantation and decidualization [48]. Notch receptors and ligands are differentially expressed during the menstrual cycle and have been detected in the luminal and glandular epithelium of the non-pregnant uterus as well as in stromal and endothelial cells [48]. Notch4 is markedly expressed during the proliferative phase, whereas Jagged1, DLL4 and Notch1 are predominantly produced during the mid-secretory phase [49–52]. Changes in the expression of Notch1, DLL1 and Jagged1 were noticed in infertile women suggesting that levels of some of the Notch receptors and ligands are critical for uterine receptivity [53,54]. Indeed, precisely regulated levels of activated Notch1 are pivotal for successful implantation and decidualization [55]. Human chorion gonadotrophin (hCG)-mediated induction of Notch1 was shown to promote survival of uterine stromal cells and initiated decidualisation, whereas downregulation of Notch1 and up-regulation of NUMB was required for completion of differentiation [56,57]. Women suffering from endometriosis have decreased levels of Notch1, Notch4, Jagged2 and DLL4 [58]. Moreover, overexpression of Notch1 ICD in the murine uterus causes infertility due to the lack of uterine glands and DNA-methyltransferase 3b-mediated hypermethylation of the prostate receptor gene [59]. During the first trimester of pregnancy Notch2 was the predominant receptor in decidual stromal cells which additionally express Jagged1, DLL1 and DLL4, whereas decidual glands produce all Notch receptors and ligands [60]. Disruption of Notch2 impaired expression of the differentiation markers prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) suggesting that the receptor is crucial for decidualization [60]. Moreover, different receptors and ligands were detected on uterine NK (uNK) cells and DLL1 and DLL4 increased interferon-γ secretion [60,61].

2.2. Notch signalling components control murine placental development

Regulation of angiogenesis represents one of the best characterised functions of Notch signalling. DLL4-Notch determines the cell fate of endothelial precursors towards an arterial or venous phenotype and controls specification of endothelial tips and stalk cells as well as vessel stability and sprouting [11]. Likewise,
homozygous mutations of Notch and its target genes mainly affect the murine placental vascular system and/or branching morphogenesis of chorionic villi which goes in hand with vessel sprouting [12]. Disruption of Notch1, Notch1/Notch4, Dll4, Hey1, Hey2 or RBPJκ inhibited chorio-allantoic branching and/or formation of an appropriate labyrinthine vascular network around midgestation [62,67]. In addition, failures in chorion-allantoic fusion and decreased numbers of proliferative trophoblasts have been reported in RBPJκ mutant mice [62,68].

Like in invasive human EVTs [69], Notch2 seems to be the predominant receptor in diverse mouse trophoblast subtypes. Notch2 has been detected in the chorion, the ectoplacental cone, in spongiotrophoblasts as well as in sinusoidal trophoblast giant cells (TGCs) and TGCs lining the maternal blood vessels [70–72]. Complementary expression of Notch2 and its putative ligands DLL1, DLL4, Jagged1 or Jagged2 has been detected in adjacent cell layers of the chorion, junctional zone and labyrinth, respectively, suggesting that the receptor could orchestrate different steps of placental development [73]. Although the receptor may not play a role in trophoblast cell type specification, it is critically involved in establishing the placental-maternal circulation. Notch2 gene knock-out prevented formation of blood sinuses and caused delayed placental perfusion [70]. The role of Notch2 in murine placenta was investigated in more detail using a homozygous deletion of Notch2 in spongiotrophoblast precursors giving rise to invasive trophoblast subtypes, spiral artery-associated TGCs and canal TGCs [74]. Conditional deletion of the gene inhibited arterial invasion and reduced the size of maternal blood canals as well as perfusion indicating a crucial role for Notch2 in trophoblast-mediated vessel remodelling and differentiation of the above mentioned TGC subtypes [72]. A similar phenotype was observed in TLE3 mutant mice suggesting that Notch2 and TLE3 operate in the same pathway [75].

2.3. Notch signalling regulates human placental development

Notch receptors and their ligands display trophoblast-subtype specific expression in the human placenta and also vary with gestational age and trophoblast differentiation [72,76,77]. Although not functionally tested, expression of Notch1, DLL1, DLL4 and Jagged1 in endothelial cells of villi suggests a role in placental vessel formation and/or angiogenesis [77]. Akin to its expression in invasive mouse trophoblasts, Notch2 was predominantly detected in different extravillous trophoblast (EVT) subtypes of first and second trimester tissues and increased during EVT formation in vivo and in vitro [69,72,76,77]. Interestingly, in distal HLA-G+ cell column trophoblasts (CCTs) Notch2 was mainly detected at the cell membrane, whereas intramural EVTs of remodelling spiral arteries additionally expressed nuclear Notch2 ICD [69]. Hence, it is anticipated that Notch2-dependent signalling is activated by adjacent maternal endothelial cells expressing DLL1, DLL4, Jagged1 and Jagged2 in first trimester decidual tissues [69]. Human Notch2 ICD might fulfill a similar role as its murine counterpart being critical for endovascular trophoblast invasion and adaption of blood flow to the placenta [72]. In contrast, disruption of Notch2 increased in vitro motility in human primary trophoblast preparations containing Notch2+ distal cell column trophoblasts [69]. Therefore, we speculate that maintenance of column integrity by cell-cell

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**Fig. 2.** Non-canonical effects of Notch. Full-length Notch receptor, NTM-ICD and NICD provide a binding interface for multiple proteins exhibiting transcriptional or kinase activity. Notch increases cell survival by activating PI3K/AKT/mTOR signalling and negatively affects Wnt-β-catenin signalling by triggering degradation of active β-catenin. Nuclear NICD interacts with different transcription factors such as HIF1α or YY1 in a complex with RBPJκ, bound to its cognate sequence, thereby integrating different environmental signals. Furthermore, Notch promotes activation of NFκB through direct binding of its p52 subunit and indirectly by promoting phosphorylation and proteosomal degradation of IκB through activation of IKKα.
adhesion could be the main function of Notch2 in the distal portion of the cell column [69].

Similar to other developing systems, the role of Notch signalling in human placenta is complex and likely depends on the specific cellular context. Notch1, Notch3 and Notch4 have been detected in cytotrophoblast (CTB) progenitors whereas Notch2 and Notch3 are expressed by invasive trophoblasts [72,76]. The overall Notch activity decreased during in vitro EVT formation suggesting that the pathway could be mainly associated with maintenance and stemness of trophoblast precursors [78]. Indeed, Notch1 was recently shown to be critical for trophoblast survival and proliferation of villous CTBs and CCT progenitors [24]. Conversely, inhibition of γ-secretase or silencing of RBPJk did not provoke apoptosis and promoted outgrowth in explant cultures and, as a consequence, EVT differentiation [24,78]. These contrasting results might be explained by the Notch-independent roles of γ-secretase and RBPJk as well as the non-canonical functions of Notch1 ICD [79], again suggesting high complexity of the pathway and individual roles of its components. RBPJk and total Notch activity could be necessary to balance rates of cell column growth and EVT differentiation. Of note, Notch1, regulated by hypoxia, seems to be critical for initiation and maintenance of the EVT lineage [24]. Notch1 appears in clusters of vCTBs at the basal side of the placenta around the 6th week of gestation and is specifically expressed in a subset of proliferative, proximal CCTs at 12th week. Besides inhibiting cell fusion and EVT formation, overexpression of Notch1 ICD in CTBs suppressed p63 and TEAD4, two markers of villous CTB self-renewal, and induced the stemness genes, myc and VE-cadherin, which are mainly expressed by CCT progenitors. Notch1 exerts its effects by direct binding to the myc gene as well as by up-regulating IRF6, a negative regulator of p63 stability [24,80,81]. Rapid down-regulation of Notch1 during differentiation might involve NUMB proteins, of which different isoforms have been detected in human placenta [82].

2.4. Notch signalling in gestational diseases and choriocarcinoma cells

Gestational diseases such as early-onset preeclampsia and severe intrauterine growth restriction (IUGR) are associated with defects in trophoblast invasion, spiral artery remodelling and placental vascularisation [83,84]. Therefore, critical pathways of placental development such as Notch signalling could be altered in these pregnancy disorders. Indeed, several studies demonstrated changes of Notch receptor and ligand expression in preeclamptic placentae using qPCR, western blotting or immunohistochemistry [85–89]. However, the majority of these expression data are contradictory which could be explained by differences in placental sampling or ethnicity, the utilisation of inadequate antibodies, technical issues in the experimental procedure or differences in gestational age of placentae. Notch1–4, DLL1, DLL3, DLL4, Jagged1 and Jagged2 were shown to be decreased in preeclamptic tissues [85,86,88,89], whereas others reported no changes of most Notch receptors and ligands [72,89], or even an increase of Notch3 [89]. Along those lines, immunohistochemical analyses suggested Notch1 expression in CTBs and the syncytiotium of normal,

![Fig. 3. The role of Notch in placental anchoring villi. Proximal cell column trophoblasts (CCTs) express Notch1 ICD promoting proliferation and cell survival. Active Notch1 also induces the extravillous trophoblast (EVT) progenitor-specific stemness markers myc and VE-cadherin and suppresses villous cytotrophoblast (vCTB)-specific TEAD4 and p63 expression, the latter by up-regulating IRF6. Notch1 ICD also suppresses formation of the syncytium (S) and EVT differentiation. One could speculate that Notch1 might also have a non-canonical role in proximal CCT by binding β-catenin to the membrane. Downregulation of Notch1 in EVTs might then liberate β-catenin from the receptor, thereby provoking its nuclear recruitment and Wnt-dependent EVT differentiation and/or migration, as recently shown [95]. In contrast to Notch1, Notch2 is present in the distal cell column and in intramural EVTs. In the latter Notch2-dependent signalling could be required for trophoblast-endothelial interactions promoting endovascular invasion, remodelling and/or short range communication with immune cells. VS, villous stroma.](image-url)
preeclamptic or IUGR placentae obtained between the 24th week and term [85,87–90]. However, at this time of pregnancy mRNA and protein of the receptor are completely absent from all trophoblast subtypes [24,72]. Nevertheless, changes in methylation of Notch4 and DLL1 were noticed in preeclamptic placentae suggesting epigenetic alterations of Notch components in this disease [88]. Finally, analyses of Notch signalling in cultured chorionic carcinoma cell lines suggested profound changes of its role in tumour cells. In these studies Notch1 promoted cell migration but inhibited proliferation [91–93], whereas downregulation of Notch2 increased cell growth [94]. These effects have not been observed upon manipulation of total Notch activity, Notch1 or Notch2 in primary CTBs [24,76].

3. Summary

In conclusion, Notch signalling is a critical regulator of murine and human placental development. In mice, different Notch pathway members control trophodectoderm formation, angiogenesis, vessel formation, giant cell differentiation and endothelial trophoblast invasion. In the human placenta, Notch plays a crucial role in cell column formation, CCT survival and EVT differentiation. Future studies should address epigenetic regulation of pathway members as cell adhesion molecules, PLoS One 9 (9) (2014) e108535.

Author’s contributions

S.H. drew the illustrations and provided support with respect to literature search and structure of the review. J.P. contributed to writing and improvement of graphical design. M.K. wrote the manuscript.

Disclosure statement

The authors have nothing to declare.

Conflict of interest

The authors do not report any conflict of interest.

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