universität wien

# DIPLOMARBEIT

Titel der Arbeit

Notch Signaling in the Human Placenta

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)


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Wien, im Mai 2010

An dieser Stelle möchte ich mich bei meinen Eltern und Schwiegereltern für Ihre Unterstützung während meines Studiums bedanken. Ich konnte Vorlesungen und Übungen durch Ihre Mithilfe bei der Betreuung meines Sohnes problemlos besuchen. Meinem Vater möchte ich auch besonders für die Motivation und finanzielle Unterstützung während meiner Studienzeit danken.

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## ABSTRACT

During my diploma thesis I focused on the expression of Notch signaling receptors and ligands in the human placenta and the role of the active Notch pathway in placental cell lines and primary cultures. Notch signaling is a highly conserved pathway that, dependent on the cellular context, can promote or suppress cell proliferation, cell death, acquisition of specific cell fates or activation of differentiation programs. Since gene chip analyses has revealed that several members of the Notch pathway are widely expressed in the human placenta I analysed mRNA expression of the four Notch receptors and five ligands in human gestational tissues and cells using semi-quantitative RT-PCR. To confirm these results, I performed immunohistochemical analyses focussing on first trimester tissue. These data revealed that all receptors and ligands are expressed in first trimester placenta, some of them being restricted to a subset of placenta cell populations. In order to study the activitiy of the Notch cascade I transiently transfected a trophoblastic cell line with luciferase vectors containing wilde type and mutated CBF1 binding sites revealing an endogenous Notch activity in this cell line. Additionally, I could further induce Notch activity by overexpressing the Notch intracellular domain. Using a co-culture model with the trophoblastic cell line and isolated primary decidual fibroblast I could show that the Notch pathway can be elucidated by the fibroblast cell population. In all transfection studies, the γ-secretase inhibitor DAPT was used to block the Notch pathway being a control for the endogenous and fibroblast-induced Notch activity. Finally, I could figure out that DAPT-induced blocking of the Notch pathway stimulates the migration not only of the trophoblastic cell line but also of villous explant culture-derived extravillous trophoblasts in a dose dependent manner.

These data not only proved the presence of Notch receptors and ligands in the human placenta but also showed specific expression patterns suggesting some regulatory roles in trophoblast proliferation, cell column formation and invasion. The induction of trophoblast migration upon inhibition of Notch signaling further corroborates the influence of the Notch activity on trophoblast differentiation and invasion. Furthermore, the pathway has endogenous activity and can be induced by fibroblasts suggesting that Notch signaling could be an important communication tool between extravillous trophoblasts and decidual cells. Taken together, these data support the idea that the Notch signaling pathway might have a functional role during the development of the early human placenta.

# ZUSAMMENFASSUNG

Im Zuge meiner Diplomarbeit konzentrierte ich mich auf die Expression der Notch Rezeptoren und Liganden in der humanen Plazenta und auf die Rolle des aktiven Notch Signalweges in plazentären Zelllinien und primären Kulturen. Der Notch Signalweg ist ein hoch konservierter Mechanismus, der, je nach zellulärem Zusammenspiel, Zellwachstum, Zelltod, zelluläres Schicksal und Zelldifferenzierungen fördert oder hemmt. Aufgrund von genomischen Chip Daten, die das Vorhandensein vieler Faktoren des Notch Signalweges in der humanen Plazenta ergaben, begann ich mit der Detektion der mRNA der vier Notch Rezeptoren und den fünf Notch Liganden in humanen schwangerschafts-assoziierten Geweben und Zellen mit Hilfe der semi-quantitativen RT-PCR. Um diese Ergebnisse zu bestätigen, führte ich immunhistochemische Analysen an Geweben des ersten Trimesters mittels validierten Antikörpern durch. Diese Daten ergaben, daß alle Rezeptoren und Liganden in der humanen Plazenta exprämiert werden, einige von ihnen auf ganz bestimmte plazentäre Zellpopulationen beschränkt. Um die Aktivität des Notch Signalweges nachzuweisen, transfizierte ich eine trophoblastäre Zelllinie mit Luziferase-Vektoren, die Wild-Typ und mutierte CBF1 Bindungsstellen trugen. Dies bestätigte eine endogene Notch Aktivität innerhalb dieser Zelllinie. Zusätzlich konnte ich die Notch Aktivität mithilfe der Überexpression der Notch Intrazellulären Domäne induzieren. Mithilfe eines Ko-Kultur Model Systems bestehend aus der trophoblastären Zelllinie und isolierten primären dezidualen Fibroblasten konnte ich zeigen, daß der Notch Signalweg auch durch die Fibroblasten aktiviert werden kann. In all den Transfektionsstudien verwendete ich den γ-Secretase Inhibitor DAPT um die Notch Signalkaskade zu blockieren, wodurch ich eine Kontrolle für die endogene und fibroblast-induzierte Notch Aktivität hatte. Schließlich fand ich heraus, daß das Blockieren der Notch Aktivität mittels DAPT, abhängig von der Dosierung, zu einer erhöhten Migration führt, und zwar nicht nur in der trophoblastären Zelllinie, sondern auch in extravillösen Trophoblasten aus villösen Explant Kulturen.

Diese Ergebnisse haben nicht nur das Vorhandensein der Notch Rezeptoren und Liganden bewiesen, sondern zeigten auch spezifische Expressionsmuster wodurch der Schluß nahe liegt, daß diese eine regulierende Rolle in der Trophoblasten Proliferation, Zellsäulenformation und Invasion spielen. Die Erhöhung der Trophoblasten Migration aufgrund der Notch Signalweg Inhibierung bestätigt weiters den Einfluß der Notch Aktivität auf die Trophoblasten Differenzierung und Invasion. Weiters zeigt dieser Signalweg endogene Aktivität und kann durch Fibroblasten induziert werden, wodurch

der Schluß nahe liegt, daß der Notch Signalweg ein wichtiges Kommunikationswerkzeug zwischen extravillösen Trophoblasten und Deziduazellen darstellt. Zusammenfassend unterstützen diese Daten den Schluß, daß der Notch Signalweg eine funktionelle Rolle während der frühen Plazentaentwicklung spielen könnte.

# Table of Content

# Figure Legend

# Table Legend

# 1. Introduction

## 1.1. The Human Placenta

The human placenta develops from the trophectoderm of the blastocyst and is the connecting organ between mother and fetus. It supplies nutrient and oxygen to the fetus and passes out waste. Additionally, the human placenta produces hormones (e.g. human choriongonadotrophin, hCG) that are necessary for the maintenance of pregnancy. In the following chapter implantation, trophoblast differentiation and invasion will be described in detail.

### 1.1.1. Implantation

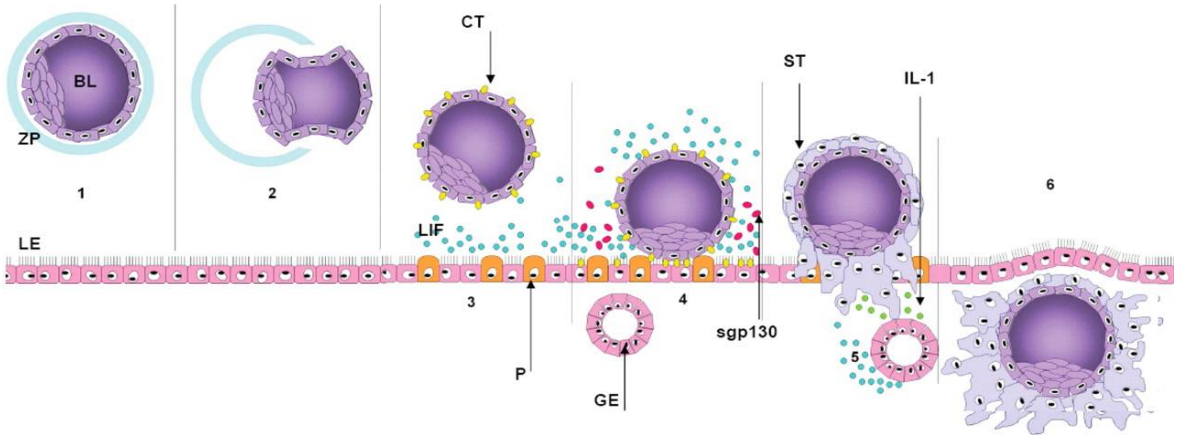


**Fig. 1: Implantation of the blastocyst (Fitzgerald et al., 2008)**
(1) Blastocyst (BL) is formed of a trophectoderm surrounding the inner cell mass, both are covered by Zona pellucida (ZP), which is removed before implantation (2). Endometrium expresses LIF (leukemia inhibitory factor) that binds to the LIF receptor on the blastocyst (3). Next, the blastocyst attaches to the luminal epithelium (LE) of the uterine endometrium and secretes LIF by itself. At the same time, endometrial protein expression of gp130 and LIF receptor together with the appearance of pinopodes (P) increases (4). After the differentiation of the trophectoderm into inner cytotrophoblasts and syncytiotrophoblasts (ST), the latter invade into the maternal uterus (5). IL-1 secretion of the blastocyst stimulates glandular epithelia (GE) to produce LIF, which in turn assists for the complete implantation of the blastocyst (6).

Implantation requires synchronous development of the blastocyst and endometrium. After fertilisation of the egg, the zygote develops from the two-cell state via the morula into the blastocyst that finally reaches the uterine endometrium. Supported by several soluble and membrane-bound proteins, the blastocyst adheres and invades into the endometrial wall. Among many influencing factors, LIF (leukaemia inhibitory factor), that is produced by

both the endometrium and the blastocyst, is found to be highly concentrated at the feto-maternal interface thereby facilitating the implantation process (Kondera-Anasz et al., 2004). A specialized cell population, the pinopodes that protrude from the endometrium, are also discussed to assist in blastocyst adhesion (Bentin-Ley et al., 1999) and are found to be coexpressed with LIF (Aghajanova et al., 2003). Already in blastocyst state, the trophoblast cell population starts to produce the pregnancy specific hormone β-HCG (human chorionic gonadotrophin) that constrains the corpus luteum to maintain the production of progesterone. Progesterone suppresses the immune reaction at the foeto-maternal interface thereby protecting the foetus from immune responses and rejections.

## 1.1.2. The human mature placenta

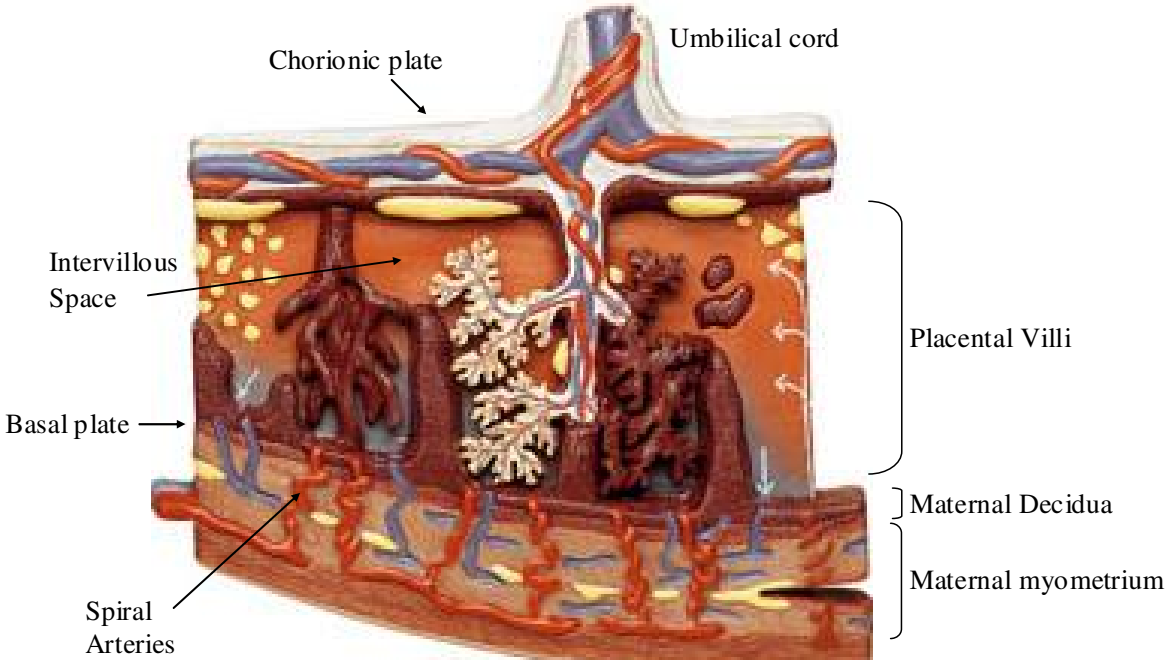


**Fig. 2: Schematic illustration of the human adult placenta (SOMSO-Plast®, placenta-model, MS 47/16)**

The mature human placenta is the connecting and supplying organ between the mother and the fetus during pregnancy. It consists of a tree like structure (=placental villi) that emerges from the chorionic plate (facing the amniotic fluid) and is to some extent attached to the basal plate (facing the decidua of the maternal uterine wall). The villi, flooded with maternal blood (intervillous space) take up oxygen and nutrients and excrete fetal waste products back into the maternal circulation. The placenta is connected to the fetus via the umbilical cord that is the transferring organ between the fetus and the placenta.

In Figure 2 the cross section of a human mature placenta is shown. This organ consists of the chorionic plate that faces the amniotic fluid, the basal plate which displays the connection area to the maternal uterine wall and the branching structure (placental villi)

that is flooded with maternal blood. The nutrients are delivered by the spiral arteries into the intervillous space where the uptake into the foetal system (villi) occurs. The transport goes on via the umbilical vessel system to the foetus. Waste products are conveyed back into the intervillous space and removed by the maternal vein system.

### 1.1.3. Trophoblast differentiation and invasion

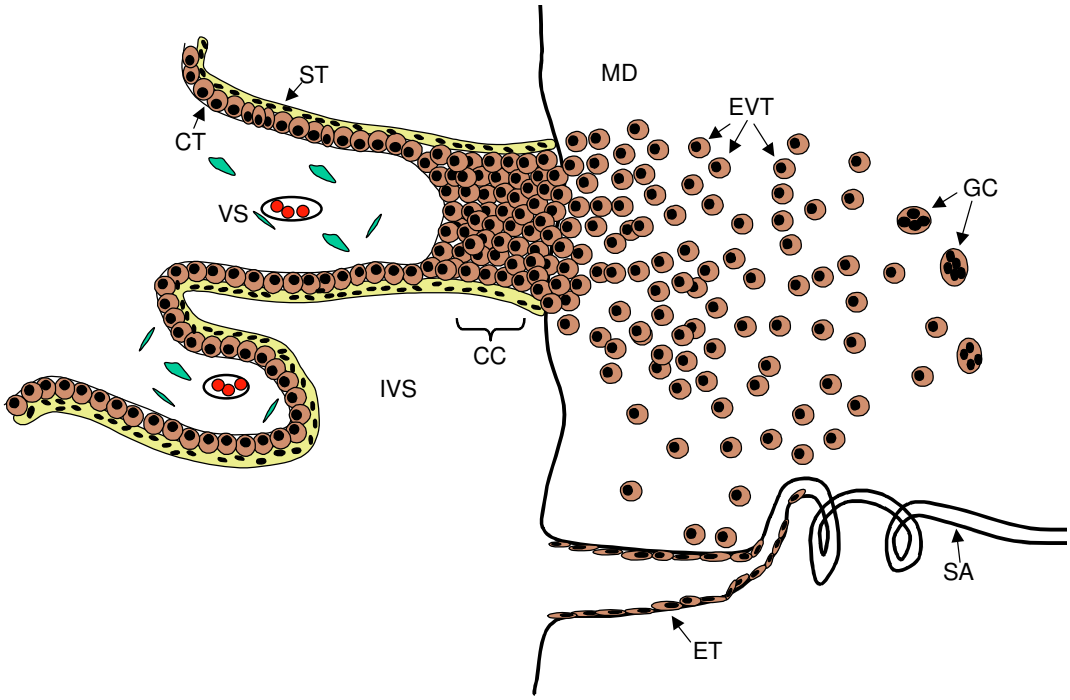


**Fig. 3: Trophoblast invasion**
In contrast to floating villi (FV), anchoring villi (AV) are attached to the maternal decidua (MD). The villous stroma (VS) containing fetal blood vessels (V), fibroblasts, and other stromal cells, is surrounded by the basal membrane which provides the basis for the cytotrophoblasts (CT) and finally the syncytiotrophoblast (ST) layer that produces hormones and is primarily responsibly for transport functions. Cytotrophoblasts can proliferate to form cell column (CC), which attaches to the maternal decidua (the converted endometrium). The now called extravillous trophoblasts (EVTs) invade into the maternal decidua and first third of the myometrium to reach spiral arteries (SA), where they displace the endothelial cells (EC) and differentiate into endovascular trophoblasts (ET). Another population of EVTs arises by endoreduplication finally forming large, multinucleated giant cells (GC). IVS; intervillous space

Figure 3 shows an enlargement of the villous structure at the feto-maternal interface of the first trimester of pregnancy. Basically, placental villi are divided into floating and anchoring villi; the latter mechanically support the attachment of the placenta to the uterine wall. The villous stroma with blood vessels, fibroblasts and other stromal cells is surrounded by a basal membrane that is coated with cytotrophoblasts (CTB). CTBs undergo two differentiation processes. On one hand, CTBs fuse to form a multinucleated cell layer, the syncytiotrophoblast layer (ST) that produces hormones and shields the villi

against the maternal blood. STs represent the transport unit of placental villi. On the other hand, CTBs proliferate and form cell columns. Their integrity might be maintained due to interaction of L-Selectin with carbohydrate ligands (Prakobphol et al., 2006). However, at their distal anchoring sites, CTBs detach from the columns and invade the maternal decidualized endometrium and inner myometrium, a process that is suggested to be initiated by extracellular matrix contact and decidual components. Additionally, oxygen concentrations might be critically involved since hypoxia promotes trophoblast proliferation whereas normoxia inhibits proliferation and induces migration (Genbacev et al., 1997). The now called extravillous trophoblast (EVT) has two further differentiation potentials. On one hand, EVTs are able to transform maternal spiral arteries into vessels of low resistance by replacing endothelial cells and mural vascular smooth muscle cells (Pijnenborg et al., 1983). Apoptosis of vascular smooth muscle cells around these vessels and displacement of maternal endothelial cells by endovascular trophoblasts (ET) thereby gaining endothelial-like functions expressing typical vascular adhesion molecules (Zhou et al., 1997) are key features of the particular invasive differentiation process (Harris et al., 2006; Pijnenborg et al., 1983). The second differentiation process of EVTs results in the generation of giant cells, large multinucleated cells whose functions still remain to be elucidated.

In the last couple of years, two diverse theories concerning the progenitors of EVTs and syncytiotrophoblasts have emerged. The first theory indicates the presence of a bipotential trophoblast progenitor cell during the first trimester placenta. This cell is supposed to form either syncytiotrophoblast or EVT (Baczyk et al., 2006). The second theory is based on the presence of two separate villous cytotrophoblast populations being differently committed to produce either EVTs or syncytiotrophoblasts (James et al., 2005).

However, differentiation from CTB to EVT plays a critical role in developing vascular connection between the mother and the fetus. Additionally, the changes in the vessel conductivity are necessary to fulfill the embryo's demands for nutrients and gases. Inadequate transformation of spiral arteries and shallow interstitial invasion were detected in the placental bed of women suffering from preeclampsia or severe intrauterine growth restriction (IUGR) (Fig. 1D) indicating that there is a defective EVT differentiation process in these pregnancies (Pijnenborg et al., 1991). Excessive invasion, on the other hand, is associated with partial and complete moles (Federschneider et al., 1980), and choriocarcinomas (Seckl et al., 2000; Shapter and McLellan, 2001).

The highly invasive behavior of EVTs in vivo and in vitro is a process that is very well observed in several types of cancer. Beside the investigation of pregnancy disorders, the trophoblast might also be a model to study a tumor-like behavior but displaying a strictly controlled invasion thereby gaining some insights into invasion processes and putative control mechanisms.

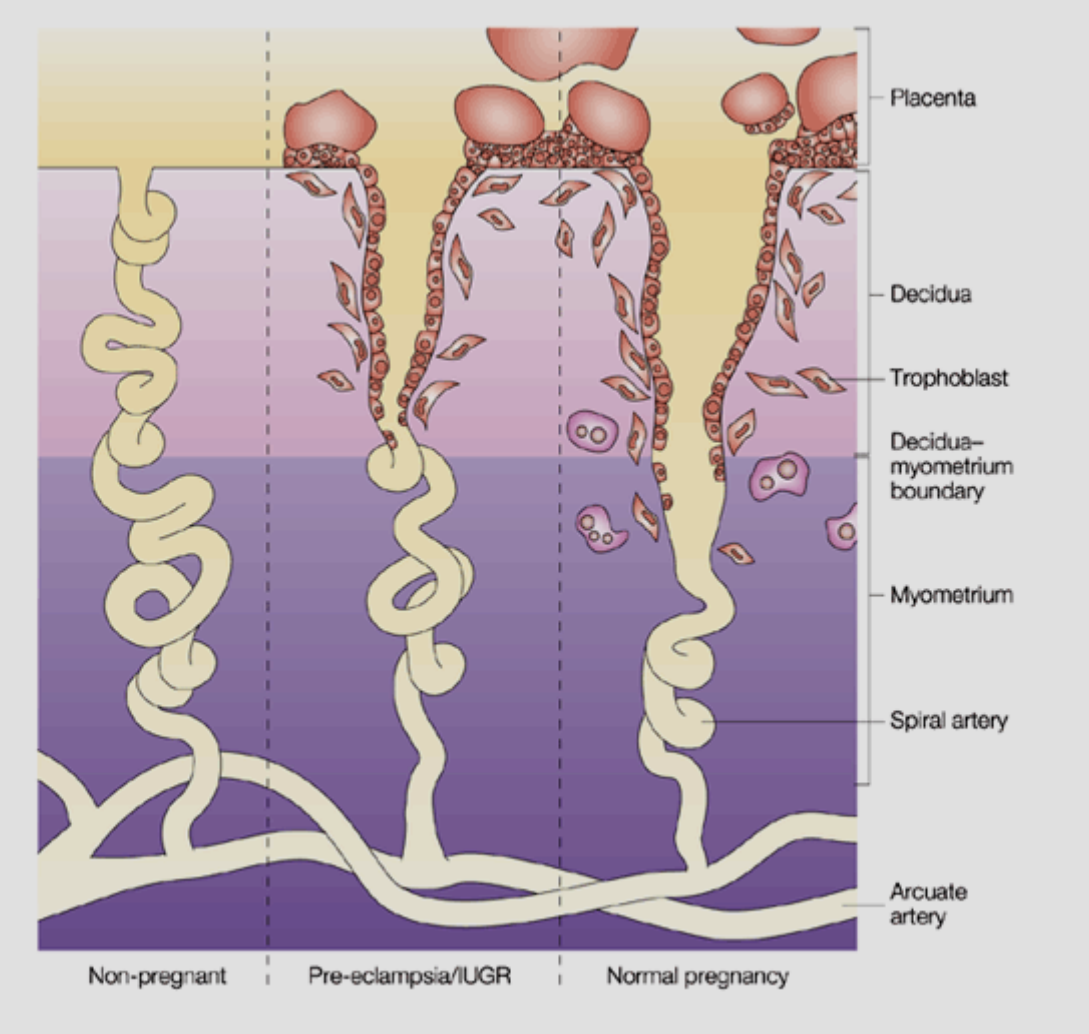


**Fig. 4: EVT invasion non-pregnant, pre-eclampsia/IUGR and normal pregnancy cases (Moffett-King, 2002)**
The invasive depth and spiral artery transformation in pregnancy disorders like pre-eclampsia and IUGR is reduced leading to inadequate nutrient and gas supply to the fetus.

## 1.1.4. Differentiation markers of cytotrophoblasts and EVTs

The following chapter shall give an overview of typical markers used in immunohistochemical analysis that allow distinguishing between different cell types and their differentiation levels. Fig. 4 represents immunohistochemistry performed of early, mid and late placenta. Cytokeratin 7 is used as a trophoblast marker while vimentin stains

stromal and decidual cells (data not shown). KI67 detects proliferative cells. For detection of fully differentiated EVTs, Kip2p57 staining is performed. Kip2p57 (cyclin-dependent kinase inhibitor) is a potential inhibitor of several G1 cyclin/Cdk complexes; overexpression of Kip2p57 arrests cells in G1 (Lee et al., 1995; Matsuoka et al., 1995; Seizinger, 1991).

In early placenta, the villous trophoblast layer is a bilayer consisting of cytotrophoblasts and syncytium (Fig. 4, A). During onset of pregnancy, the cytotrophoblast layer disappears; only few cytotrophoblasts can by found in third trimester placenta (Fig.4, B). The cell column of early placentae is composed of proliferative cells (KI67) emerging from the villous tip and differentiated trophoblasts (Kip2p57) that form the distal part of the column. Note that some trophoblasts have overlapping expression (Fig. 4, C and D). In the midgestation placenta (22nd weak of gestation) villi have attached to the decidua and differentiated EVTs invade the maternal uterine layers (Fig. 4, E). EVTs displace endothelial cells of spiral arteries (Fig. 4, F) and a multi-nucleated subpopulation (giants cells) can be found deep in the placenta bed (Fig. 4, G).

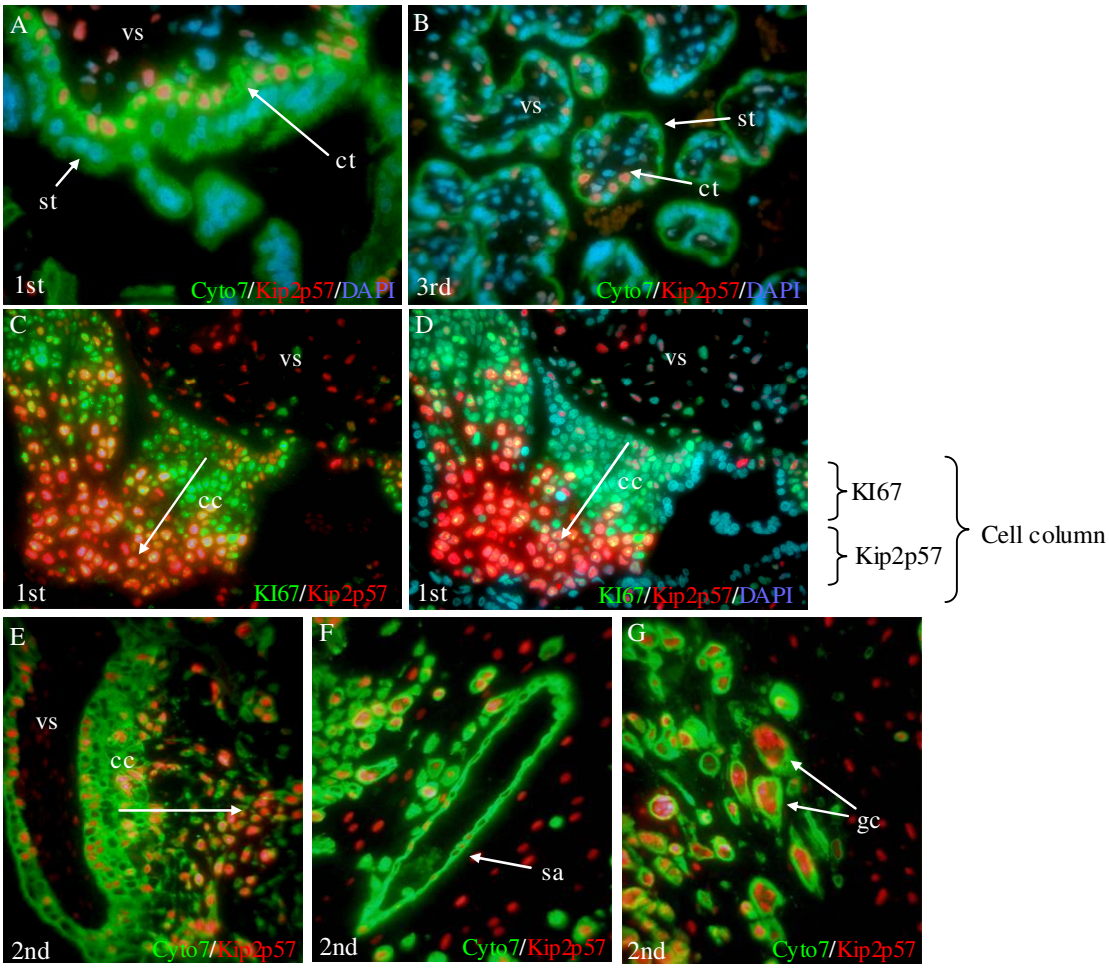


**Fig. 5: Representative immunohistochemistry of human placental tissues**
Pictures A (400f magnification) and B (200f magnification) show villous parts of a 10weak and 39weak of gestation, respectively. Tissues are stained with cytokeratin 7 (trophoblast cells), Kip2p57 (differentiated cells) and DAPI (nuclei). Note that all cytotrophoblast nuclei of the late gestation are Kip2p57 positive whereas several CTBs of the early pregnancy are not fully differentiated. Panel C and D represent a cell column (10weak of gestation, 200f magnification) stained with KI67 (proliferation), Kip2p57 (C) and DAPI (nuclei) (D). Note the switch from proliferation to differentiation of trophoblasts along the cell column. Picture E shows staining of an anchored villus (22weak of gestation) with cytokeratin7 and Kip2p57. Note that the cell column trophoblasts are now mainly positive for Kip2p57. Picture F represents a decidual area with a spiral artery and surrounding EVTs. Note that endothelial cells are fully displaced by EVTs (positive for Cyto7). Picture G shows giant cells containing several nuclei positive for Kip2p57. Cytokeratin7 negative cells in pictures F and G represent decidual cells.

# 1.2. The Notch signalling pathway

## 1.2.1. The canonical Notch signalling pathway

Notch signalling is a highly conserved signalling pathway with different regulatory functions during development including lateral inhibition, boundary formation and cell fate assignation (Bray, 1998). The Notch pathway is a short-range communication

signalling event requiring cell-to-cell contact between a signal-sending cell carrying the ligand and a signal-receiving cell expressing the receptor. In mammals, genes encoding four Notch receptors (Notch 1-4) and five Notch ligands (Jagged1; Jagged2; Delta-like ligand 1, DLL1; Delta-like ligand 2, DLL2; Delta-like ligand 3, DLL3) are present allowing complex control and regulatory mechanisms (Fig. 2). In Drosophila, only one Notch receptor and the two different ligands, Delta and Serrate are expressed, while in C. elegans, two genes encode for Notch (lin-12 and glp-1) and several Delta/Serrate/Lag-2 (DSL) homologues (Maine et al., 1995). The mammalian Notch receptors display both, redundant and unique features. Depending on the context, Notch signalling can promote or suppress cell proliferation, cell death, acquisition of specific cell fates or activation of differentiation programs (Kopan and Ilagan, 2009). The Notch signalling pathway has some unique features resulting finally into signal transduction. First, the receptor has to undergo some proteolytic events after ligand-receptor recognition to generate an active intracellular Notch fragment (NICD = Notch Intracellular Domain). Second, the Notch receptor displays both, the role of a membrane bound receptor and, in the form of the NICD, a transcriptional co-activator that translocates into the nucleus to transactivate the transcription of several target genes. Thus, this particular signalling pathway does not include any second messengers or signal amplification capacities. Hence, this signalling pathway is highly sensitive and operates with very little amounts of active signal transducers.

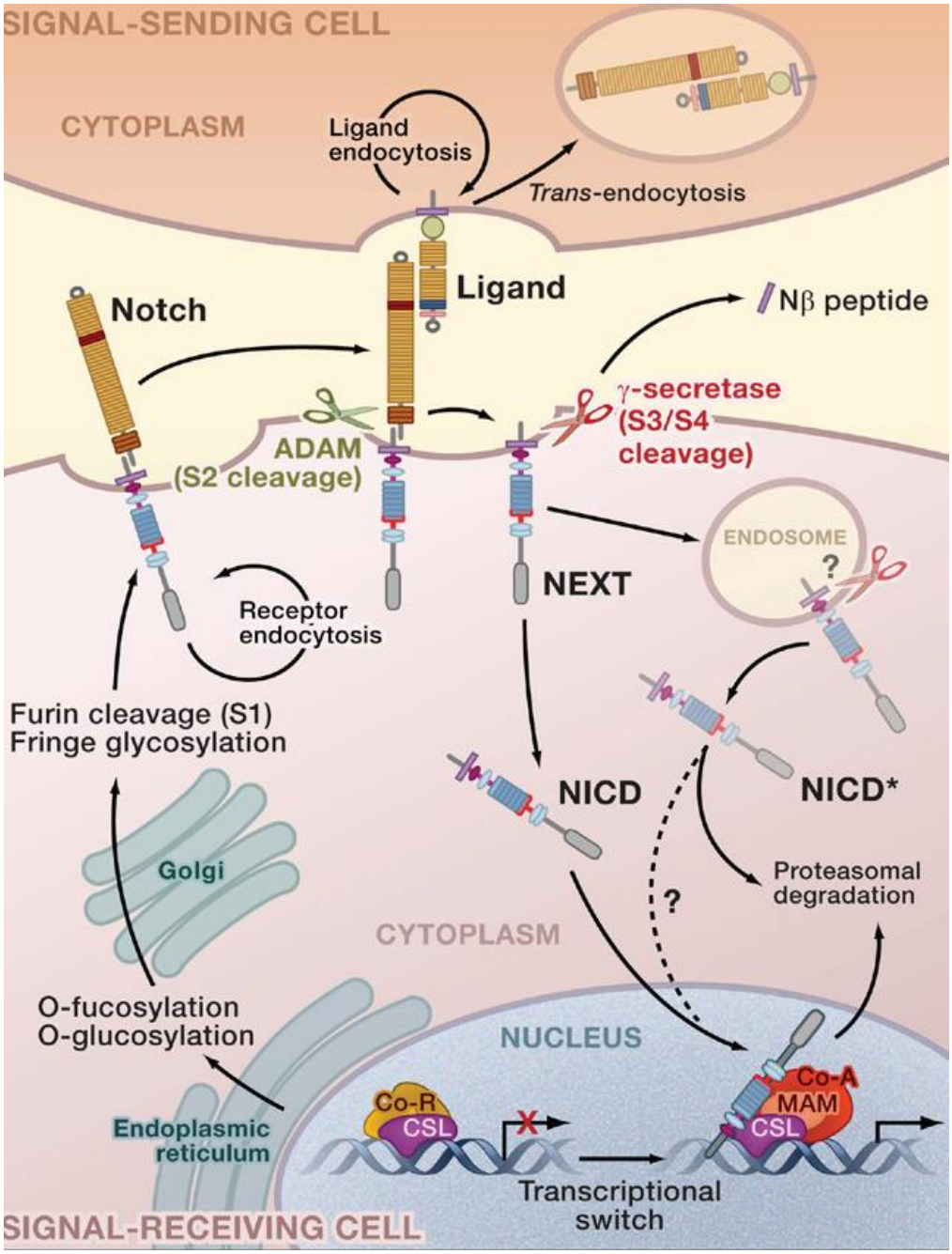


**Fig. 6:The canonical Notch signalling pathway (Kopan and Ilagan, 2009)**

On its way to the cell surface, the Notch receptor gets fucosylated, glucosylated, cleaved at S1 by Furin-like convertases and the now fully mature receptor is anchored at the cell surface. After binding to a ligand, the receptor is cleaved at S2 by enzymes of the ADAM family creating the NEXT fragment (Notch extracellular truncation fragment), the perfect substrate for an enzyme complex called the γ-Secretase. This intramembrane cleaving protease cleaves at S3, resulting in the NICD (Notch intracellular domain) and at S4, which leads to the release of Nβ into the intercellular space. The NICD translocates into the nucleus and binds to the nuclear binding protein CSL [CBF1/RBPJk/Su(H)] thereby replacing Co-Repressors, recruiting MAM and Co-Activators which finally leads to the transcription of target genes including Hes and Hey. B: The arrows indicate the different cleavage positions and resulting products produced by the γ-Secretase. Cleavage at S3 can result in 4 different NICD products with different life spans according to the N-end rule. S4 cleavage releases two different Nβ fragments into the intercellular space

During each Notch receptor lifetime, several post-translational modifications and proteolytic events occur (Fig. 1A, 1B) (Bray, 2006). After translation the Notch receptors are fucosylated and glucosylated by the enzymes O-fut and Rumi. During the secretory pathway, the Notch polypeptide is cleaved by furin-like convertases within the secretory pathway at site 1 (S1) which is located within an unstructured loop of the heterodimerisation domain. This very first proteolytic event produces the mature Notch heterodimer (Notch extracellular domain – Notch transmembrane and intracellular domain) that is connected via non-covalent interactions. Cells that do express the glycosyltransferase Fringe can prolong the O-fucose thereby influencing the ligand specifity mediating the Notch Receptor activation. The Notch receptor is than anchored as a single-pass type I transmembrane protein (Fig. 2A, 2B) in the cytoplasm membrane. The binding of a ligand to the Notch Receptor leads to conformational changes of the cleavage site 2 (S2) being now accessible for an enzyme family called ADAM (a disintegrin and metalloprotease). Two members of this family are known to cleave at S2 of the Notch receptors, ADAM10/Kuzbanian (Deuss et al., 2008) and ADAM17/TACE (tumour necrosis factor alpha converting enzyme), the particular ADAM required for the Notch Receptor activation is context dependent (Bozkulak and Weinmaster, 2009). The cleavage at S2 generates NEXT (Notch extracellular truncation fragment) which is a perfect substrate for another enzyme complex called γ--Secretase. This enzyme complex belongs to the growing family of intramembrane cleaving proteases (Selkoe and Wolfe, 2007; Wolfe and Kopan, 2004). This protein complex that contains presenilin as the catalytic component (Kopan et al., 1996) cleaves NEXT (Fig. 1B) at site 3 (S3) and site 4 (S4) either at the cell surface or in endosomal compartments, but it seems that the cell surface processing preferentially releases a more stable form of NICD. The γ--Secretase activity at S4 relieves the remaining Notch fragment (Nβ) into the intercellular space (Okochi et al., 2002) and the NICD into the cytoplasm by cleaving the NTMIC at S3. This cleavage is preferentially performed at valine 1744 (NICD-V) (Schroeter et al., 1998), but recently, other cleavage product variants, NICD-L (lysine 1745 or 1746) and NICD-S (serine), have been identified (Fig. 6) (Tagami et al., 2008). According to the N-end rule, the NICD-S and NICD-L are less stable and rapidly degraded by the 26S proteasome (Blat et al., 2002; Tagami et al., 2008). The NICD translocates into the nucleus and acts as a coactivator of the DNA-binding transcription factor suppressor of hairless Su(H) (CBF1/CSL/RBPJκ/Lag1) (Fortini and Artavanis-Tsakonas, 1994). In the absence of NICD, CBF1 is associated with co-repressor proteins and histone deacetylases

preventing transcription of the target genes. NICD binding leads to the co-repressors dissociation and recruitment of co-activators such as MAM1-3 and the MED8 mediator transcription activation complex (Kovall, 2008). This event upregulates gene expression of target genes such as the members of the bHLH repressor family ESR (Enhancer of Split Related), HES (Hairy and Enhancer of Split), or Hey (Hairy and Enhancer of Split Related) (Fischer and Gessler, 2003). Several proteins involved in the Notch signalling activity are listed in table 1.

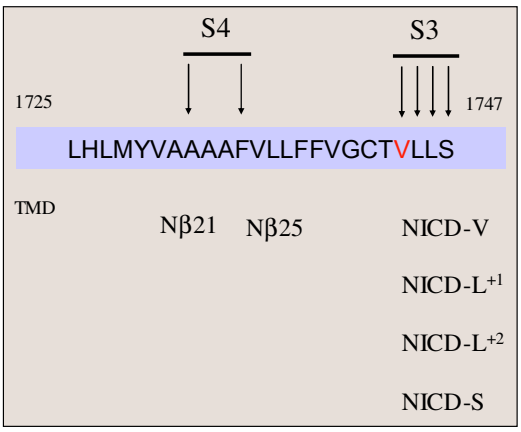


**Fig. 7: Proteolytic cleavage sites performed by γ--Secretase complex**
The γ-Secretase activity has two cleavage possibilities at S4 relieving Notch fragments (Nβ21, Nβ25) into the intercellular space. The cleavage at S3 can produce 4 different NICD fragments: The NICD-V (valine 1744), which is the most stable one and additionally the product variants NICD-L (lysine 1745 or 1746) and NICD-S (serine) that are rapidly degraded

| Component class | Drosophila | Mammals | Function |
|---|---|---|---|
| Notch Receptors | Notch | Notch 1-4 | Single transmembrane receptor and also transcription factor |
| Ligands | Delta, Serrate | DLL1, DLL3-4, Jagged1 and 2 | Single transmembrane ligands of the Notch receptor |
| Nuclear Effectors | Su(H) | RBPJk/CBF1/CSL | DNA binding transcription factor |
| | Mastermind | Mastermind1-3 | Transcriptional Co-activator |
| | Hairless, SMRTR | Mint/Sharp/SPEN, NcoR/SMRT/Kyot2 | Transcriptional Co-repressors |
| Canonical Target bHLH Repressor genes | E(spl) | HES/ESR/HEY | Target genes of the Notch signalling pathways |

**Table 1: proteins involved in the canonical Notch Signalling Pathway (Fiuza and Arias, 2007; Kopan and Ilagan, 2009)**
Four Notch Receptors can bind to 5 different Notch ligands. Upon NICD translocation into the nucleus, it binds to RBPJk, replaces Co-Repressors, recruits Mastermind 1-3 and finally induces the transcription of target genes Hes, Hey, Esr (Hairy and Enhancer of Split, Hairy and Enhancer of Split Related, Enhancer of Split Related).

## 1.2.2. The CBF1 independent Notch signalling

This particular signalling works without interaction with the nuclear binding protein CBF1. Shawber and colleagues have shown, that Notch signalling can inhibit muscle cell differentiation in a CBF1-independent manner. A truncated form of Notch lacking the CBF1-binding domain was still able to stop myoblast differentiation (Shawber et al., 1996). Studies in drosophila finally revealed two kinds of alleles of Notch, both mutants with gain of function phenotypes independent of Su(H). Abruptex (Ax; point mutants concerning the EGF-like repeats 24-29) and Microchaetae defective (Mcd; deletions of protein domains C-terminal to the ANK repeats) depend on shaggy, that encodes the Drosophila homologue of GSK3β and plays a role in the Wnt pathway (Brennan et al., 1997). Further studies in drosophila confirmed a cross link between Notch activity and the Wnt pathway indicating that Notch influences Wnt signalling by setting a threshold for the function of β-Catenin (Hayward et al., 2005). In vertebrates, two groups could show a direct effect of Notch on the β-Catenin activity, thereby acting as a tumour suppressor or influencing cell fate decisions (Deregowski et al., 2006; Nicolas et al., 2003).

## 1.2.3. Structure of the Notch Receptors and Notch Ligands

### *1.2.3.1. Structure of the Notch Receptor*

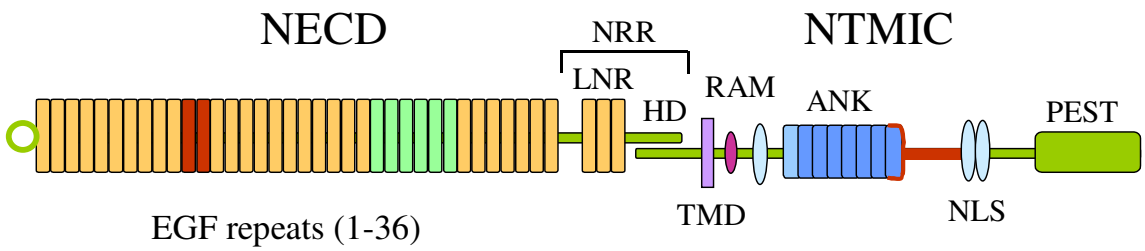


**Fig. 8: Structure of the mammalian Notch Receptor 1**
The Notch Extra-Cellular Domain (NECD) consists of 1-36 EGF repeats important for ligand interactions and the Negative Regulatory Region (NRR) which is formed by three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerisation domain (HD). The NRR hides the site 2 cleavage site until a ligand binds to the receptor somehow provoking the accessibility of ADAM to S2. Several domains are located on the Notch Trans-Membrane Intracellular domain. The transmembrane domain (TMD) harboring the cleavage sites S3 and S4 is followed by the RBPJk-association module (RAM) and an ANK domain, which is surrounded by nuclear location sequences. Finally, there is a region called PEST (proline, glutamic acid, serine, threonin), which is important for NICD degradation after the signal activity to ensure low levels of NICD present in the cell thereby raising the sensitivity to Notch response.

The mature Notch Receptor is a heterodimer consisting of a Notch extracellular domain (NECD) and a Notch trans-membrane intracellular domain (NTMIC). NECD: The extracellular domain mainly consists of up to 36 epidermal growth factor (EGF) repeats that are involved in ligand interactions. The EGF repeats 11 and 12 are responsible for trans-interactions (de Celis and Bray, 2000) with the ligands of signal sending cells while the EGF repeats 24-29 mediate the cis-inhibition that prevents the auto-activation performed by ligands of the same cell (Glittenberg et al., 2006). The ability of EGF repeats to bind calcium ions influence the Notch structure and affinity to ligands (Cordle et al., 2008) and may affect the signalling performance (Raya et al., 2004). The next region on the extracellular domain is the Negative Regulatory Region (NRR) which is composed of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerisation domain (HD). The NRR deeply buries the ADAM cleavage site S2 (located 12 amino acids before the transmembrane domain) until a ligand is bound to the receptor. This region displays a structural regulation mechanism preventing auto-activation of the receptor in the absence of a ligand. There are some theories how the accessibility to S2 can be gained upon ligand binding and several mechanisms that regulate the closed and open state of this cleavage site are discussed. Conformational changes due to the interaction with the ligand (Gordon et al., 2007) or a mechanical force pulling the receptor in ligand direction due to ligand-NICD transendocytosis performed by the signal sending cell (mechanotransduction model) (Parks et al., 2000). NTMIC: The transmembrane domain of the Notch receptor ends with 3-4 arginine/lysine residues. This is followed by 12-20 amino acids centred on a conserved WxP motif, which represents the RBPJk-Association-Module (RAM). Nuclear location sequences embrace an ANK domain (seven ankyrin repeats). The last structure of the C-terminal end of NTMIC is formed by conserved proline/glutamic acid/serine/threonin-rich motifs that harbour degradation signals (degrons).

### *1.2.3.2. Structure of the Notch Ligands*

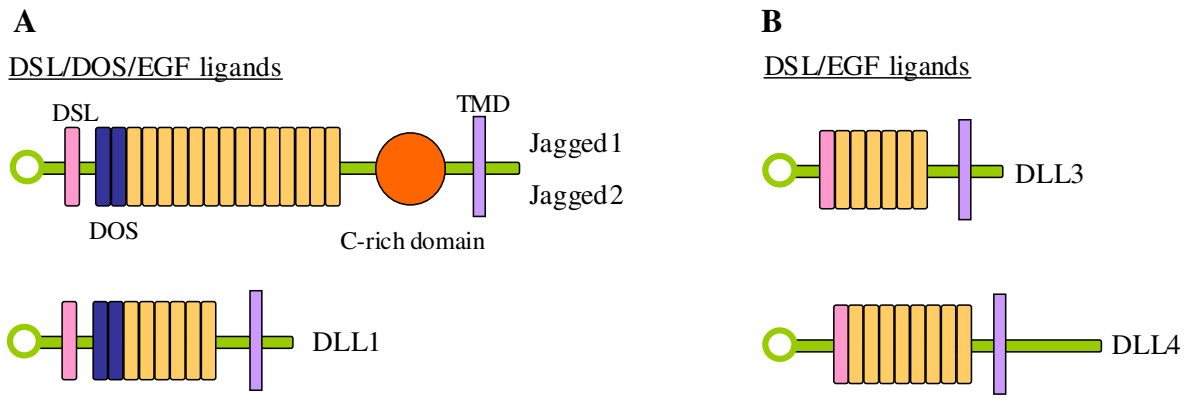


**Fig. 9: Structure of mammalian Notch Ligands**
**T**he mammalian Notch ligands can be divided into ligands containing Delta/Serrate/LAG-2 (DSL); Delta and OSM-11-like (DOS) and endothelial growth factor EGF repeats which are called Jagged1, Jagged2 and DLL1 (Delta like ligand 1). DLL3 and DLL4 lack the DOS domain and belong to the DSL/EGF ligand group.

Reviewed by D'Souza and colleagues, most notch ligands are type I transmembrane proteins (D'Souza et al., 2008). The largest family of Notch ligands contains three related structural motifs including the N-terminal DSL (Delta/Serrate/LAG-2) motif, tandem EGF repeats called the DOS domain (Delta and OSM-11-like proteins) (Komatsu et al., 2008) and EGF-like repeats (Fig. 8). DSL ligands are split into cysteine-rich domain containing (Jagged-1, Jagged-2, DLL-1) and cysteine-rich domain lacking (DLL-3, DLL-4) ligands. Ligands without DOS and DSL domains are considered to act in the non-canonical Notch signalling pathway (Takahashi et al., 2008).

## 1.2.4. Functions of Notch

Notch function and control mechanisms was recently reviewed by Borggreve and Oswald (Borggrefe and Oswald, 2009). In general, Notch signalling is important for binary cell fate decisions during development (lateral inhibition and induction) and differentiation processes (stem cell maintenance and induction of terminal differentiation). Examples for Notch signalling in differentiation processes are given by the human intestine and skin. Notch signalling is known to sustain an undifferentiated state of crypt progenitor cells in the intestine (Stanger et al., 2005). In human skin Notch signalling induces cell cycle arrest in keratinocytes which enforces differentiation of keratinocytes (Okuyama et al., 2008).

#### *1.2.4.1. Functions of Notch target genes*

Several studies in mammals have revealed that the transcriptions factors of the Hes and Hey family are the best-described Notch target genes. Hes and Hey proteins are helix-loop-helix transcriptions factors that mainly act as functional repressors (Davis and Turner, 2001). The investigation of Hes and Hey gene deficient mice have revealed different functions of Hes and Hey proteins during development (reviewed by Andreas Fischer and Manfred Gessler) (Fischer and Gessler, 2007). In general, Hes proteins play important roles in development of the nervous system, pancreas, endocrine cells and lymphocytes while Hey proteins critically influence the cardiovascular system. A summary of several Hes and Hey target genes is also listed in the review Andreas Fischer and Manfred Gessler. With respect to placental gene expression some findings were interesting. In pancreatic progenitor cells Hes1 inactivation resulted in the upregulation of $p57^{Kip2}$ expression leading to cell cycle arrest, precocious differentiation and depletion of the progenitor pool (Georgia et al., 2006). Additionally, Murata et al could show that Hes1 controlled proliferation through the transcriptional repression of the $p27^{Kip1}$ and Hes1 -/- mice had increased levels of this particular cdk inhibitor (Murata et al., 2005). In fibroblasts, inhibition of Hes1 caused a higher susceptibility of these cells entering a senescence and terminal differentiated state (Sang and Coller, 2009). Considering these results, Hes1 seems to influence the proliferation and differentiation of distinct cell populations. However, $p57^{Kip2}$ is highly expressed in differentiated, non-proliferative EVTs and CTBs and its expression could be under the control of the Notch target Hes1.

## 1.2.5. Notch and human Pathologies

Since the Notch signalling pathway plays a critical role in many fundamental processes, it is not surprising that aberrant gain or loss of notch signalling components lead to multiple human disorders. Basically, these mutations cause developmental syndromes, adult-onset diseases and cancer.

Developmental syndromes: Mutations in both, the Jagged1 gene (Li et al., 1997; Oda et al., 1997) and the Notch-2 gene (Samejima et al., 2007) are associated with the Alagill syndrome. Normally, it is diagnosed in the first 2 years and has multiple defects, thereby affecting kidney, heart, skeleton, liver and eye. Another disease caused by a missense mutation (G274D) in Jagged-1 is the Tetralogy of Fallot, whose clinical signs are four heart malformations (Eldadah et al., 2001). Mutations in DLL3 lead to spondylocostal

dysostosis, where vertebral segmentation defects are associated with rib anomalies (Gridley, 2003). Mutations in Notch-1 cause the familial aortic valve disease, which is accompanied with a spectrum of developmental aortic valve anomalies and severe valve calcification (Garg et al., 2005).
Adult-onset disease: CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), an autosomal-dominant vascular disorder, is caused by mutations of Notch3 gene (Gridley, 2003). In CADASIL patients, a degeneration and loss of vascular smooth muscle cells can be observed leading to a variety of symptoms, including recurrent subcortical ischemic strokes, progressive cognitive decline, dementia and premature death (Chabriat et al., 1995; Ruchoux et al., 1995).

#### *1.2.5.1. Notch and Cancer*

Notch deregulations due to mutations in the Notch heterodimerisation domain or the PEST domain cause T-cell acute lymphoblastic leukaemia (Weng et al., 2004). Finally, inhibition of the Notch signaling in certain tumours had effects on tumour growth and differentiation: Inhibition of the Notch signaling pathway turned proliferative crypt cells into goblet cells indicating that colon adenomas might need the concerted activity of the Notch signaling pathway (van Es et al., 2005). Ridgway and colleagues found out, that the blocking of the Notch pathway by neutralizing DLL4 stopped tumour growth via deregulated angiogenesis (Ridgway et al., 2006).

Nicholas and colleagues found out that Notch1 deficiency in skin and primary keratinocytes caused the development of basal-cell carcinoma-like tumours. Furthermore, they could observe that Notch1 was able to inhibit β-Catenin-mediated signaling suggesting that Notch1 may function as a tumour suppressor gene in mammalian skin (Nicolas et al., 2003). In one form of lung cell cancer, the small cell lung cancer (SCLC), the constitutively active Notch signaling caused a growth arrest associated with the up-regulation of $p21^{waf1/cip1}$ and $p27^{kip1}$ supporting the idea that Notch activation can be associated with a reduction in neoplastic potential (Sriuranpong et al., 2001).

In another study performed by Li and colleagues, the authors found out that the Notch ligand DLL4 acts as a positive driver for tumour growth in human glioblastoma and

prostate cancer. They showed an up-regulation of DLL4 in tumour cells and tumour endothelial cells of human glioblastoma. DLL4 elevation in the tumour cells induced Notch signaling in the stromal/endothelial cells thereby increasing the blood vessel size and the vascular function within tumours. This finally leads to the promotion of tumour growth which was, to some extent, caused by the reduction of tumour hypoxia and apoptosis (Li et al., 2007).

Another study investigating meningiomas found out that increased Notch1 and Notch2 signaling correlates with the tetraploidy in meningiomas thereby leading to chromosomal instability suggesting that abnormal Notch signaling may promote tumour development (Baia et al., 2008). A further study supporting the idea of Notch being a tumour-promoting factor was performed by Pannequin and colleagues. Using a colorectal cancer cell line, a mouse model and the human colorectal cancer samples they could show a correlation between Notch pathway inhibition and increased goblet cell differentiation. Furthermore, the authors found out, that the Notch ligand Jagged-1 was under the control of β-catenin/TCF-4 indicating a cross talk between the two pathways thereby promoting cancer progression (Pannequin et al., 2009). Taken together, the Notch signaling pathway seems to have a pivotal role: it can act as tumour suppressor as well as a tumour promoter depending on the different expression pattern, the cellular context and the potential to interfere with other signaling pathways, e.g. the Wnt-signaling cascade

### 1.2.6. The role of Notch signaling in the placenta

Components of the Notch pathway are widely expressed in the mouse placenta and were shown to be critically involved in murine placental development. In particular, homozygous mutation of Notch receptor 1, DLL4, Hey1/Hey2 and CBF1 resulted in the inhibition of chorioallantoic branching (Gasperowicz and Otto, 2008). Mutations in the ankyrin repeat of the Notch receptor 2 lead to malformation of maternal blood sinuses in the developing mouse placenta (Hamada et al., 2007) while Notch3 does not seem to be essential for embryonic development or fertility in mice (Krebs et al., 2003). The distribution of Notch proteins and their ligands was also analyzed in normal and preeclamptic human placentas (Cobellis et al., 2007; De Falco et al., 2007). Immunohistochemical analysis was performed detecting Notch receptor 1, 4 and Jagged1 in CTB, ST and EVT. In preeclamptic villi, those Notch isoforms were decreased

suggesting a malfunction of the Notch signaling pathway in placental development and angiogenesis. Similar findings were observed by Sahin and colleagues investigating the placentae of pregnancies with intrauterine growth restriction (IUGR) or pregnancy induced hypertension (PIH). Furthermore the expression of several Notch isoforms and functionality of the Notch signaling pathway was detectable in the endometrium and endometrial stromal cells (Cobellis et al., 2008; Mazella et al., 2008; Mikhailik et al., 2009).

# 2. Goal of the Study

The Notch pathway seems to play many roles in different types of cells and tissues dependent on the developmental state and cellular context. Additionally, several diseases including the influence of cancer formation and progression are provoked by deregulation of the Notch pathway. The placenta is an organ that develops rapidly thereby undergoing several differentiation and invasion processes that are strictly regulated in a time- and dose-dependent manner. The interplay of the differently skilled trophoblasts with other cell types found in pregnancy tissues are of crucial importance to provide an uncomplicated pregnancy including the optimal supply of the fetus with nutrients and gases. However, gene chip analysis data revealed, that several mRNAs of the Notch receptors and ligands are expressed in different human placental cell types. This occurrence supports the idea that the Notch signaling might play a role within some of the differentiation and invasion processes in the human placenta.

According to this, the compilation of the expression profile of all Notch receptors and ligands in the human placenta will be first determined. The exact expression of receptors and ligands are supposed to give some insights into the potential interaction of different cell populations or developmental control mechanisms. Since $p57^{Kip2}$ seems to be regulated by Notch signaling in several human cell types, co-expression of Notch members with proliferation or differentiation markers will be determined. Furthermore, the activity of the Notch pathway will be demonstrated using the trophoblastic cell line SGHPL-5 to ensure that the present Notch members are not only expressed but can also elicit the Notch pathway. Stimuli with the active Notch intracellular domain and the activation with a different cell line that provides a ligand-dependent activation will be performed. Additionally, the functional influence of the Notch cascade activity on the trophoblast migratory potential will be tested. Finally, a co-culture model using villous explants and first trimester primary decidual cells will be tested for the communication of different cell types with each other.

Taken together, Notch signaling might have some putative roles in the human placenta. It could be involved in the decision of CTBs forming either syncytiotrophoblasts or EVTs. Furthermore, this signaling pathway might control the switch of proliferation and invasion in cell columns. Additionally, the interaction of decidual cells with EVTs could influence the invasive depth of trophoblasts. Using the experiments described above I will try to gain more insights into the importance of Notch signaling in the human placenta.

# 3. Results

## 3.1. Expression of Notch receptors and ligands

### 3.1.1. mRNA expression of Notch receptors and ligands

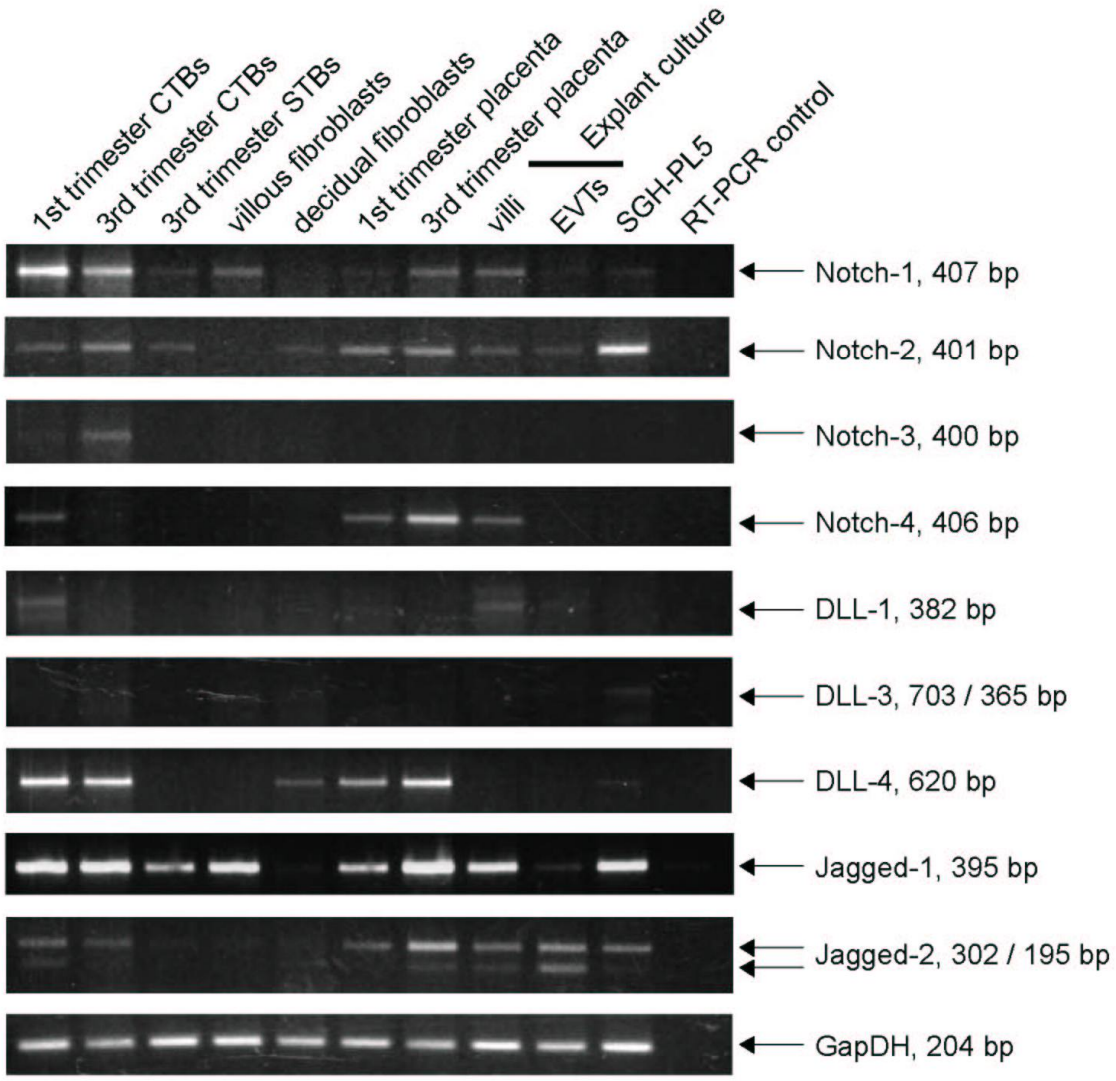


**Fig. 10: mRNA detection of Notch family members using semiquantitative RT-PCR**
Several cDNAs of different trophoblast and fibroblast subpopulations of first and third trimester placentae were used in RT-PCR detecting Notch receptors and ligands. The primer sequences, cycle numbers and annealing temperatures are listed in table 2; the fragment sizes are indicated.

In the first experiments I used RT-PCR for the detection of all Notch pathway receptors and ligands to determine the mRNA expression of the Notch family members throughout pregnancy. In Figure 10 the whole table of the PCR results is shown. The Notch receptor 1 seems to be predominantly expressed in the cytotrophoblasts of first and third trimester

pregnancy while it is weaker in all other cell or tissue preparations. Similar results could be obtained for the Notch receptor 2, which was also strongly expressed in the trophoblastic cell line SGHPL-5. Notch-3 could only be detected in the first and third trimester cytotrophoblasts. Notch-4 receptor showed expression in the tissue preparations of the first and third trimester. DLL-1 was not strongly expressed but showed a weak staining in trophoblast and fibroblast cells. DLL-3 showed mRNA expression only in third trimester trophoblasts and SGH-PL5. The Jagged-1 ligand could be detected in all cDNAs except the decidual fibroblasts. Interestingly, it was the only ligand that was also expressed in syncytialized trophoblasts. Jagged-2 was expressed in cytotrophoblasts of the first and third trimester, the tissue preparations and clearly in extravillous trophoblasts, where the expression of the small fragment (the second splice variant) was stronger expressed than in other cell populations tested. Expression of Glycerinaldehyd-3-phosphat-Dehydrogenase (GapDH) was used as a loading control.

All following experiments are performed with cell and tissue preparations of the first trimester of pregnancy to focus on trophoblast differentiation and invasion.

### 3.1.2. Protein expression of Notch receptors and ligands

For the detection of protein expression and localisation of the Notch receptors and ligands I performed immunohistochemistry of first trimester placenta. Beside the particular receptor and ligand antibodies I additionally used specific antibodies to detect trophoblasts (cytokeratin7), stromal cells (vimentin), proliferative cells (KI67) and differentiated cells ($p57^{Kip2}$) for tissue orientation (cytokeratin7, vimentin) and EVT differentiation (Ki67, $p57^{Kip2}$). All pictures display representative areas and photographs are taken at 400-fold magnification. The used primary and secondary antibodies, dilutions, species and providing companies are listed in table 5 and 6 (materials and methods).

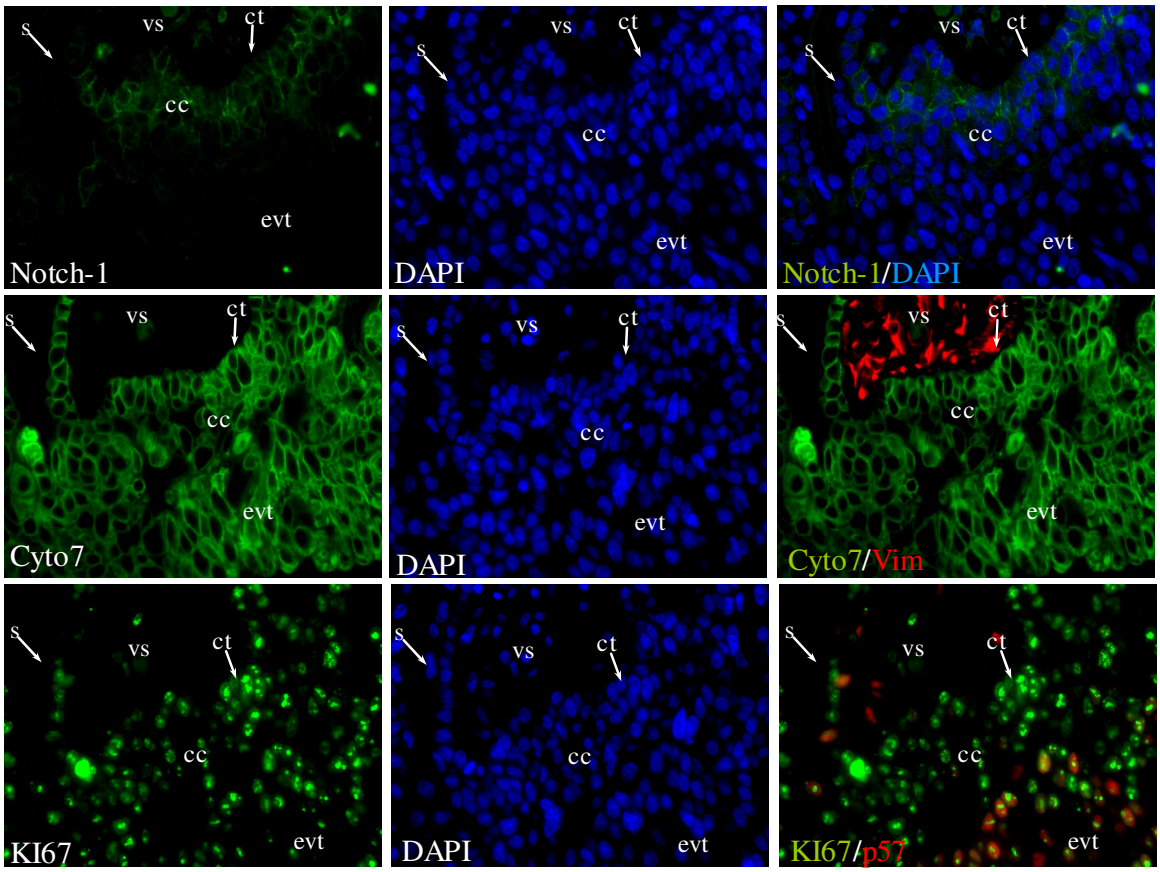


**Fig. 11: Notch-1 expression in cell columns of 1st trimester placentae**

Staining of Notch-1 (upper panel from right to left) with the corresponding nuclei staining and the overlay picture. Note that the proximal cell column trophoblasts express Notch-1 without any correlation of the receptor expression with proliferation and differentiation. The picture series in the middle (from right to left) shows the detection of cytokeratin 7 with the corresponding nuclei staining and an overlay picture with cytokeratin7 (green) and vimentin (red). Staining of KI67 is shown in the lower panel with the corresponding nuclei staining and a costaining with KI67 (green) and detection of $p57^{Kip2}$ (red). s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

Notch receptor 1: The Notch receptor 1 stained the cytoplasmic membranes and was predominantly expressed in trophoblasts of the proximal cell column and the cells of the villous stroma (Fig. 11). Regarding Notch-1 positive trophoblasts, the staining started at the apical surface of first row of cell column trophoblasts and was not present luminal to the villous stroma. After two to four rows of Notch-1 positive cell column trophoblasts, the detection vanished abruptly even before the differentiation of the trophoblasts to the invasive phenotype has been completed. Regarding the proliferative area of the cell column, the expression of Notch-1 does not seem to correlate with the cell cycle. There is no staining visible in other trophoblast subpopulations or cells that are associated with vessel formation, including endothelial cells and smooth muscle cells (Fig.12).

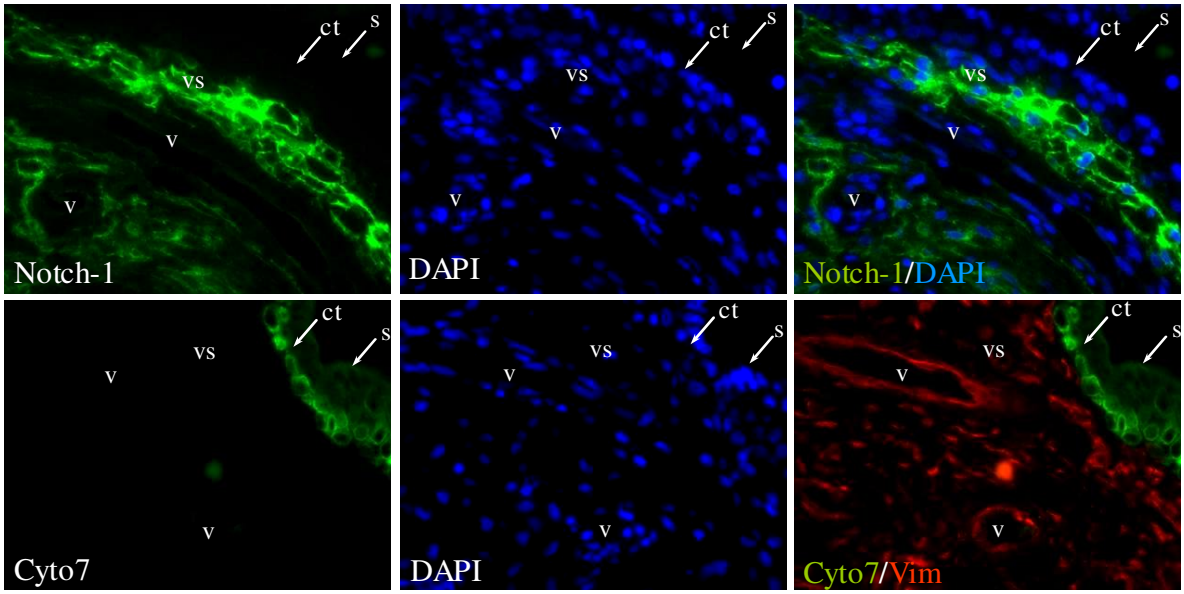


**Fig. 12: Notch-1 expression of floating villi of 1st trimester placentae**

Staining of Notch-1 (upper panel, from right to left) is shown with the corresponding nuclei staining and the overlay picture. Detection of cytokeratin 7 (lower panel) with the corresponding nuclei staining and an overlay picture with cytokeratin7 (green) and vimentin (red) is shown. Note that villous stromal cells, but not vessels and surrounding muscle cells, strongly express Notch-1; ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; v, vessel

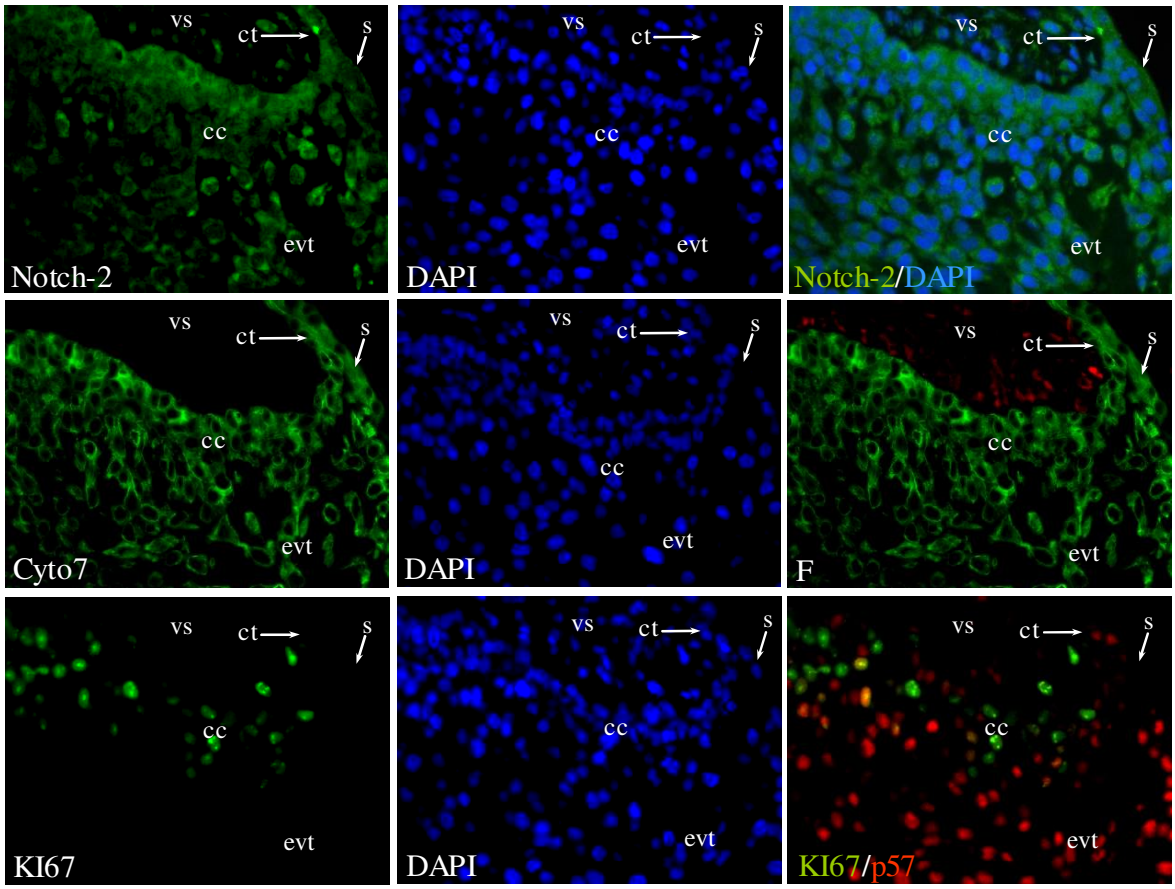


**Fig. 13: Notch-2 expression of cell columns of 1st trimester placentae**
Staining of Notch-2 (upper panel) is shown with the corresponding nuclei staining. Note, that all placental cell types express Notch-2. The middle panel shows detection of cytokeratin 7 (middle panel) with the corresponding nuclei staining and an overlay picture with cytokeratin7 (green) and vimentin (red). KI67 staining is shown in the lower panel with the corresponding nuclei and a costaining with KI67 (green) and $p57^{Kip2}$ (red). ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast;

Notch Receptor 2: The Notch receptor 2 was expressed in nearly all cells of the placenta including syncytiotrophoblasts, cytotrophoblasts and villous stroma cells (Fig. 13). The staining pattern changed along the cell column from a uniform staining of the cytoplasmic membranes (proximal cell column) to a patchier staining (distal cell column).

Notch Receptor 3: This receptor could be detected in cytoplasmic membranes of cytotrophoblasts, trophoblasts of the proximal part of the cell column and villous stromal cells (Fig. 14). The antibody also strongly reacted with endothelial cells of the villous stroma and foetal blood cells. Compared to Notch1 expression, the cell column expression of Notch3 was even more distal but still vanished within the proliferative cell population and was absent from EVT proliferation.

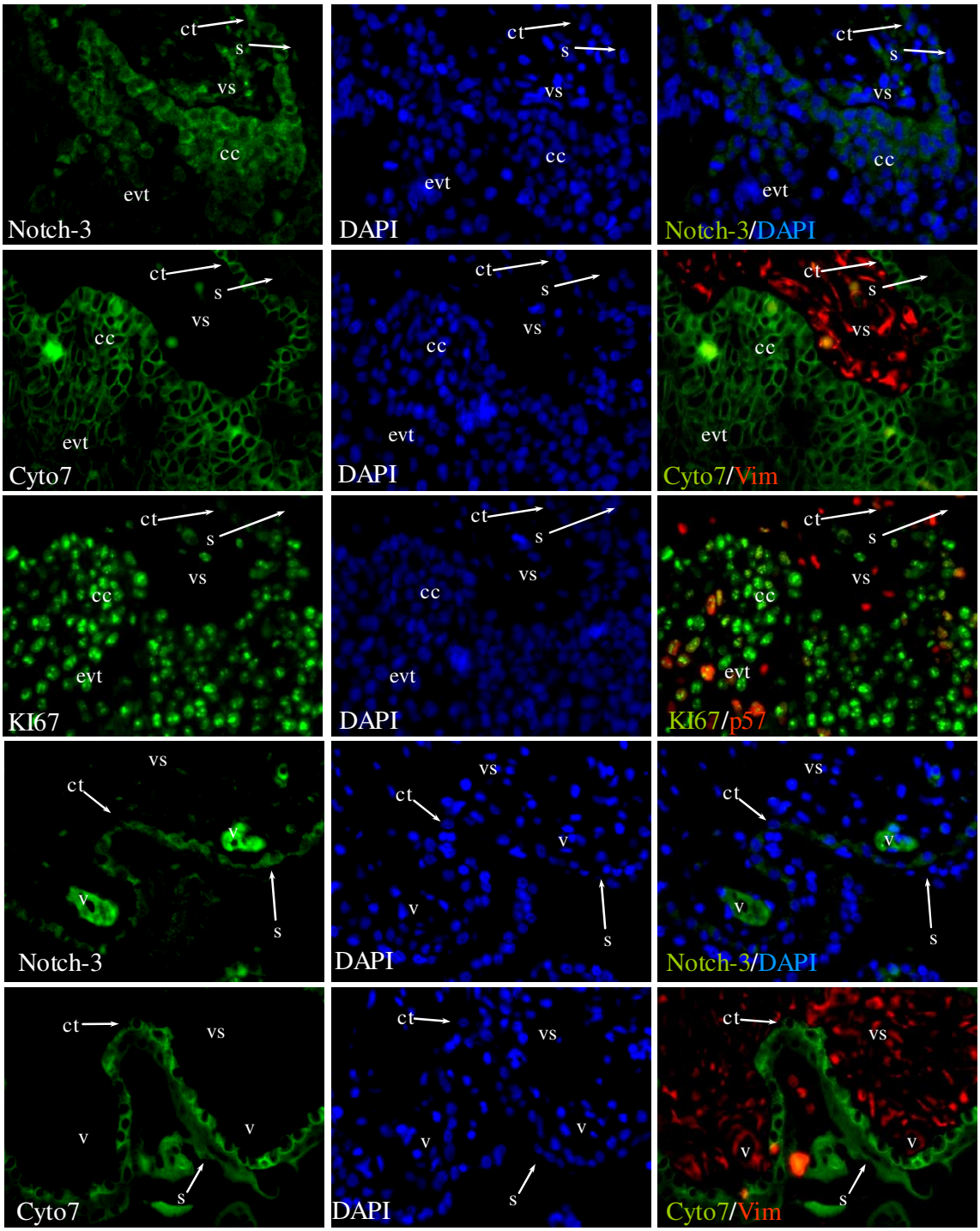


**Fig. 14: Notch-3 expression in cell columns and floating villi of 1st trimester placentae**
Panels one to three represent sections of a cell column; the last two panels show a floating villus. Staining of Notch-3 are shown in the first and forth panel with the corresponding nuclei staining and overlays (from right to left). Note, that Notch-3 is expressed in villous cytotrophoblasts, proximal cell column trophoblasts, stromal cells and stromal vessel. Cytokeratin 7 expression with the corresponding nuclei staining and overlay pictures with cytokeratin7 (green) and vimentin (red) are shown in the second and last panel. Detection of KI67 with the corresponding nuclei staining and a costaining with KI67 (green) and $p57^{Kip2}$ (red) are shown in the middle panel. ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast; v, vessel

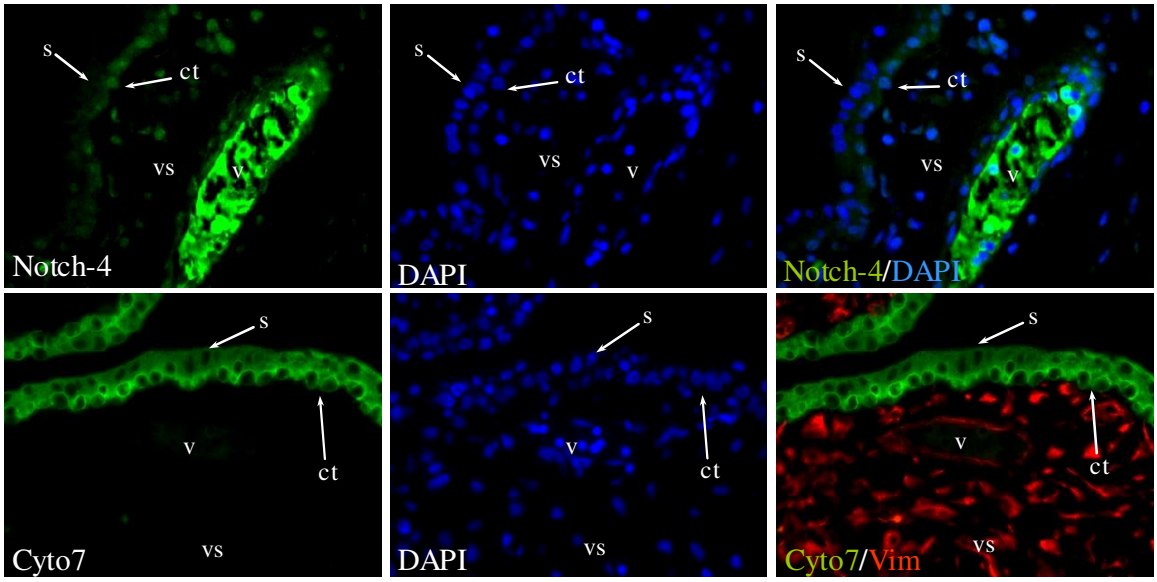


**Fig. 15: Notch-4 expression in floating villi of 1st trimester placentae**
Staining of Notch-4 (green) is show in the upper panel with the corresponding nuclei staining and the overlay picture. Note that Notch4 is strongly expressed in stromal vessels and endothelial cells and very weakly in cytotrophoblasts. The lower picture series shows detection of cytokeratin 7 with the corresponding nuclei staining and an overlay picture with cytokeratin7 (green) and vimentin (red). ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; v, vessel

Notch Receptor 4: The Notch receptor 4 was very weakly expressed in villous cytotrophoblasts and strongly stained the villous endothelial cells and foetal blood cells (Fig. 15).

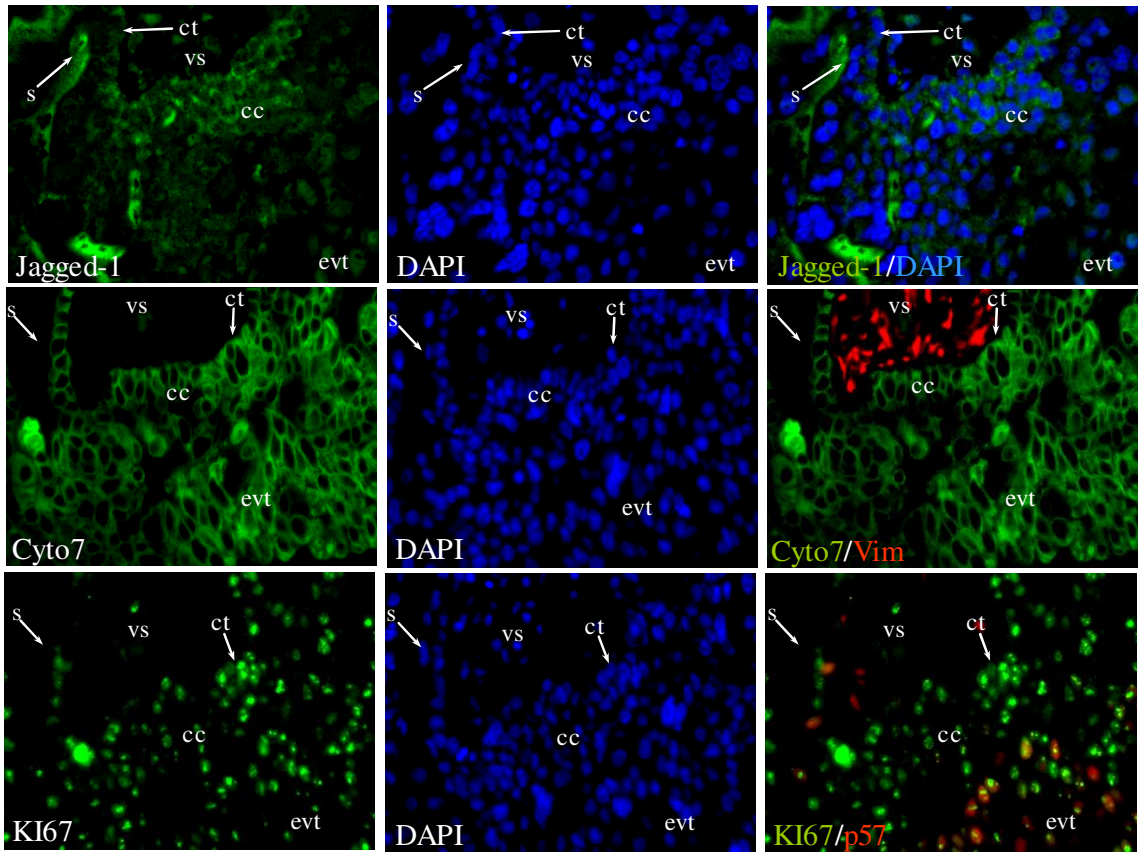


**Fig. 16: Jagged-1 expression in cell columns of 1st trimester placentae**
Staining of Jagged-1 (upper panel) is shown with the corresponding nuclei staining and the overlay picture. Note that Jagged-1 in expressed in cytotrophoblasts, proximal cell column trophoblasts and villous stromal cells. The middle panel represents detection of cytokeratin 7 (middle panel) with the corresponding nuclei staining and an overlay picture with cytokeratin7 (green) and vimentin (red). Staining of KI67 is shown in the lower panel with the corresponding nuclei staining and a costaining with KI67 (green) and detection of Kip2p57 (red). ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

Jagged-1: The antibody against Jagged-1 detected the villous stromal cells, cytotrophoblasts, proximal cell column trophoblasts and, similar to Notch1 and Notch3, disappeared in distal cell column trophoblasts (Fig. 16). Once again, the loss of Jagged-1 did not correlate with the cell cycle of the cell column trophoblasts. Interestingly, Jagged-1 could also be detected in the apical surface of selected syncytial parts and seems to be associated with contact points of distinct syncytial membranes. Fig. 17 shows different regions of the placenta highlighting the areas of interest with red arrows.

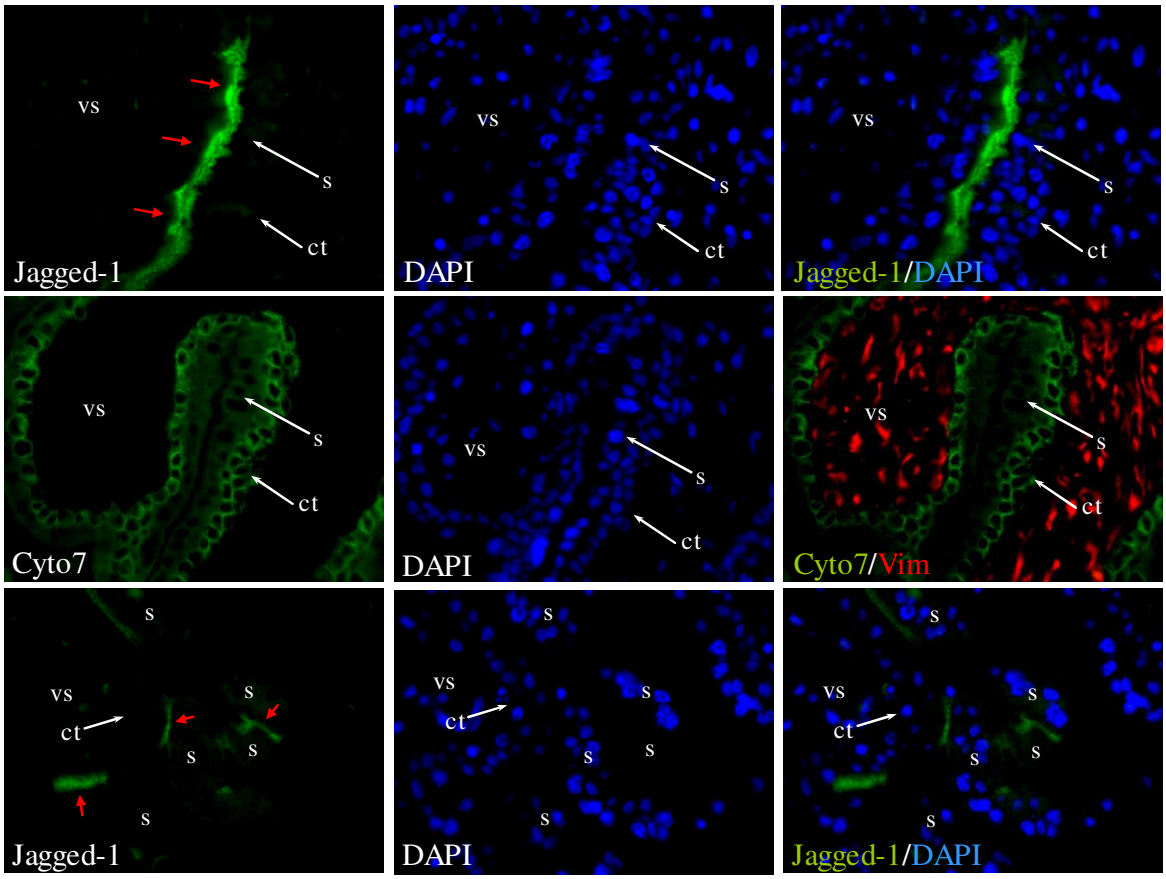


**Fig. 17: Jagged-1 expression in syncytia of 1st trimester placentae**
The upper and lower panel (from right to left) shows staining of Jagged-1 with the corresponding nuclei staining (DAPI) and the overlay pictures. Note that the contact areas of syncytia strongly express Jagged-1 (red arrows). The middle panel represents the staining with cytokeratin 7, DAPI and vimentin corresponding to the first row of pictures. ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

Jagged-2: Jagged-2 was expressed in villous stromal cells, cytotrophoblasts, proximal cell column trophoblasts and distal extravillous trophoblasts (Fig. 18). Similar to the expression of Notch-2, this particular ligand had a different, more patchy staining pattern in distal EVTs with a strong perinuclear expression (highlighted with red arrows) compared to the expression profile in the other trophoblasts and stromal cell populations.

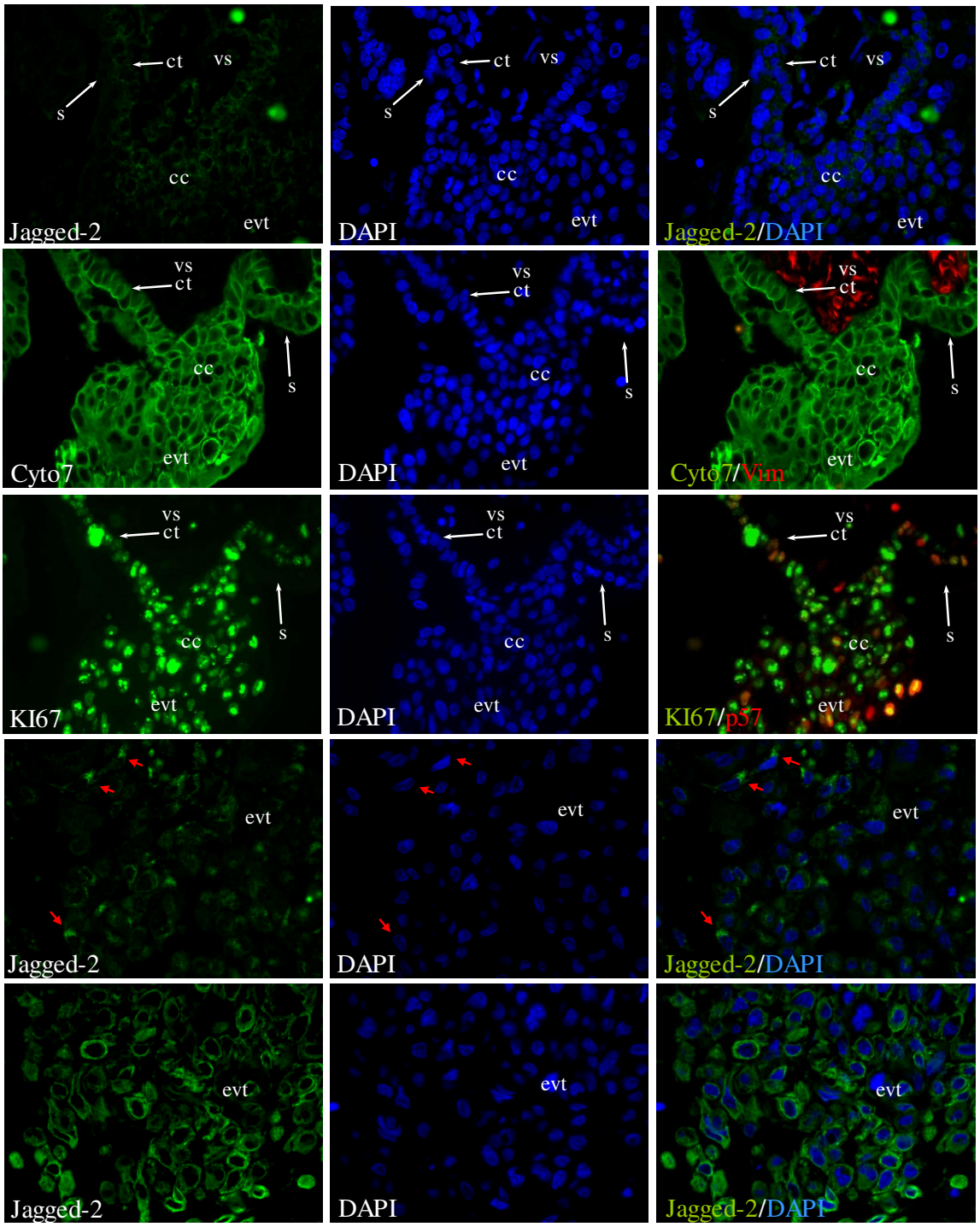


**Fig. 18: Jagged-2 expression in cell columns and EVT-areas of 1st trimester placentae**

The first three rows show a cell column stained with Jagged-2 and the corresponding expression profiles with Cytokeratin7/Vimentin and KI67/p57$^{Kip2}$. Note, that Jagged-2 was expressed in cytotrophoblasts, proximal cell column trophoblasts and villous stromal cells. The last two rows of pictures represent Jagged-2 and the corresponding cytokeratin expression in distal EVT areas, respectively. Note, that the receptor showed a patchy expression with perinuclear enrichment (red arrows). ct, cytotrophoblast; s, syncytium; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

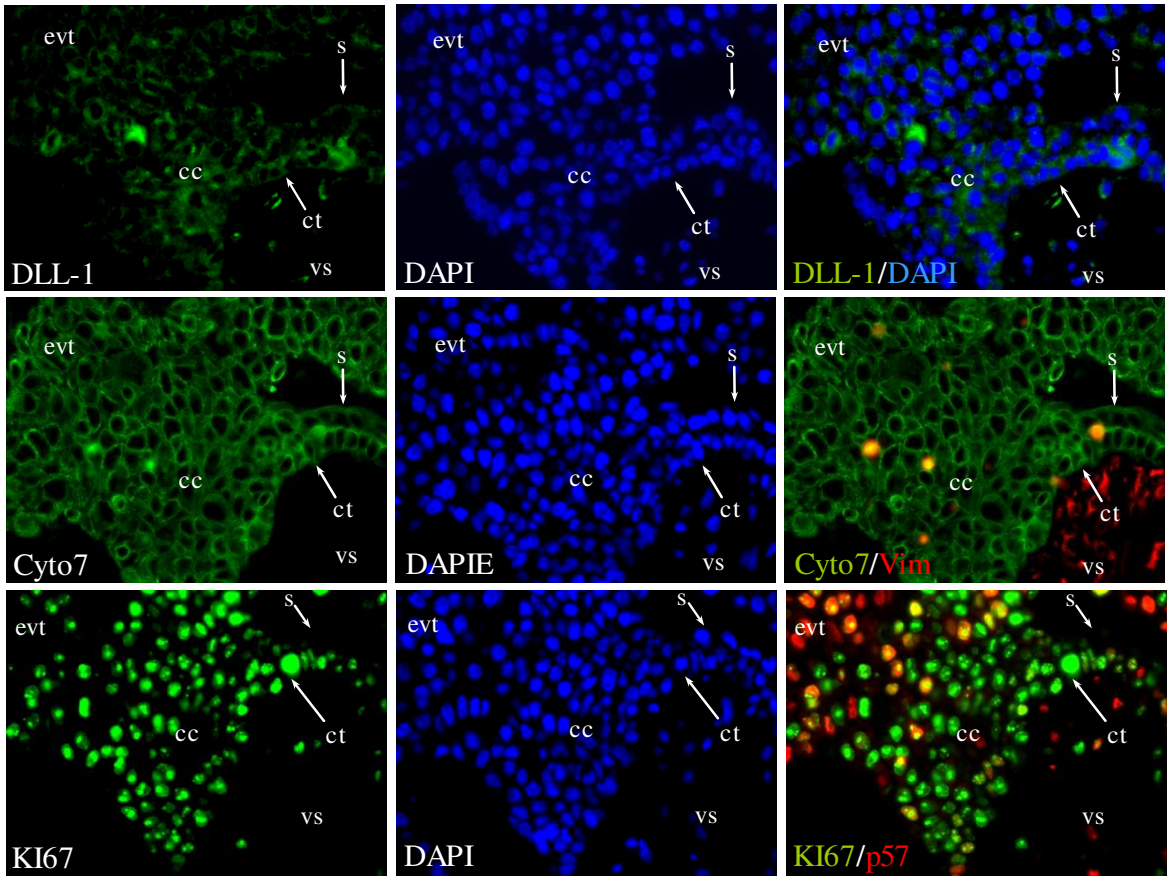


**Fig. 19: DLL-1 expression in cell columns of 1st trimester placentae**

The upper panel (from right to left) shows staining of DLL-1 with the nuclei staining (DAPI) and the overlay pictures. Note that DLL-1 was expressed in all placental cell types. The middle panel represents the corresponding staining with cytokeratin 7, DAPI and vimentin. The last row of pictures represents proliferation (KI67) and differentiation ($p57^{Kip2}$) of this cell column. ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

DLL-1: The antibody against DLL-1 showed continuously staining throughout the first trimester placenta, including the syncytiotrophoblasts and seems not to be regulated by proliferation or differentiation of trophoblasts (Fig. 19).

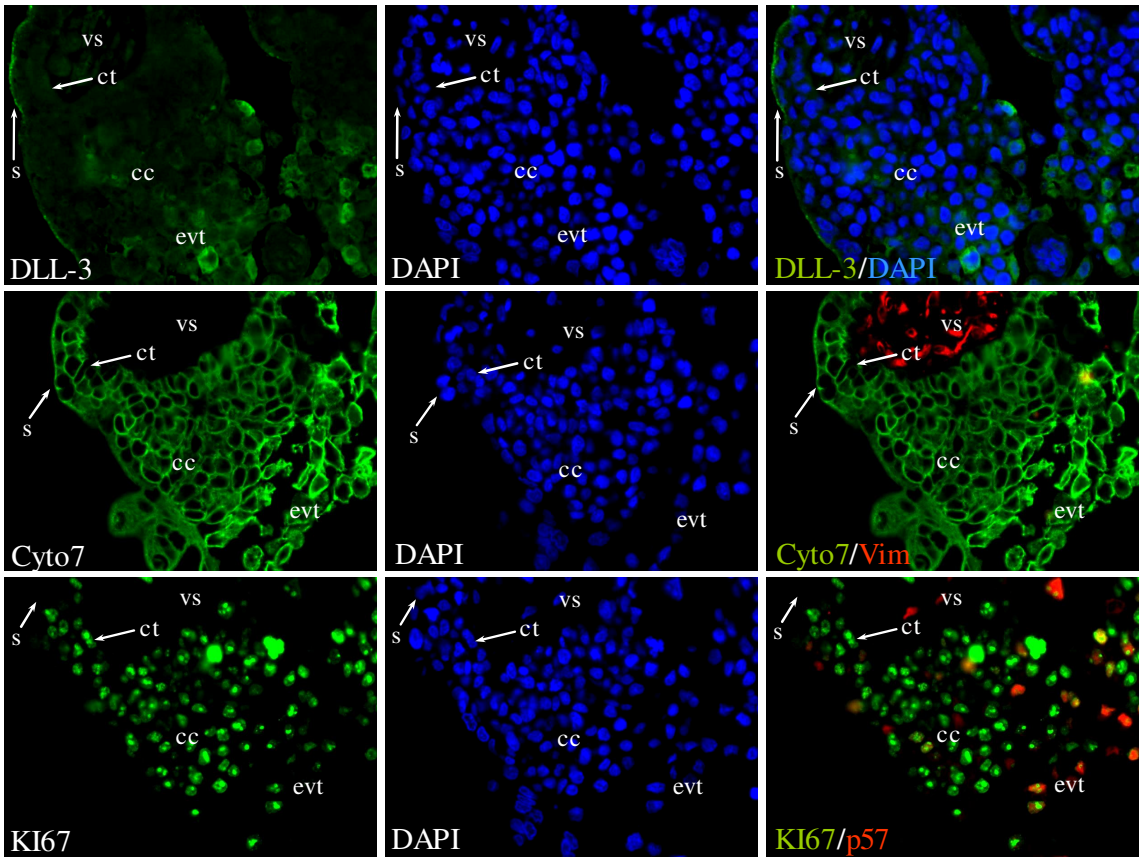


**Fig. 20: DLL-3 expression in cell columns of 1st trimester placentae**
The upper panel (from right to left) shows staining of DLL-3 with the nuclei staining (DAPI) and the overlay pictures. Note that DLL-3 was expressed in distal cell column trophoblasts. The middle panel represents the corresponding staining with cytokeratin 7, DAPI and vimentin. The last row of pictures represents proliferation (KI67) and differentiation ($p57^{Kip2}$) of this cell column. ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

DLL-3: DLL-3 was expressed at the apical surface of the syncytiotrophoblasts and in distal EVTs (Fig. 20). The expression of this ligand shows a correlation with the exit of the cell cycle in extravillous trophoblasts since the expression starts in those EVTs that express $p57^{Kip2}$ and have widely lost their proliferative potential.

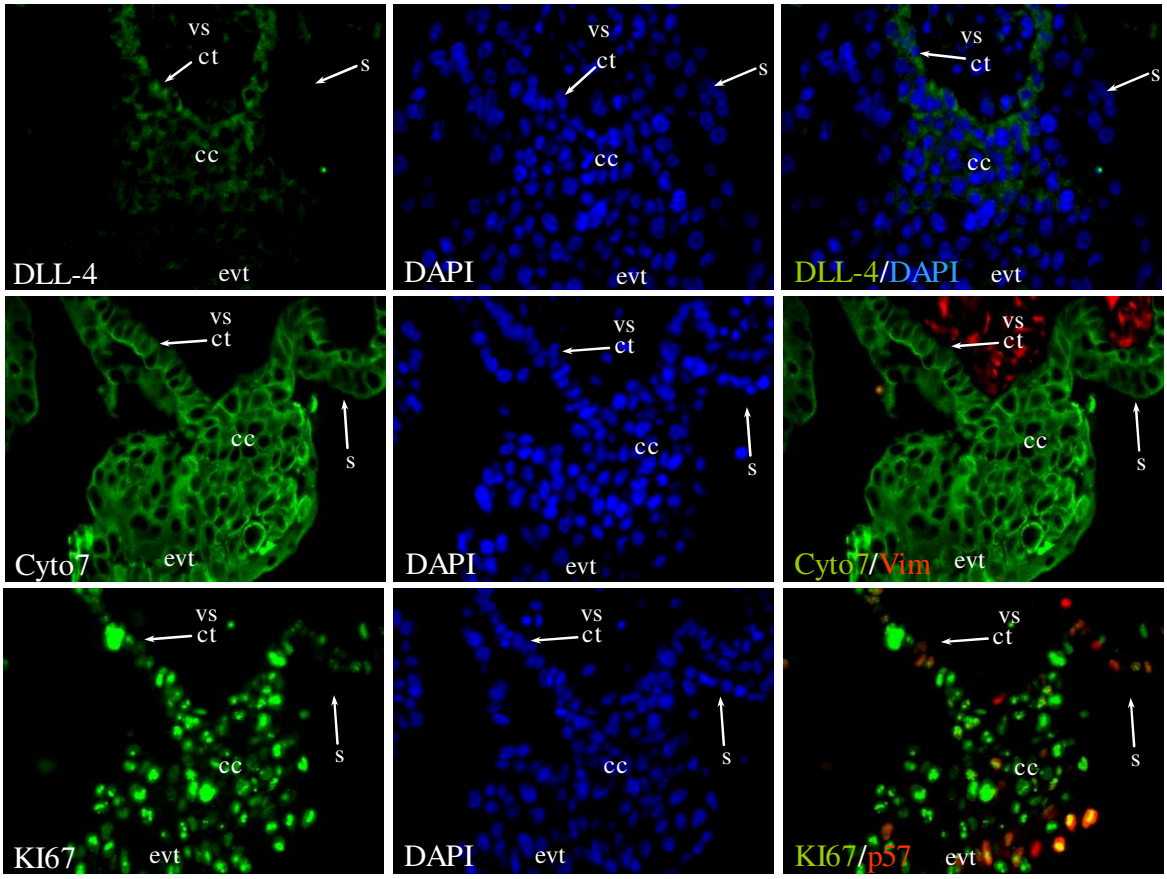


**Fig. 21: DLL-4 expression in cell columns of 1st trimester placentae**
The upper panel (from right to left) shows staining of DLL-4 with the nuclei staining (DAPI) and the overlay pictures. Note that DLL-4 is expressed in villous stromal cells, cytotrophoblasts and proximal cell column trophoblasts. The middle panel represents the corresponding staining with cytokeratin 7, DAPI and vimentin. The last row of pictures represents proliferation (KI67) and differentiation ($p57^{Kip2}$) of this cell column. ct, cytotrophoblast; s, syncytiotrophoblast; vs, villous stroma; cc, cell column; evt, extravillous trophoblast

DLL-4: The staining pattern of DLL-4 had similarities to the expression profile of the Notch receptor 3. The ligand was expressed in villous stromal cells, cytotrophoblasts and proximal EVTs (Fig. 21). The expression vanished with the distance to the villous along the cell column. Once again, the expression was not associated with the proliferation/differentiation of the extravillous trophoblasts.

## 3.2. Notch pathway activity

In this chapter, I transiently transfected the first trimester trophoblastic cell line SGHPL-5 with reporter plasmids (4mtCBF1Luc, 4wtCBF1Luc) to measure the Notch signaling activity. Furthermore, I used an eukaryotic expression plasmid carrying the functional part of the Notch receptor 1 intracellular domain (NICD) to overexpress this particular

NICD thereby stimulating the Notch pathway. All three plasmids were kindly provided by Diane Haywarth (University of Otago, New Zealand). In order to obtain a mock control for the NICD overexpression, I removed the NICD fragment using the restriction endonucleases EcoR1 and XbaI. Graphs and descriptions of the used plasmids are shown in materials and methods. All experiments were repeated at least three times.

### 3.2.1. Notch pathway activity among the SGHPL-5 trophoblastic cell line

To assess the Notch signaling activity I transiently transfected the trophoblastic cell line SGHPL-5 with a reporter construct containing 4 CBF1 binding sites upstream of the luciferase gene (4xwtCBFLuc) (Fig. 22). A plasmid with mutations in the CBF1 binding sites (4xmtCBFLuc) displayed the background level and the plasmid pSG5-Flag-NICD that overexpresses the Notch receptor 1 intracellular domain (NICD) was used to activate the Notch signaling pathway. The white bars represent the cells that were transfected with the vector containing the mutated CBF1 binding site displaying the threshold level of the transfection experiments. The upregulation of the luciferase activity with the wild type CBF1 reporter construct (black bars) was doubled compared to the mutant control plasmid thereby indicating an ongoing Notch activity among SGHPL-5 cells without any stimulation. Blocking of the γ-Secretase activity (with 10µM DAPT) had no effect on the mutant CBF1 reporter plasmid. In the wild type studies, the Notch activity could be reduced significantly in the presence of DAPT additionally confirming the endogenous Notch activity of the trophoblastic cell line. Overexpression of the NICD domain strongly upregulates luciferase activity indicating a high induceability of the Notch cascade in the trophoblastic cell line. Take together, the transfection of the 4wtCBFLuc indicates that there is a Notch cascade activity among SGHPL-5 cells. Overexpression of the NICD domain in combination with the wild type CBF1 reporter plasmid further increased the Notch cascade activity.

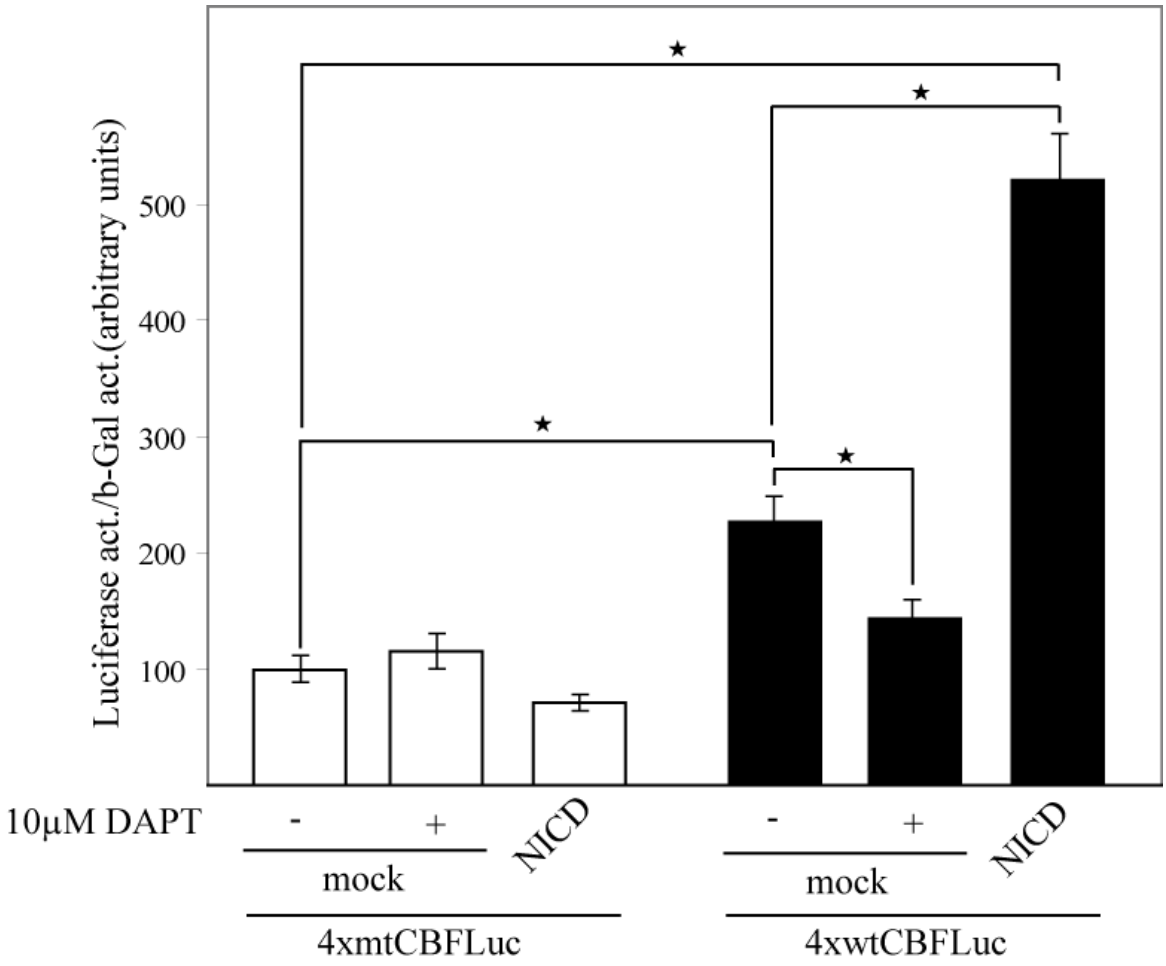


**Fig. 22: Notch pathway activity among the trophoblastic cell line SGHPL-5**
Transient transfection with the 4wtCBFLuc (black bars) without any stimulation show a two-fold induction compared to the 4xmtCBFLuc (white bars). Treatment with 10µM DAPT reduces the Notch activity and overexpression of the NICD domain strongly upregulates the luciferase activity. Mean values ± SD of four experiments performed in duplicates are shown. Normalized value of unstimulated control (4xmtCBFLuc) was arbitrarily set at 100%. *, $P < 0.05$

To control the overexpression efficiency with the pSG5-NICD I performed Western blot analyses using antibodies against the NICD domain and against the Flag-tag, respectively (Fig. 23). Both antibodies detect a protein with the protein size of approximately 72kDa indicating a high expression of the active form of the Notch receptor 1 intracellular domain. The GapDH protein expression verifies the equal amounts of protein in both samples, the NICD overexpression and the mock control. Furthermore, I determined the expression of Hes1, a typical Notch signaling target gene (Fig. 24) to verify the influence of the enhanced Notch pathway activity on the cellular transcription potential. Using RT-PCR analyses I could detect an induction of the Hes1 mRNA upon expression of NICD compared to the mock control.

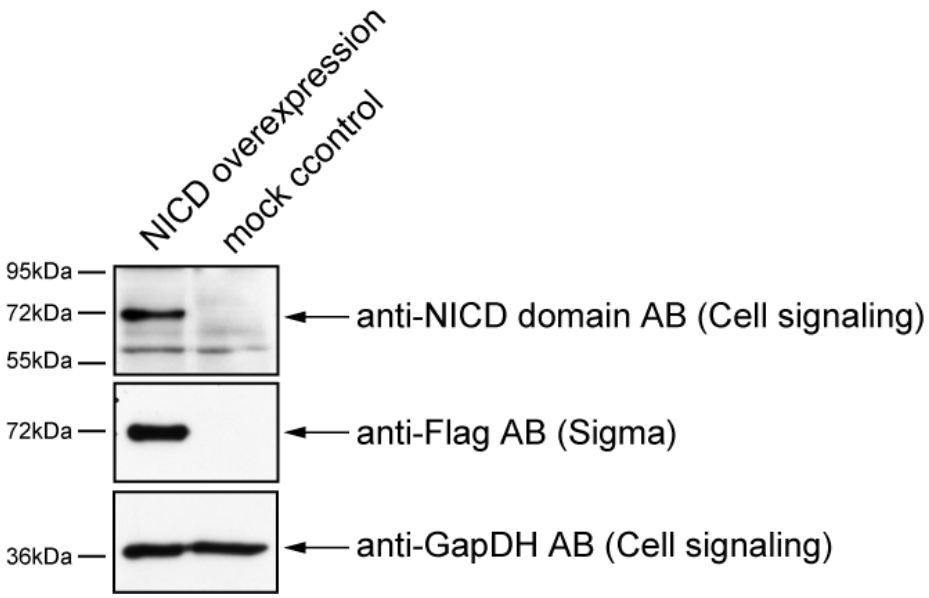


**Fig. 23: Protein detection of the overexpressed NICD domain**
After transient expression of the NICD domain the transfection efficiency was verified using Western blot analyses. Both antibodies, the antibody against the Notch1 intracellular domain (NICD-V) and the antibody against the Flag tag strongly detected a protein band at 72kDa, which was absent in the protein lysate of the mock control. A specific antibody against GapDH (37kDa) verified similar loading amounts.

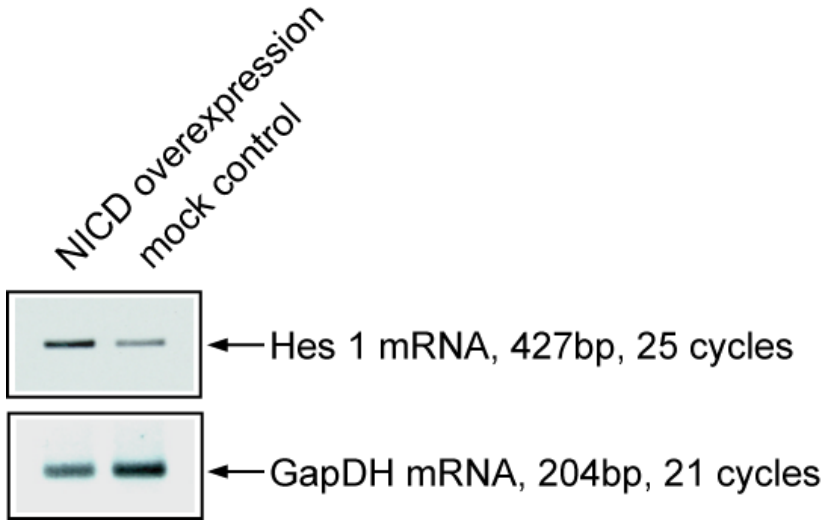


**Fig. 24: Hes1 mRNA detection**
Semi-quantitative RT-PCR shows the upregulation of the Hes1 mRNA upon NICD overexpression (ethidium bromide-stained band at 427bp). The RNA quantity is verified using primers for GapDH mRNA detection (204bp).

Since this pathway needs cell-to-cell contact, I further wanted to test, if different cell densities might influence the basal and inducible Notch activity in SGHPL-5 cells. Fig. 25 shows similar luciferase activities in a low-density cell population (60%, light grey bars) and high density cell population (95%, black bars) indicating that, at least in these ranges, the cell density does not influence the Notch activity.

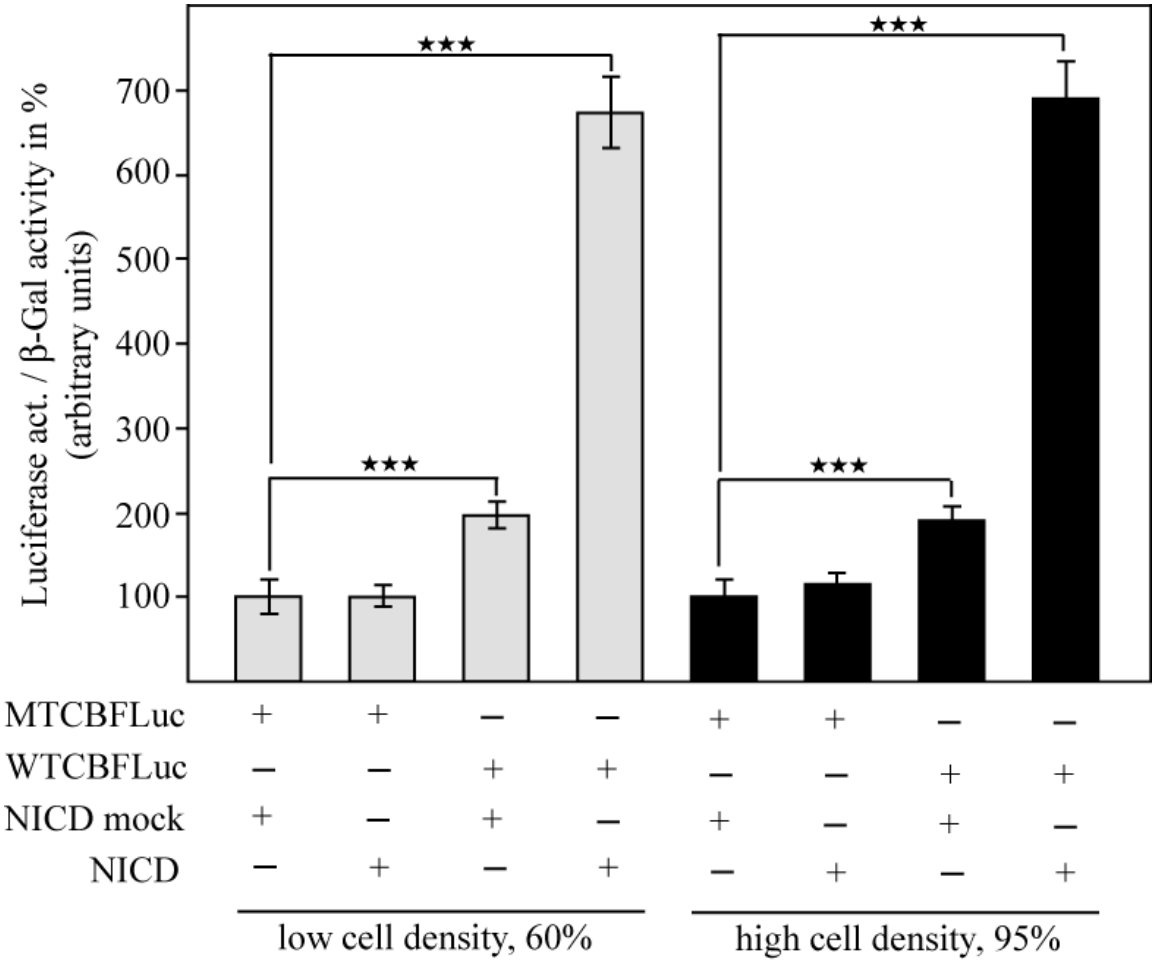


**Fig. 25: Notch activity in low cell density and high cell density cultures**
Using two different cell densities (60%, light gray bars; 95%, black bars) the luciferase activities were measured after transient transfection with mtCBFLuc, wtCBFLuc, NICDmock and NICD. As expected, the SGHPL-5 cells had an endogenous Notch activity, which could be further, induced upon NICD transfection, both independent of the cell density. Mean values ± SD of three experiments performed in duplicates are shown. Normalized value of unstimulated control (4xmtCBFLuc) was arbitrarily set at 100%. ***, $P < 0.001$

### 3.2.2. Notch pathway activity between SGHPL-5 cells and decidual fibroblasts

Since in vivo the extravillous trophoblasts do have contact to decidual fibroblasts I wanted to figure out, if the trophoblastic cell line SGHPL-5 might communicate with first trimester decidual fibroblasts via the Notch pathway. Therefore, I used a modified transfection approach illustrated and described in materials and methods (Fig. 40).

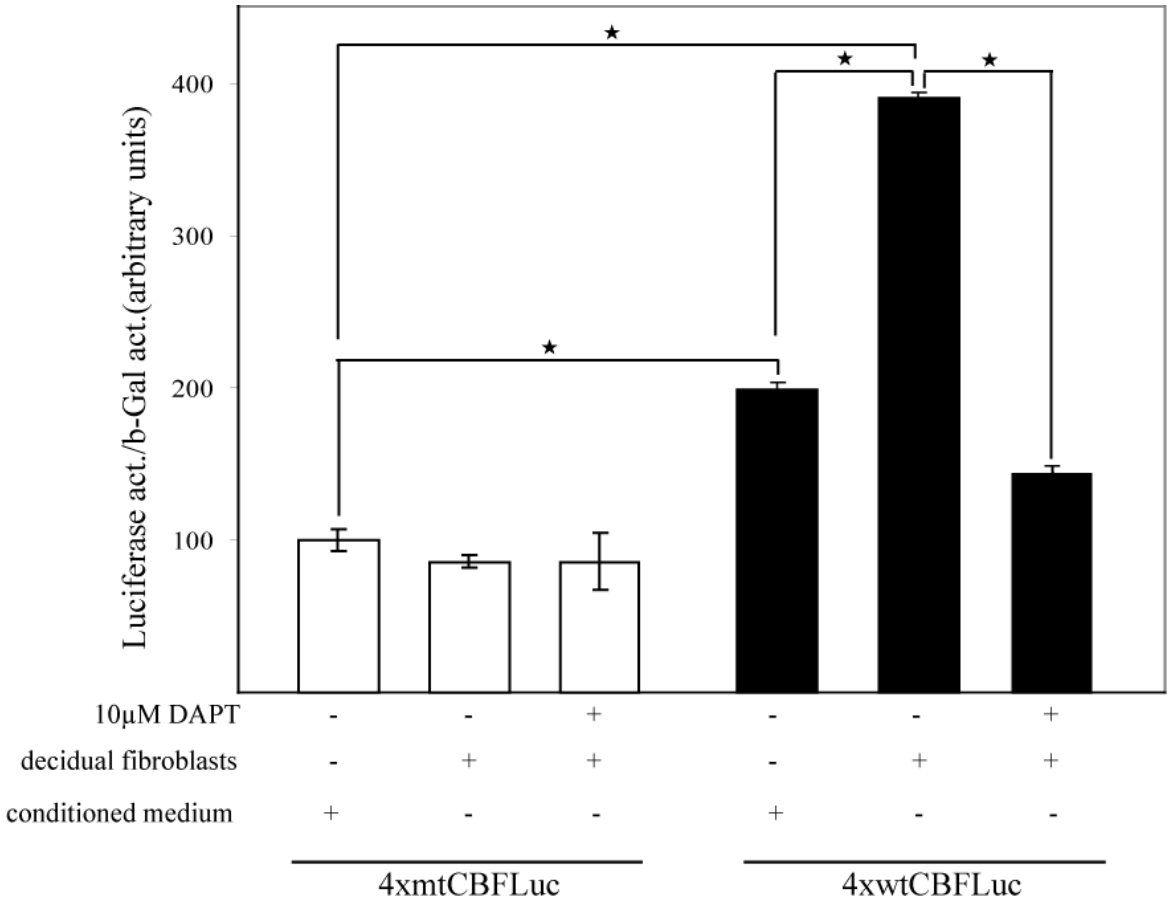


**Fig. 26: Stimulation of the Notch signaling with decidual fibroblasts**

The white bars represent the usage of the mutated CBF binding site (4xmtCBFLuc), the black bars show the transfection with the wild type CBF binding site (4xwtCBFLuc). The luciferase activity was enhanced in the presence of conditioned medium (endogenous Notch activity). In the presence of decidual fibroblasts, the Notch activity was further increased being reduced by DAPT. Mean values ± SD of three experiments performed in duplicates are shown. Normalized value of unstimulated control (4xmtCBFLuc) was arbitrarily set at 100%. *, $P < 0.05$

Briefly, SGHPL-5 cells were transiently transfected with 4xwtCBFLuc und 4xmtCBFLuc for 6 hours and then stimulated with decidual fibroblasts or conditioned medium. The black bars represent the wild type CBF1 binding site; the white bars are used to show the basic levels of the luciferase activity (mutant CBF1 binding site). Fig 26 shows a 2fold induction of the luciferase activity in SGHPL-5 cells in the presence of conditioned medium. The same results were obtained in previous transfection experiments with normal cell culture medium suggesting, that the conditioned medium had no effect on the Notch activity. This result confirms that soluble factors in the conditioned medium could not stimulate the Notch communication among SGH-PL5 cells. The addition of fibroblasts strongly increased the luciferase activity compared to the medium control. The presence of the γ-Secretase inhibitor DAPT reduced the Notch activity to the base levels. The addition of fibroblasts did not stimulate the mutant CBF binding site verifying the

specificity of the experiments. This assay showed, that the decidual fibroblasts were able to stimulate the Notch pathway in the trophoblastic cell line. Furthermore, the physical contact was essential since the influence of secreted fibroblast specific factors on the endogenous Notch activity among the SGHPL-5 cells could be excluded.

## 3.3. Notch signaling and migration

Finally, I wanted to verify, if the Notch signaling has an effect on the migratory behaviour of the trophoblastic cell line SGHPL-5 and explant-derived EVTs. In both experimental approaches I used different concentrations of the γ-Secretase inhibitor DAPT to turn off endogenous Notch signaling.

### 3.3.1. The inhibition of the Notch signaling stimulates the migration of SGHPL-5

To test the migratory behaviour of SGHPL-5, I used a transwell assay system and performed the experiments with 0, 1, 10 or 100µM DAPT, respectively. After 24h, an increase of migration could be observed upon downregulation of the Notch signaling pathway in a dose-dependent manner (Fig. 27). Statistical significant results were obtained with 10µM and 100µM DAPT.

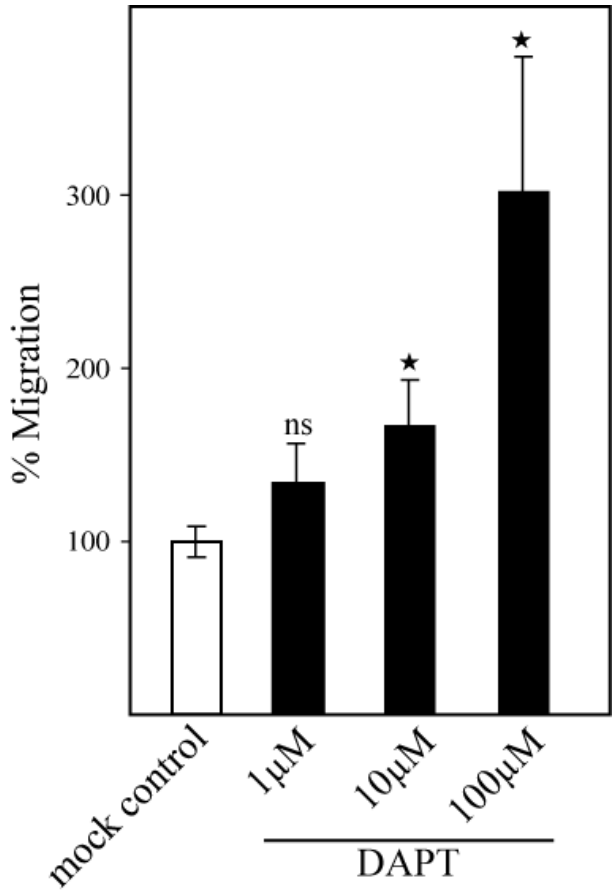


**Fig. 27: Increase of migration upon downregulation of the Notch signaling in SGHPL-5 cells**
After 24h of migration, rising concentrations of DAPT caused an increase in the migratory behaviour of the trophoblastic cell line compared to the control (DMSO). Mean values ± SD of three experiments performed in triplicates are shown. Normalized value of unstimulated mock control was arbitrarily set at 100%. ns, not significant; *, $P < 0.05$

## 3.3.2. The inhibition of the Notch signaling stimulates the migration of explant-derived EVTs

Since the inhibition of the Notch cascade provoked an increase of migration, I wanted to verify, if primary cells do react similarly. For this purpose, I prepared villous explants cultures on rat-tail collagen I droplets and treated the organ cultures with 0.1, 1 and 10µM DAPT. In the first two concentrations, an increase of EVT migration was clearly detectable. The 10µM concentration had a toxic effect (data not shown) and was omitted for further studies. I used 12 explants each condition and repeated the experiment three times. All explants were monitored and photographed directly after seeding (0h), after 24h and 48h. A representative sample is shown in figure 28A. Already after 24h the first differences could be observed. Turning off the Notch pathway leads to an enhanced EVT migration. After 48h, the EVTs reached a higher distance. Additionally, pictures at a higher magnification of the EVT population after 48h display a slightly different

phenotype. In the presence of 1µM DAPT the EVTs seem to achieve a more migratory phenotype having an elongated shape and a more loosened structure proximal to the cell column compared to the mock control EVTs.

To objectively verify the outgrowth differences, I measured the distance that the EVTs had covered. For this purpose, I paced three arrows on each explant sample beginning at the villous tip and ending at the most distal EVT. The average distance of the EVTs was then calculated for each condition. The plot with the results is shown in Fig. 28B and confirms the previous findings. With rising concentrations of DAPT the migration of EVTs increases up to 1.8 fold in the presence of 1µM of the γ-Secretase inhibitor. Similar to the results obtained with the trophoblastic cell line SGHPL-5, the primary EVTs have an enhanced migratory behaviour upon downregulation of the Notch signaling activity.

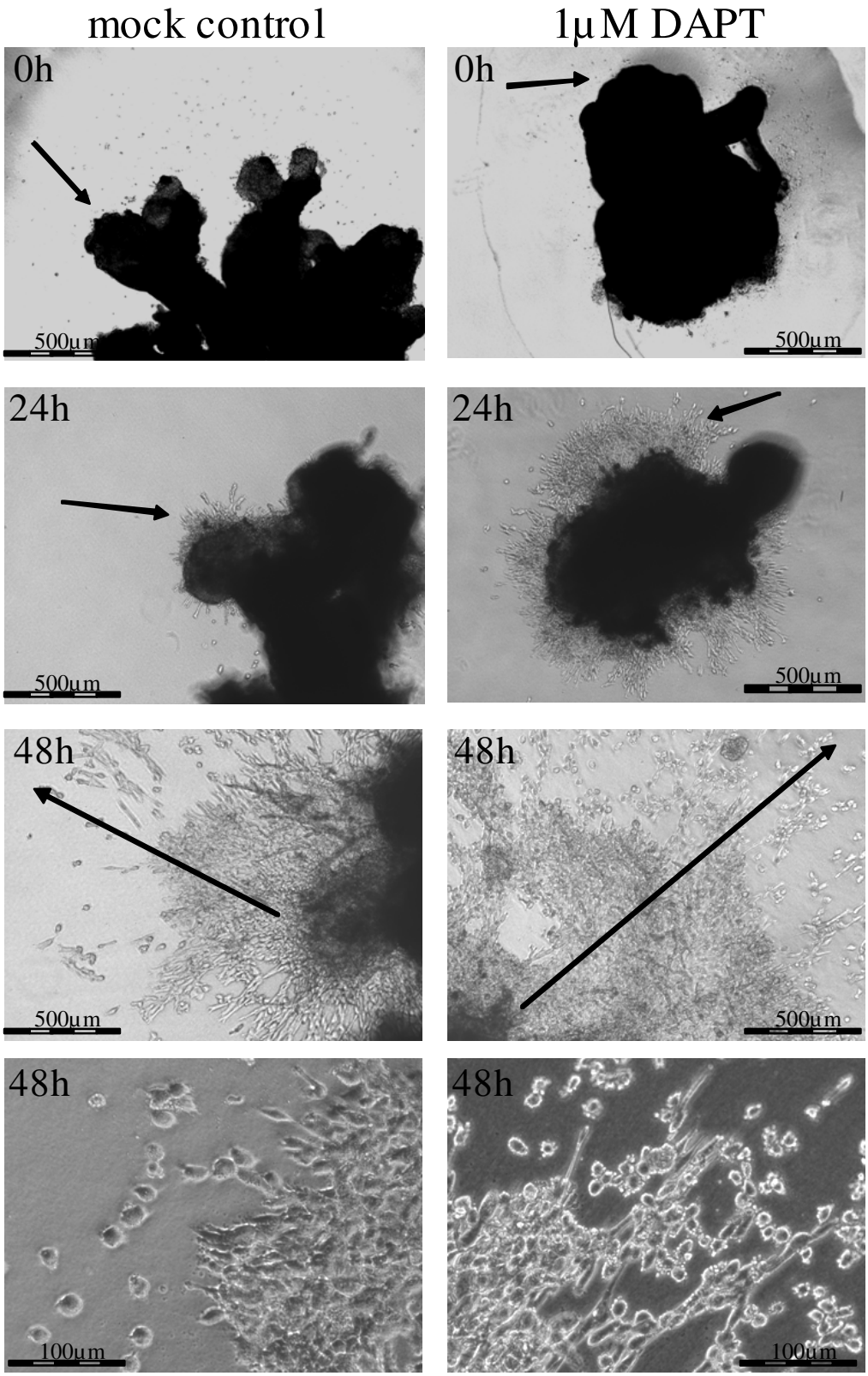


**Fig. 28A: Increased migration of explant-derived EVTs in the presence of DAPT**

Explants seeded on collagen were untreated or treated with 1µM DAPT were monitored and photographed at 0h, 24h and 48h. After 24 and 48h an enhanced EVT outgrowth could be observed. Higher magnifications after 48h shows also differences in the morphology of the EVT population to a more migratory phenotype compared to the mock control.

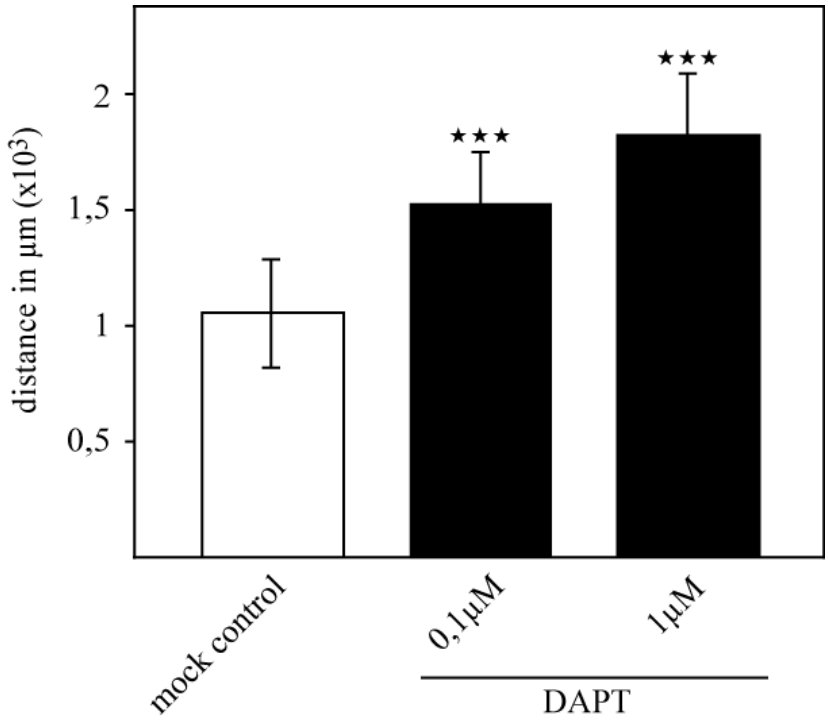


**Fig. 28B: Rising concentrations of DAPT increase EVT migration**
After 48h of incubation the EVT outgrowth was verified. In the presence of 0.1µM and 1µM DAPT migration was significantly enhanced 1.5 and 1.8 fold, respectively. The distance represents the most distal EVTs and is measured in µm from the villous tissue. Mean values ± SD of three experiments performed with 12 explants each condition are shown. ***, $P < 0.001$

## 3.4. Coculture experiments of EVTs and decidual fibroblasts

Finally, I performed coculture experiments using isolated decidual fibroblasts and explant-derived extravillous trophoblasts. The aim was to test behaviour of EVTs when reaching the fibroblast layer and further to verify the expression of Notch2 since this receptor was expressed in distal EVTs. Fig. 29 shows phase contrast pictures of the coculture system (df=decidual fibroblasts, evt=extravillous trophoblasts). Picture A displays the fibroblast layer (df) with a part of the villous tissue with the outgrowing extravillous trophoblasts (evt) nearly reaching the end of the collagen. Figure B shows the border, where the EVT population starts to contact the fibroblasts. Picture C and D finally show the EVTs overgrowing the fibroblasts.

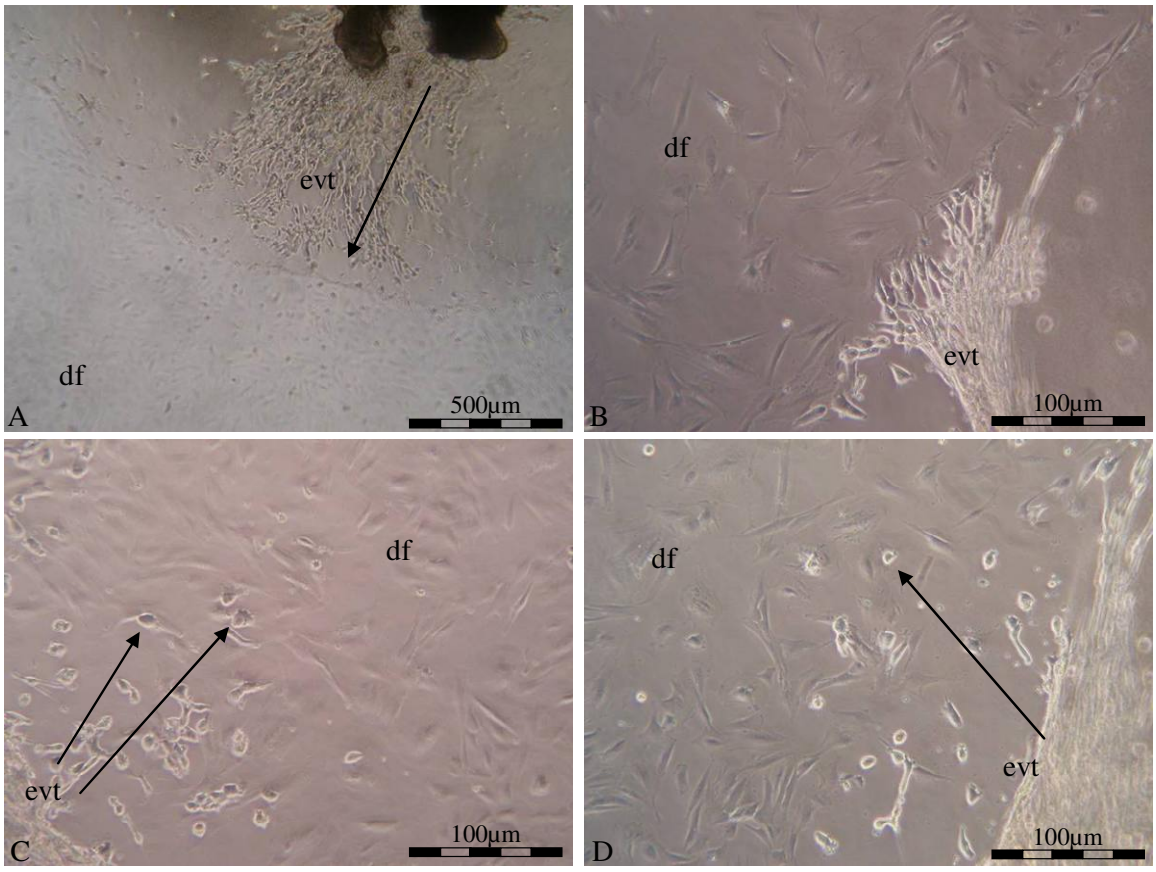


**Fig. 29: Coculture system between EVTs and decidual fibroblasts**
Picture A displays a 40 fold magnification of the fibroblast layer with the villous tissue on top of the collagen matrix with the outgrowing EVTs toward the fibroblasts. Higher magnifications of the contact points are shown in figures B-C.

The cocultures were stained with specific markers in order to identify the different cell types. The left picture of the first panel (Fig. 30) displays the co-staining with cytokeratin7 (EVTs, green) and vimentin (fibroblasts, red). The EVTs have got into contact with the decidual fibroblasts. The right picture shows the co-staining with α5β1 integrin (EVTs, green) together with vimentin (fibroblasts, red). The fibronectin receptor α5β1 is a marker for non-proliferative EVTs and clearly identifies this cell population in the coculture system. In the second and third row of pictures co-staining with Notch-2 and vimentin was performed. The pictures are taken at a 1000f magnification and show three cells, two are vimentin positive (red) and one has a strong Notch-2 expression (green). The Notch-2 positive cell is supposed to be an extravillous trophoblast since it lacks vimentin expression. This experiment has two interesting outcomes. First, the positive staining for Notch-2 in those differentiated EVT verifies the immunohistochemically obtained data; secondly, the fact that the in vitro grown EVTs express the same Notch receptor compared to the in vivo situation supports the idea that this model system might

mimic the in vivo situation correctly. However, the coculture system might be a helpful tool to study the Notch signaling not only in EVTs but also in decidual fibroblasts, which could give more insights into the communication and interaction between these two different cell types.

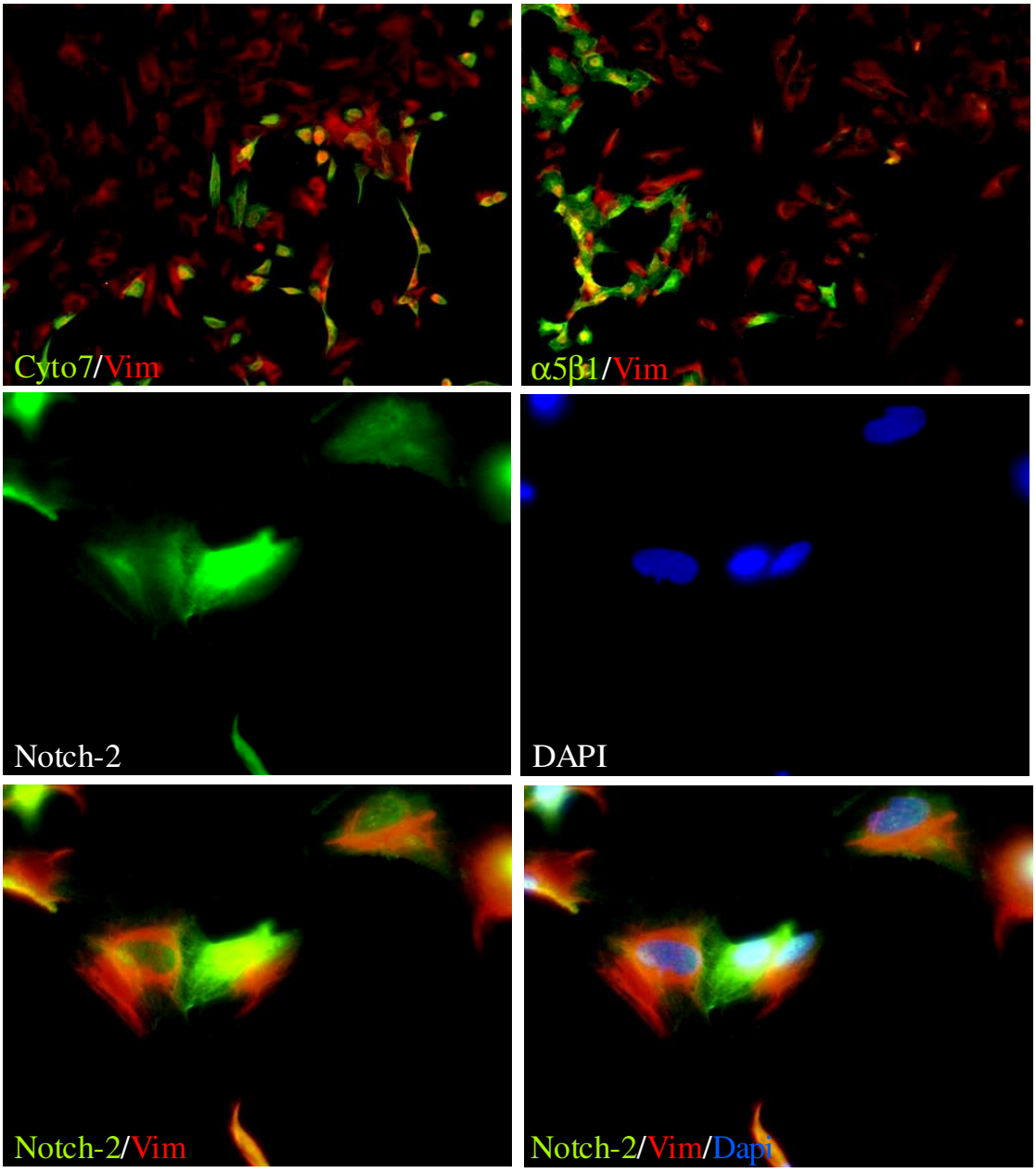


**Fig. 30: Immunocytochemistry of cocultures of decidual fibroblasts and EVTs**
The first row of pictures is photographed at a 100f magnification; the left picture shows the border between EVTs (green, cytokeratin7) and decidual fibroblasts (red, vimentin), the right picture represents the EVT expression of α5β1 integrin (green) in the coculture system with fibroblasts (red, vimentin); The second and third panels show the expression of Notch-2 in EVTs (green), the corresponding nuclei staining in blue and the overlay pictures. Note, that Notch-2 is strongly expressed in the cell, that has no vimentin expression and is therefore supposed to be an EVT

# 4. Discussion

The Notch signaling pathway plays important roles in cell biology such as cell proliferation, differentiation and apoptosis. Since there is very little known about the Notch pathway in the human placenta, this work is focused on the expression profiles, activity studies and functional experiments.

## 4.1. Expression of the Notch receptors and ligands

The immunohistochemical expression of Notch receptors and ligands are summarized in Fig. 31. Regarding the expression of Notch family members in the human placenta, only little information could be excerpted from literature. Cobellis and others have shown expression data of Notch1, Notch4 and Jagged-1 in human preeclamptic and age matched healthy term placentas finding a wide-spread appearance of all three members in the placental cell types with changes in preeclamptic samples (Cobellis et al., 2007; De Falco et al., 2007). The RT-PCR data (Fig. 10) have first revealed the occurrence of nearly all Notch receptors and ligands in the examined tissue and cell preparations. In the next paragraph I want to discuss the expression match between the RT-PCR and immunohistochemistry data since some of them seem to be slightly discordant.

*Notch Receptor 1:* The mRNA of Notch1 (Fig. 10) is strongly detected in first trimester cytotrophoblasts ($1^{st}$CTBs), weaker in $3^{rd}$ trimester cytotrophoblasts and can be found in villous fibroblasts and the total tissue preparations of the first and third trimester and to some extent in the SGHPL-5 cells. The immunohistochemical detection was restricted to the trophoblasts of the proximal cell column (Fig. 11) and to the villous stroma (Fig. 12). This difference between the $1^{st}$CTBs and cell column expression between mRNA and protein could be possibly explained by the seeding method of $1^{st}$ trimester CTBs. After trophoblast isolation, the cells are seeded on an extracellular matrix (Matrigel$^{R}$) that rapidly induces the differentiation of the CTBs to EVTs. Although the mRNA of $1^{st}$CTBs is isolated already 16h after seeding, this incubation time could be enough for the beginning differentiation into EVTs thereby gaining some characteristics of the proximal cell column trophoblasts that show a strong Notch1 protein expression. However, the staining of proliferative and differentiated cells on parallel slides could show that the expression of this particular receptor does not correlate with the cell cycle. Hence, the upregulation of the receptor in this particular area could have a function in cell column

formation. The question which signal or intrinsic program initiates cell column formation still remains to be elucidated. Two putative theories of bipolar CTBs or differently committed CTBs have been compiled (Baczyk et al., 2006; James et al., 2005). Regarding the Notch-1 expression profile, Notch signaling might be involved in the determination towards cell column formation. The villous stromal expression of Notch1 protein is reflected by the mRNA expression of villous fibroblasts and is also reported by others (De Falco et al., 2007) but the total lack of Notch1 in the villous vessels and their surrounding muscle cells (Fig. 12) was not described by others and needs to be further elucidated.

*Notch Receptor 2:* The mRNA of Notch2 (Fig. 10) could be found in all placental cell types and tissue preparations except the villous fibroblasts. This expression profile is reflected by the immunohistochemical data (Fig. 13) but the Notch2 protein was also expressed in villous stromal cells suggesting that the isolated fibroblasts somehow lost the requirement for Notch2 in culture. A damage of the Notch2 RNA could also be a reason for the lack of mRNA detection in PCR analyses. Additionally, Notch-2 was the only Notch receptor that could be found in the syncytiotrophoblast layer. However, the localisation of Notch2 was not regulated in any way regardless of the cell type and differentiation state. Interestingly, the trophoblastic cell line had a strong mRNA expression of Notch2 concluding that this cell line predominantly uses this particular receptor during Notch signaling.

*Notch Receptor 3:* The mRNA of Notch3 (Fig. 10) was only found in 1st and 3rd trimester cytotrophoblasts. The staining pattern produced a slightly different pattern. The Notch3 protein was detected in villous stromal cells with a strong staining of villous vessels, their surrounding muscle cells and foetal blood cells (Fig. 14). Additionally, the protein was found in cytotrophoblasts and cell column trophoblasts with a decline along with the distance from the villous tip. Once more, the extraction possibilities of cDNA pools for the mRNA detection limit the consistence of RNA and protein data. The proximal part of the cell columns can hardly be isolated but might at least be partially reflected by the 1st CTBs that seems to be a mixture between 1st cytotrophoblasts and proximal cell column trophoblasts (EVT population) due to the seeding feasibility. Therefore, the mRNA signal in 1$^{st}$ CTBs comes from both, the CTBs and proximal EVTs. The EVT pool used for RT-PCR is gained after the culture of villous explants and reflects

very distal EVTs that are fully differentiated. This might explain, why there was no Notch1 and Notch3 mRNA expression in this particular cell population. The lack of any mRNA signal in villous fibroblasts could be again explained be the loss of receptor expression due to the culture conditions. Damaged RNA in the fibroblast pool could also be a reason for failed Notch3 mRNA expression.

*Notch Receptor 4:* The mRNA of Notch4 (Fig. 10) could be found in 1st CTBs, tissue preparations of 1st and 3rd trimester, in villi obtained from explants and very weakly in 3rd trimester CTBs. This finding correlates to the expression of the Notch4 protein (Fig. 15) that was detected weakly in villous CTBs and very strongly in villous endothelial cells and foetal blood cells.

*Jagged-1:* The mRNA of Jagged-1 (Fig. 10) could be found in all placental cell types and tissue preparations except the decidual fibroblasts. The protein expression and localisation (Fig.16) shows a similar pattern with an interesting protein expression profile in syncytiotrophoblasts (Fig. 17). The Jagged-1 protein could be detected in CTBs and cell column EVTs with a decrease along with the distance to the villous. Furthermore, those syncytia that had contact points to other syncytial layers had a very strong Jagged-1 protein expression at the apical surface. This unprecedented staining pattern offers room for many speculations including a regulatory role in the syncytialisation process, the communication between syncytiotrophoblasts of different villi and possible mechanisms for repair machineries. The placental architecture has, similar to colon villi, the advantage, providing an enormous large surface important for the exchange of nutrients and gases between the mother and the foetus. The regulated expression of Jagged-1 could therefore be a mechanism to maintain the villous structure by inhibiting auto-fusion of different syncytial layers with each other since this would result in the reduction of surface area leading finally to a reduced exchange rates and malnutrition of the foetus. In fact, researchers of another field found out that Jagged-1 transduced stroma strongly inhibited the fusion of mononucleated myoblasts into multinucleated myotubes (Jaleco et al., 2001). The signaling could then presumably be transduced by Notch-2 since this receptor is the only one expressed in the syncytial epithelium. However, this finding should be further investigated regarding the intracellular domain of Notch2 and the Notch activity in syncytial layers that have contact to each other.

*Jagged-2:* The mRNA of Jagged-2 (Fig. 10) could be found in 1st and 3rd CTBs, in the tissue preparations and firstly, it was strongly expressed in EVT derived from villous explants that represent the most distal, non-proliferative and fully differentiated proportion of this cell type. Furthermore, compared to all the other Jagged-2 mRNA expression profiles, these cells expressed the mRNA of both splice variants in equal amounts suggesting that it might play a distinct role in distal EVTs. The mRNA expression correlated strongly with the protein expression and localisation (Fig. 18). Interestingly, the protein expression pattern changed from a uniform membranous staining found in the proximal cell column EVTs to a patchy but strong perinuclear expression profile in distal EVTs supporting the idea that the latter reflects the second splice variant found in mRNA studies. However, the expression of this ligand might play a role in the communication between decidual cells and trophoblasts since the mRNA and protein was expressed in distal EVTs and, additionally, seems to be differentially regulated.

*DLL-1:* The mRNA of DLL-1 (Fig. 10) could only be found in 1st and weakly in 3rd CTBs and in the villous tissues of explant cultures. The protein expression profile shows a fully different expression profile suggesting that the RT-PCR should be improved for this particular ligand. The protein was expressed throughout the placenta with a weaker expression in syncytiotrophoblasts and distal EVTs (Fig.19).

*DLL-3:* The mRNA of DLL-3 (Fig. 10) was nearly not detectable at all but the immunohistochemical protein detection revealed the DLL-3 protein expression on the apical surface of the syncytiotrophoblast layer and very distal EVTs. Similar to the RT-PCR data obtained for DLL-1, the conditions for DLL-3 RT-PCR should also be improved. This ligand was the first, that shows some correlation to the EVT cell cycle exit since the areas of EVTs that express the ligand do overlap with the EVT parts that are widely negative for KI67 (proliferative cells) and positive for Kip2p57 (differentiated cells) (Fig. 20).

*DLL-4:* The mRNA of DLL-4 (Fig. 10) was found in 1st and 3rd CTBs, decidual fibroblasts and the tissue preparations. These results perfectly correlate with the immunohistochemically obtained protein expression pattern where DLL-4 was expressed in villous stromal cells, cytotrophoblasts and the cell column EVTs with a decline along

with the distance from the villous tip (Fig. 21). The DLL-4 mRNA was absent in villous fibroblasts which could be explained be the different behaviour of cells in vivo and in vitro. Beside Notch-2, the ligand DLL-4 was the only one, whose mRNA was expressed in decidual fibroblast. I do not have any protein expression data of decidual cells since those are not present in the tissue pools of the first trimester placentae. However, these two Notch members might play a role in distal EVTs and decidual cells suggesting the investigation of Notch-2 and DLL-4 in coculture models with decidual cells and EVTs.

Figure 29 shows a summary of the protein expression profile of the four Notch receptors and five Notch ligands. Regarding this summary Notch receptors are largely expressed in the proximal part of the placenta and could therefore play a role within the trophoblast population hence regulating the differentiation and invasion processes. However, the ligands have a steady expression all over the placenta supporting the idea that these could also be responsible for the communication and interaction with uterine cell types. Notch-2 and DLL-1 were expressed in all cell types with no regulatory expression changes. Notch-3, Jagged-1, Jagged-2 and DLL-4 were expressed in villous stromal cells, in cytotrophoblasts and cell column extravillous trophoblasts with an expression decline along with the distance to the villous tip. Notch-1 protein expression was only expressed in the proximal first few rows of cell column EVTs and the villous stroma, Notch-4 had a weak cytotrophoblast and a strong endothelial expression, which was also found by others (Uyttendaele et al., 1996) and DLL-3 showed expression in syncytium and distal EVTs.

Taken together, one of the most promising receptors in this expression profile seems to be Notch-1 suggesting a regulatory role in cell column formation and/or maintenance of the EVT progenitor pool. Among the ligands, Jagged-1 is upregulated in contact sites of syncytial surfaces supporting the idea that it could influence syncytialization processes. Finally, the ligands Jagged-2 and DLL-3 show an enhanced expression in distal EVTs thereby potentially displaying a possibility of interaction between foetal EVTs and maternal decidual cell. Along their invasion into the maternal decidua and myometrium the EVTs get in contact with many different cell types including decidual fibroblasts, vessel associated smooth muscle cells, endothelial cells of spiral arteries, natural killer cells and others. The communication with each cell type might have different effects on the EVT behaviour and vice versa. The contact and communication between decidual fibroblast and EVTs, for example, could control the invasive depth of trophoblasts.

Enhanced trophoblast invasion is associated with pregnancy disorders including partial and complete mole placentae and chorion carcinomas. On the other hand, the reduced trophoblast invasion would result in pregnancy complications such as pre-eclampsia (Fig. 4) and IUGR.

It is also well known, that EVTs lead to vascular remodelling of the maternal spiral arteries by eliciting apoptosis in smooth muscle cells and endothelial cells (Ashton et al., 2005; Cartwright et al., 2002). This process that could also be supported by signaling via the Notch cascade since Notch induces apoptosis in neural progenitor cells (Yang et al., 2004). Summarizing the expression data, the multiplicity and locally restricted expression of the Notch receptors and ligands in the human placenta supports the idea that among others, Notch activity might play an important role in placental differentiation and regulatory processes that provides physiological adaptation of the placenta to the foetus' demand along the pregnancy.

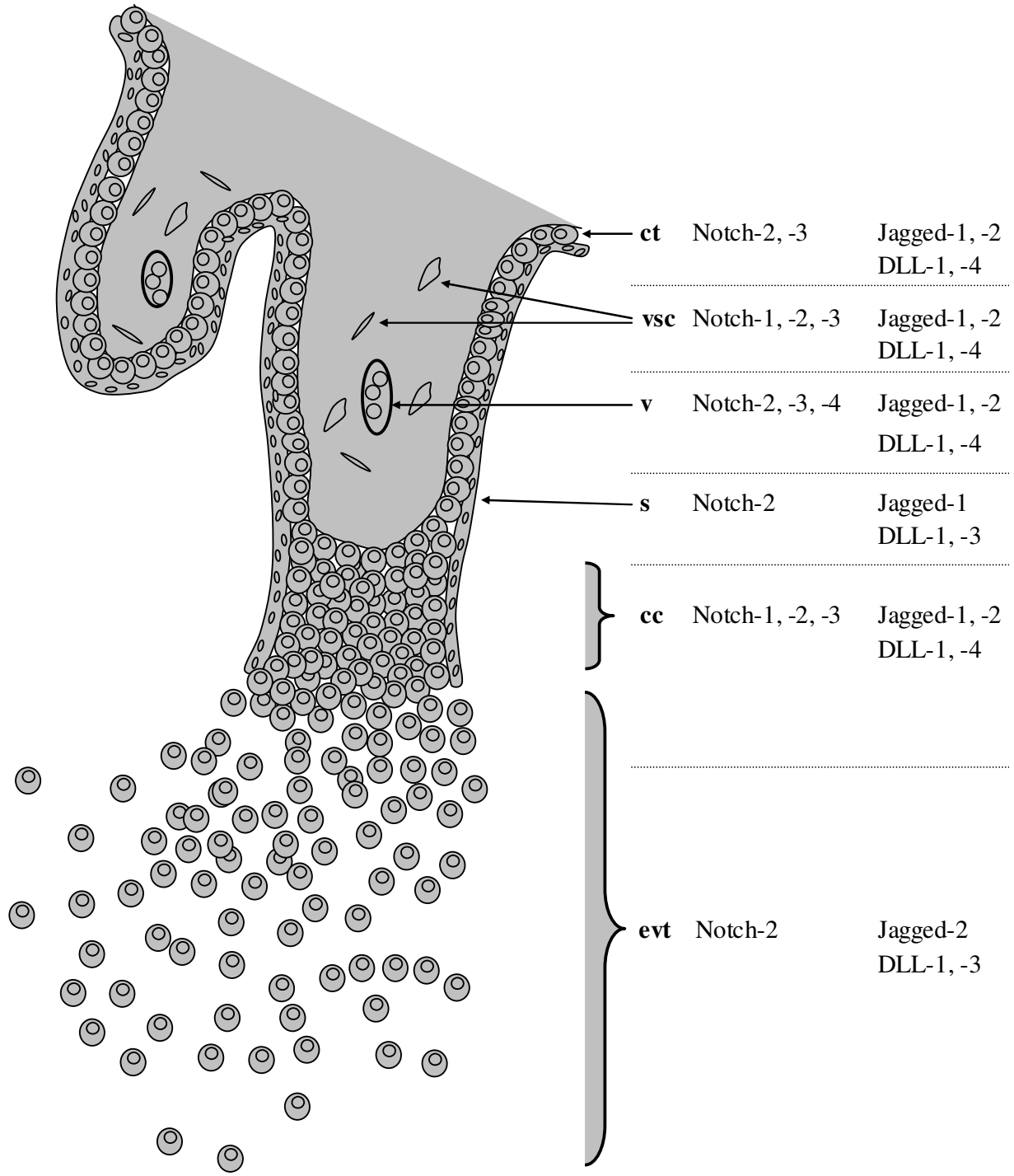


**Fig. 31: Overview of the Notch receptor and ligand expression profile**

The CTBs express Notch2, Notch3, Jagged-1, Jagged-2, DLL-1 and DLL-4. Syncytiotrophoblasts express Notch2, Jagged-1, DLL-1 and DLL3; Villous stroma has all members except Notch4 and DLL-3; Fetal endothelial cells do not have Notch1 and DLL-3; Proximal cell column trophoblasts display all factors except Notch 4 and DLL-3 and distally, only Notch-2, Jagged-2, DLL-1 and DLL-3 are expressed

## 4.2. The Notch cascade activity in SGHPL-5

Transfection experiments using the extravillous trophoblastic cell line SGHPL-5 gave some insights into the Notch activity of this particular cell line. Fig. 32 gives an overview of the Notch pathway and the used tools to perform Notch activity experiments.

Two facts have proven the endogenous Notch signaling occurring between trophoblastic cells. First, the 2 fold induction of the luciferase activity of the wild type CBF1 Luciferase vector compared to the mutated CBF1 luciferase vector and, secondly, the reduction of the luciferase activity to basal levels using the γ-Secretase inhibitor DAPT (Fig. 22). The inhibitor specifically blocks the γ-Secretase activity whereupon the Notch intracellular domain cannot be cleaved from the cytoplasmic membrane and Notch signaling is inhibited. Furthermore, overexpression of the NICD resulted in another 2.5 fold increase of luciferase activity demonstrating that the endogenous Notch activity has not reached the top levels and can be further induced by exogenous stimuli.

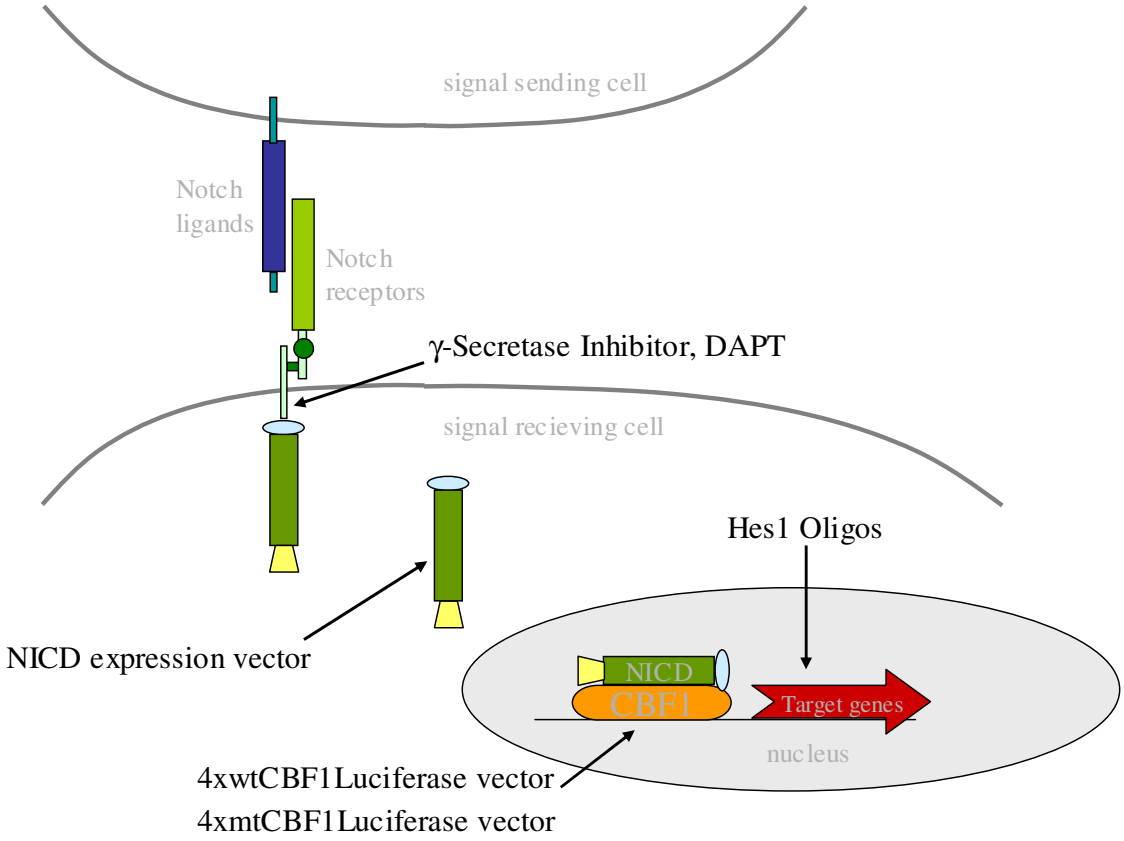


**Fig. 32: Schematic display of used tools in Notch activity**
The 4wtCBF1Luciferase and 4mtCBF1Luciferase vectors carrying 4 copies of the CBF1 binding site have been used to detect endogenous Notch activity. γ-Secretase inhibitor DAPT blocked cleavage of the Notch intracellular domain from the cytoplasmic membrane thereby blocking Notch signaling. NICD overexpression induced Notch signaling and Hes1 oligos were used to detect the Notch target gene expression.

In control experiments, the specificity of the overexpressed NICD was verified using specific antibodies against the NICD-V (valine-cleaved Notch Intracellular Domain) and against the Flag tag (Fig. 23) confirming that the enhanced luciferase activity is stimulated by NICD overexpression. Furthermore, enhanced expression of Hes1 mRNA (Fig. 24) finally proved not only the activated Notch signaling due to transfected vectors but also revealed the cellular reactions due to the NICD stimulus. Final experiments with different cell densities confirmed that the confluence of the cells does not influence Notch activity at least in the range of 60-95% of confluency.

These experiments using the extravillous trophoblastic cell line confirmed Notch activity in trophoblasts and support the idea that extravillous trophoblasts in vivo could exhibit a similar behaviour. Comparing the Notch receptor and ligand mRNA expression profile of the SGHPL-5 cells with CTBs suggests that there is a high communication potential

present that possibly controls a diversity of regulatory mechanisms in primary trophoblasts.

In the next step I tested the ability of a different cell type to stimulate this particular pathway. Since EVTs contact decidual cells during the invasion process, I used fibroblasts isolated from maternal decidua in combination with SGHPL-5 and observed, that these cells were also able to stimulate Notch activity in trophoblast cells up to 2 fold measured from the endogenous activity level. This value nearly reached the luciferase activity levels obtained after overexpression of the NICD suggesting that decidual fibroblasts could provoke a maximal stimulation of Notch activity.

These data gave some insights into the communication between trophoblasts and fibroblasts strongly supporting the idea that EVTs might cross-talk with decidual fibroblasts via the Notch signaling pathway. Considering the expression data of the Notch members in EVTs, only Notch-2 could be responsible for eliciting Notch activity in EVTs. With respect to the expression profile of the ligands in EVTs, it is possible that EVTs might activate the Notch signaling in decidual cells (Fig. 33). This could control proliferation and/or differentiation of decidual cells. Alternatively, activation of Notch activity could stimulate decidual fibroblasts to produce factors that in turn influence EVT invasion and differentiation. To confirm these speculations, transfection experiments of fibroblasts with a subsequent stimulation with EVTs should be performed. Furthermore, several receptors should be knocked out to verify if there are some candidates with unique regulatory features controlling EVT differentiation and invasion.

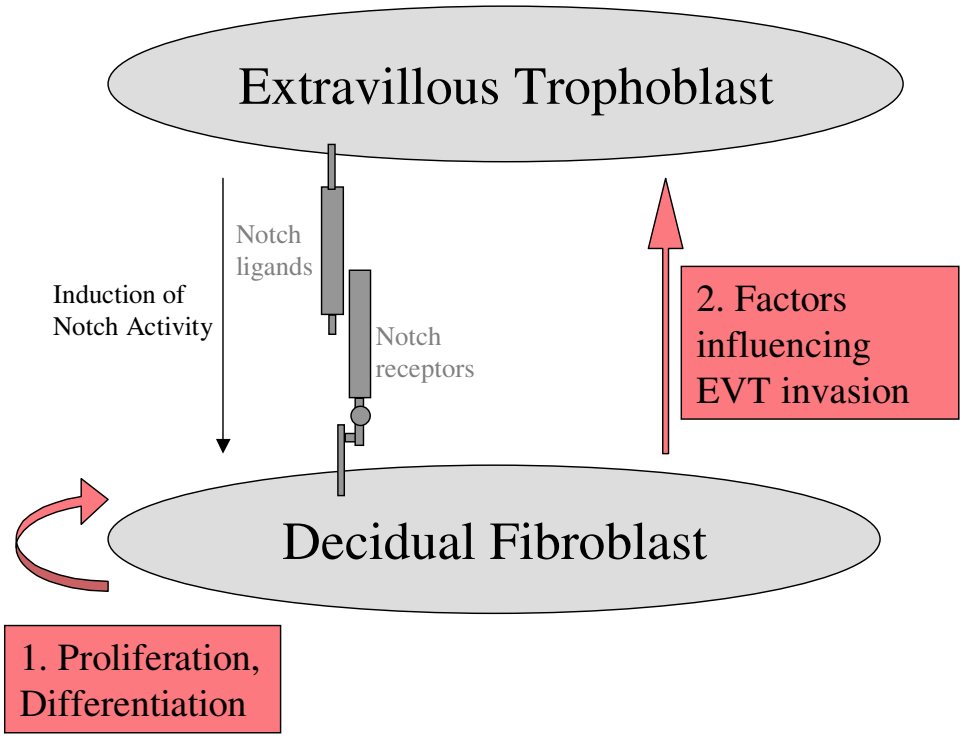


**Fig. 33: Model for the interaction between EVTs and decidual fibroblasts**
The Model displays a possible communication way of the EVTs and decidual fibroblasts where the Notch signaling is induced in the fibroblast population by EVTs whereupon 2 possible effects are shown. First, EVT control decidual fibroblast proliferation and/or differentiation. Second, decidual fibroblasts produce and secrete factors that influence the trophoblast invasion and differentiation

## 4.3. Notch activity influences trophoblast motility

In this set of experiments I tested the influence of the Notch signaling cascade on trophoblast function. First, I performed migration assays with the extravillous trophoblastic cell line SGHPL-5 in the presence or absence of rising concentrations of γ–Secretase inhibitor DAPT. In fact, migration was induced upon inhibition of the Notch pathway in a dose-dependent manner suggesting that active Notch signaling has an anti-migratory effect on SGHPL-5 cells. Further studies using explant cultures showed a similar effect although the dosis of DAPT had to be reduced. Regarding EVT outgrowth from day to day, the first difference was already visible at 24h after seeding the explants on top of the collagen I matrix. The outgrowth area has nearly doubled compared to the DMSO control explants. After 48h, the different migration distances were still clearly detectable. Not only the distance of EVTs from the villus tip was twice as big as in control explants but also the morphology of EVTs has changed to a more elongated, invasive shape. Additionally, the whole EVT population seems to have a loosened contact to each other. These data leaves room for many speculations since most of the data

obtained from literature report a pro-migratory phenotype upon Notch activation (Wang et al., 2006; Wang et al.). In contrast, the inhibition of Notch signaling leads to an increase of migration in my experiments.

However, these contrary results could be explained by the fact that most of the other studies are performed with tumor cells and we do observe the behaviour in a physiological cell population that has strictly regulated invasive characteristics. The question which role the active Notch pathway might have in the placental tissue needs to be further adressed. Since downregulated Notch activity has a pro-migratory effect, the active Notch signaling might influence events that support the cell-to-cell contact or maintain proliferation in EVTs.

## 4.4. Coculture experiments of EVTs and decidual fibroblasts

Finally, I used culture conditions where a confluent layer of fibroblasts was confronted with villous explant-derived EVTs (Fig. 29, 30). Phase contrast microscopy showed that the differentiated EVTs left the collagen drop and migrated towards the fibroblasts thereby contacting them (Fig. 29). Immunocytochemical detection of cytokeratin and vimentin clearly distinguished between EVTs and decidual fibroblasts (Fig. 30). The staining against the $\alpha 5\beta 1$ fibronectin receptor further proved the differentiation state of EVTs.

Most promising for all future experiments is the combination of decidual fibroblasts and differentiated EVTs in vitro since this particular approach perfectly mimics the in vivo situation and could finally give some insights into their mutual influence. Further experimental approaches using smooth muscle cells or endothelial cells instead of fibroblasts could further clarify the questions how the EVTs might interact with those cell types thereby helping to clarify the mechanisms of spiral artery remodelling.

Even more interestingly, Notch-2 detections revealed that vimentin negative cells were positive for Notch receptor 2. This finding verified that Notch-2 expression in vitro was similar to the one in vivo, which is important for all further in vitro experiment studying Notch communication between EVTs and fibroblasts or other cell types. Regarding PCR data (Fig. 10), DLL-4 seems to be expressed moderately in decidual fibroblasts and its expression should be tested in co-culture experiments. Further approaches using the γ-

Secretase inhibitor DAPT should be performed to control EVT and fibroblast behaviour upon Notch cascade inhibition.

# 5. Future Aspects

Since some of Notch proteins display a very promising expression pattern, an approach with floating explants and DAPT should be performed to gain some insights into the importance of individual receptors. The proliferation and differentiation behaviour should be investigated in detail by the means of cell column formation and EVT differentiation. Since some of the Notch members are also specifically expressed in the syncytial layer, syncytialization processes could be investigated with the help of two different methods (Leisser et al., 2006). The resyncytialisation after a denudation step of villous explants could be compared between DAPT treated and untreated floating explants. Additionally, isolated cytotrophoblasts tend to syncytialize under certain conditions, which could also be tested in the presence or absence of DAPT. Furthermore, the proliferation rate should be measured in villous explants and cell cultures in the presence and absence of the γ-Secretase inhibitor. In contrast to the inhibition studies, experiments based on Notch activation using soluble or membrane bound ligands should be performed. The co-culture model system should be further developed using different cell types, including trophoblasts, fibroblasts, smooth muscle cells, endothelial cells and NK cells thereby testing the communication potential of different placental cell types. Transfection experiments using fibroblasts and their Notch cascade activation potential due to EVT addition should be analysed. Furthermore, knock down experiments targeting several receptors and ligands should be included to verify if there are some candidates with unique regulatory features controlling EVT differentiation and invasion. The Notch pathway may control the proliferation and differentiation of placental cell types in many ways supporting the idea of the importance of this particular pathway in human placental development and function.

# 6. Materials and Methods

## 6.1. Cell Culture

### 6.1.1. Tissue collection

Placental tissues of uncomplicated early pregnancies (between 6th and 12th week of gestation) were obtained by evacuation from legal abortions, with the permission of the ethical committee of the Medical University of Vienna. Informed consent of patients was obtained. The tissue was washed with ice-cold PBS (phosphate buffered saline) and further processed according to the following experimental demands.

### 6.1.2. Cell Culture of SGHPL-5

The immortalized trophoblastic cell line SGHPL-5 exhibit features of EVTs and behave similarly as primary trophoblasts with respect to invasion and vascular remodelling (Cartwright et al., 2002; Harris et al., 2006). They were cultivated in DMEM/Ham's F-12 (1:1), supplemented with 10% foetal growth serum (FBS) and gentamycin, under standardized conditions in a humidified chamber at 37°C, 5% CO2 and 21% O2 until they reached passage 15. Splitting of the cells were performed using 0.8ml 0.25%Trypsin/EDTA per 10cm culture dish for 3min at 37°C. The reaction was stopped with culture medium.

### 6.1.3. Purification and cultivation of first trimester cytotrophoblasts, villous and decidual fibroblasts

Cytotrophoblasts of first trimester placentae were isolated by enzymatic digestion and density gradient centrifugation as described previously (Kliman et al., 1986; Knofler et al., 2004). Briefly, tissue was digested two times (30 min each) in Hanks′ balanced salt solution containing 25 mM HEPES, 0.125% trypsin and 250 IU/ml DNase I in a shaking water bath (37°C). After each digestion step, the supernatant was removed and neutralized with foetal bovine serum to a final concentration of 10%. The supernatants were pooled and filtered over a nylon sieve with the pore size of 80µm. Cells were then fractionated on a 5–70% discontinuous Percoll gradient. Trophoblast cells were isolated from the middle layer of the gradient (density of 1.048–1.062 g/ml). After centrifugation,

cells were immunopurified by depleting contaminating human leukocyte antigen (HLA)-I positive cells with anti-human HLA (clone W6/32; 0.2 µg/106 cells) conjugated to anti-mouse immunoglobulin G (IgG) magnetic beads (Dynal, Oslo, Norway). Pure trophoblasts (>98% cytokeratin-7-positive cells) were seeded on rat-tail collagen I coated dishes at a density of $5 \times 10^5$ cells/cm2 and cultivated in DMEM/HamF12 containing 10% FCS gold and gentamycin.

Villous fibroblasts of different first trimester placentae were isolated after gradient centrifugation of trypsinized placental material (between 25% and 35% Percoll) and passaged two times in DMEM/HamF12 supplemented with 10% FCS gold and gentamycin.

Decidual fibroblasts were gained by enzymatic digestion as described (Selam et al., 2002). First trimester decidua was washed with ice-cold PBS and minced into $2mm^3$ pieces. Digestion was performed in DMEM/HamF-12 containing 2mg/ml Collagenase I (484IU/ml) and 0,5mg/ml DNAse I. 10g of tissue per 10ml digestion solution was incubated in a shaking water bath at 37°C for 45 minutes. The reaction was stopped with 10 % FBS and the supernatant was filtered through an 80mm nylon sieve to remove undigested material. A second digestion step was performed with the remaining tissue for another 30 minutes. Both supernatants were centrifuged for 10 minutes at 1500 rpm and cells were pooled. After two washing steps with 1x HANKS balanced solution the cells were resuspended with prewarmed DMEM/HamF12 (1:1) supplemented with 10%FBS gold and gentamycin, and cultivated under standard conditions. The following day, the cells were splitted and further cultivated until they reached passage 8. All experiments were performed between passage 3 to 5.

### 6.1.4. First trimester villous explant culture

Villous placental explants were grown in DMEM/HamF12 containing gentamycin unless otherwise noted. Placental tissues were processed as described elsewhere with minor modifications (Bauer et al., 2004; Genbacev et al., 1992; Vicovac et al., 1995). Villous pieces were cut under the stereo-microscope (20fold magnification) and put into pre-warmed culture medium. The pieces were incubated at 37°C under standard culture conditions over night. On the next day villous explants were seeded on top of rat-tail collagen I (Fig. 30). After 4h, the villous explants were covered with serumfree culture medium and experiments were performed as described.

During the first 12-24h in culture, the cytotrophoblasts proliferate and form a cell column. Then, EVTs start to migrate on the surface of the collagen, which facilitates the monitoring of the migratory behaviour from day to day. Since the EVTs of the explant system undergo the same differentiation processes as they are observed from in vivo investigations (integrin switch, cell cycle exit), this cell culture model is a very useful tool to study the differentiation behaviour of the EVTs under different conditions.

The collagen droplets were prepared as following: rat-tail collagen I was first gently mixed with 10xDMEM. The transparent colour of the collagen switched to light yellow. A 7.5% sodium bicarbonate solution was added and mixed carefully to avoid any air bubbles, the colour switched to pink. Droplets were placed in the centre of 24well plates and left for 30 minutes at 37°C to polymerise. The drops were flooded with 0.5 ml culture medium and left for another 10 minutes at 37°C. Next, the medium was removed, one explant was placed in the centre of each drop and proceeded as described above. For experiments with the DAPT [N-(3,5-difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-ButylEster] (Calbiochem), explants were covered with culture medium either with or without different concentrations of DAPT or with an appropriate amount of DMSO (vehicle for DAPT; mock control). The outgrowth of the EVTs was observed day by day and every explant was photographed with an Olympus inverse X71 microscope directly after seeding, after 24h and 48h.

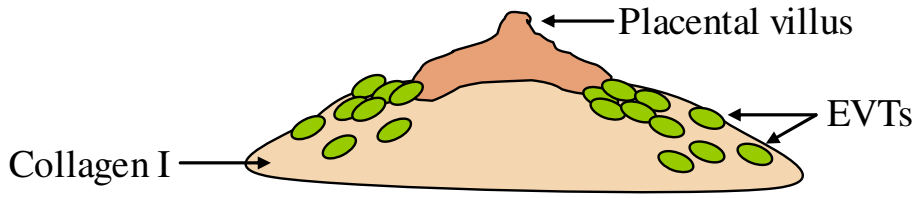


**Fig. 34: EVT outgrowth on rat tail collagen I**
Outgrowing extravillous trophoblasts proliferate and migrate on the surface of the collagen droplet thereby undergoing typical differentiation processes (integrin switch, cell cycle exit)

**Collage drops:**

1ml rat-tail collagen (3.7mg/ml)

0.1ml 10xDMEM

0.2ml 0.75% Sodium Bicarbonat solution

### 6.1.5. Coculture experiments of EVTs and decidual fibroblasts (Fig. 32)

The coculture model was performed on the basis of a method published by Cohen and colleagues with some modifications (Cohen and Bischof, 2009). Decidual fibroblasts (passage3) were grown in a 100mm culture dish until they reached confluency. Then the cells were washed with prewarmed PBS and the solution was removed. 12 collagen drops (the protocol is described in the 5.1.4.) were placed onto the fibroblast layer and incubated in the incubation chamber for 30min. Afterwards, the fibroblasts were covered with medium leaving collagen drops dry. The explants were placed in the centre of each drop and left for 4h in the incubation chamber for the first attachment. Subsequently, the cultures plates were filled with culture medium until the explant tissues were fully covered. The outgrowth of the EVTs was controlled every day and after 72h, the plates were carefully washed three times with ice-cold PBS and further processed for immunocytochemistry.

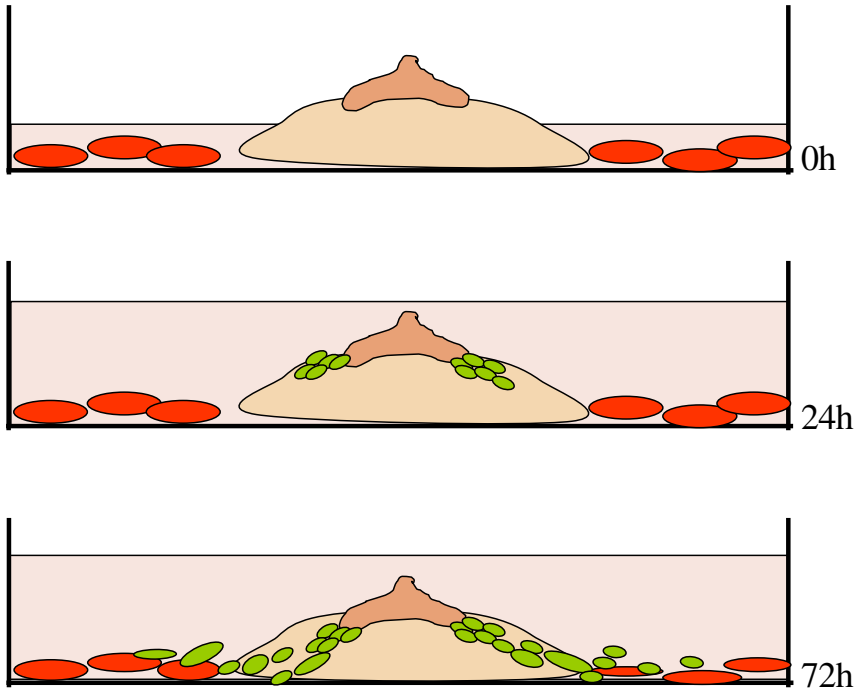


**Fig. 35: Coculture Model System**
The red cells (decidual fibroblasts, endothelial cells, smooth muscle cells or NK cells) form the confluent layer. The villous explants are seeded on top of the collagen drop and the EVTs (green) proliferate and migrate towards the cell layer until they have contact to the cells of the confluent layer. Subsequent staining with antibodies against the cleaved intracellular domain of the Notch members might confirm the active Notch signaling and the signal receiving cell.

### 6.1.6. Immunocytochemistry of 100mm culture dishes

The coculture dishes of villous explants and fibroblasts were covered with 4% Paraformaldehyd/PBS for 15minat RT, followed by a permeabilization step with 0.1% TritonX-100/PBS for another 5min at RT. After 3 washing steps with ice-cold PBS, the villous tissues were carefully removed with forceps. The individual coculture areas were surrounded with an ImmEdge$^{Tm}$ pen (Vector) to define the staining areas and the staining was performed according to the protocol described in 2.7. After the counter stain with DAPI, each area was mounted with mounting medium and covered with suitable cover plates. The staining was analysed using an inverse microscope (Olympus IX71) and representative areas were digitally photographed.

### 6.1.7. Migration assay

Transwell migrations assays were performed using transwell inserts (8µm pore size, Costar) with SGHPL-5 cells. 50.000 cells were seeded in the presence or absence of 1, 10, or 100 µM DAPT or MOCK (DMSO) in culture medium. After 24 hours of incubation under standardized conditions, the cells were washed with prewarmed PBS and fixed with ice-cold methanol for 10 min at room temperature. After 3 washing steps with cold PBS the nuclei were stained with DAPI (4',6-Diamidin-2'-phenylindoldihydrochlorid, 1:1000 in PBS) for 10min at RT. The cells on the upper side of the inserts were removed with a cotton swap. The filters were excised and mounted in mounting medium for fluorescence microscopy. For evaluation, five areas per filter were digitally photographed and the number of migrated cells was evaluated using the Olympus Cell Imaging Software.

## 6.2. RNA and Protein expression

### 6.2.1. RNA extraction and semi-quantitative RT-PCR

RNA and was extracted from frozen tissue samples (9.SSW, Villi and EVT) or cultured cells (cytotrophoblasts, villous and decidual fibroblasts, SGHPL-5) with TriFast Reagent (Peqlab) according to the manufacturer's instructions. Villi and EVT were mechanically separated under the microscope and further processed. The tissue samples were first reduced to powder. Briefly, tissues were frozen in liquid nitrogen and homogenized in a microdismembrator at 2000rpm for 1,5min. The resulting powder was put into TriFast

and the RNA isolation protocol was further processed. Quantity and Quality of RNA was evaluated using the Nanodrop elisa. 1µg of total RNA was reverse transcribed in 20µl reaction volume using 1µl MMLVSuperScript and 0.4µl Hexanucleotide Mix (62.5 A260U/ml) according to the manufacturer's instructions. Semi-quantitative PCR amplification was performed with PCR Reagent System in a RoboCycler Gradient 96 (Stratagene). Cycle numbers were optimized within the linear range of individual PCR reactions. Oligonucleotide primers, annealing temperatures, product sizes and cycle numbers are listed in table 3. In all experiments, possible DNA contamination was checked by negative control RT–PCR in which reverse transcriptase was omitted in the RT step. The PCR products were analysed on 1.5% agarose gels containing ethidium bromide and photographed under UV radiation.

| Name | Sequence 5'→ 3' | Start | Tm °C | Cycle # | size, bp |
|---|---|---|---|---|---|
| Notch-1 1s | TAC AAG TGC AAC TGC CTG CT | 2437 | 51 | 30 | 407 |
| Notch-1 1a | TCG TTG ATC TCC TCC TCA CA | 2843 | | | |
| Notch-2 1s | TGA ACA ACT GCT CCA GGA TG | 4921 | 53 | 27 | 401 |
| Notch-2 1a | TGA TGA CAA CAG CAA CAG CA | 5321 | | | |
| Notch-3 1s | GCC TGC CTC TAC GAC AAC TT | 4424 | 49 | 35 | 400 |
| Notch-3 1a | TTA CTA CCG AGC CGA TCA CC | 4823 | | | |
| Notch-4 1s | TGG ATG AGT GCC TGA GTG AC | 1908 | 52 | 40 | 406 |
| Notch-4 1a | GTG GGT CCT GTG TAG CCT GT | 2313 | | | |
| Jagged-1 1s | GTG GCT TGG ATC TGT TGC TT | 3751 | 50 | 27 | 395 |
| Jagged-1 1a | CTC TGG GCA CTT TCC AAG TC | 4145 | | | |
| Jagged-2 1s | ACA TCG ATG AGT GTG CTT CG | 1558 | 55 | 30 | 302/190 |
| Jagged-2 1a | CAC ACA CTG GTA CCC GTT CA | 1859 | | | |
| DLL-1 1s | AGA CGG AGA CCA TGA ACA AC | 2227 | 47 | 35 | 382 |
| DLL-1 1a | TCC TCG GAT ATG ACG TAC AC | 2608 | | | |
| DLL-3 1s | GAC CCT CAG CGC TAC CTT TT | 1517 | 48 | 35 | 703/365 |
| DLL-3 1a | CAC CAC CGA GCA AAT ACA AA | 2219 | | | |
| DLL-4 1s | TGA CCA CTT CGG CCA CTA TG | 896 | 50 | 40 | 620 |
| DLL-4 1a | AGT TGG AGC CGG TGA AGT TG | 1515 | | | |
| Hes-1 1s | GG CTG ATA ACA GCG GAA TC | 22 | 52 | 27 | 427 |
| Hes-1 1a | CGC GAG CTA TCT TTC TTC AG | 448 | | | |
| GapDH 1s | CCA TGG AGA AGG CTG GGG | 413 | 52 | 25 | 204 |
| GapDH 1a | CAA AGT TGT CAT GGA TGA CC | 607 | | | |

**Table 2: Oligo sequences and PCR conditions**
Names of the primer nucleotides, the sequences, the start and end points on the different mRNAs, the Annealing temperature (Tm), the PCR cycle number and the cDNA fragment sizes in base pairs (bp) are listed in table 3.

## 6.2.2. Western blot analyses

For protein detections, equal concentrations (normalized to RNA amounts) were loaded on 8.5% polyacrylamide (PAA) gels and blotted onto nitrocellulose membrane
For the detection of NICD expression the SGHPL-5 cells were lysed by freezing and thawing in the protein lyses buffer. The protein concentrations were verified by Bradford assay. 30µg protein lysate was mixed with 5xSDS-sample buffer, reduced with 1% β-mercaptoethanol, heated for 5min at 98°C and separated on a 10% SDS-PAA Gel. The proteins were then blotted onto nitrocellulose membrane (Protran, Schleicher/Schüll) overnight in protein transfer buffer with constant 20mV. Staining of the proteins with Ponceau'S solution controlled blotting outcome. After blocking with 5% non-fat milk in TBS-T for 1h at room temperature, the membranes were incubated with rabbit anti-human cleaved Notch1 intracellular domain (cell signaling, 1:1000 in BSA/TBS-T), rabbit anti human Flag (cell signaling, 1:1000 in BSA/TBS-T) and rabbit anti-human GAPDH (cell signaling, 1:5000 in BSA/TBS-T) overnight at 4°C. After 3 washing steps with TBS-T, secondary antibodies (goat anti rabbit, 1:50000) were diluted in 0.5% non-fat milk/TBS-T and added to the membranes for 1hour at RT. The detection was performed with Enhanced Chemiluminescence System (Amersham Pharmacia Biotech) and signals were visualized on autoradiography films.

**Solutions:**

Protein Lyses Buffer

20mM Hepes, pH 7.9
1% TritonX-100
0.4M NaCl
2.5% Glycerol
1mM EDTA
1mM PMSF
0.5mM DTT
0.5% Protease Inhibitor Cocktail

4xSeparating Gel Buffer, pH 8.8

1.5M Tris Base
0.4% SDS

4xStacking Gel Buffer, pH 6.8
0.5M Tris Base
0.4% SDS

10xSDS Page running buffer
0.4M Tris Base
1.92M Glycine
1% SDS

Ponceau S
0.5% Ponceau
5% acetic acid

10xTris-Buffered Saline (TBS), pH7.6
0.2M Tris Base
1,45M NaCl

Tris-buffered Saline (TBS-T)
1xTBS
0.1% Tween20

5x SDS sample buffer
100mM Tris HCl, pH 6.8
5% SDS
25% Glycerol
0.01% bromophenol blue

Protein transfer buffer, pH 8.3
25mM Tris Base
192mM Glycine
0.01% SDS
20% MeOH

BSA/TBS-T
5% BSA
TBS-T

## 6.2.3. Immunohistochemistry

First trimester tissue was fixed in 4.5 % formalin for at least 4h and embedded in paraffin. Serial sections (3µm) of paraffin-embedded first trimester placental tissues were prepared with a microtome as previously described (Bauer et al., 2004; Pollheimer et al., 2006). Slides were dried for 15min at 56°C, dewaxed in xylol for 10min and slowly rehydrated in decreasing concentrations of ethanol until Aqua destillata (A.d.) was reached. The antigen retrieval was performed in a suitable chamber using a 0.5% citraconic anhydride solution and heating the slides in a water bath at 98°C for 20min. Next, the slides were left for 20min at RT to cool down and were then washed with PBS twice. Then the slides were clamped with plastic cover slips (shandon) and placed into the staining chamber (shandon). Using this technique, the slides are permanently covered with an 80µl liquid film that can be replaced easily by pipetting another solution on top of the sections; the changing is performed by gravity flow. The volume of blocking and staining solutions could be reduced to 100 µl. Unspecific antibody reactions were minimized using 0.05% fish skin/PBS for 30minutes. The primary antibodies were incubated over night at 4°C. Subsequently a washing step was were performed with 100mM TrisCl (pH7.5), 50mM NaCl, 0.05%Tween (=washing buffer) by filling the gap between the slides and the cover plates with approximately 3ml. The secondary antibodies were added for 1 hour at RT and after an additionally washing step, the counterstain of the nuclei was done with DAPI (1:1000) for 10 min at RT. Finally, the slides were removed from the staining chamber and the sections were embedded with fluorescence fitting mounting medium and suitable cover plates. All used primary and secondary antibodies and their dilutions are listed in table 4. The sections were analysed by fluorescence microscopy (Olympus, BX50) and representative areas were digitally photographed.

**Solutions:**

Washing Buffer
0.1M TrisCl (pH7.5)
0.15M NaCl

0.05% Tween20

Antigen retrieval

0.05% citraconic anhydride (pH 7

10xPhosphate Buffered Saline (PBS), pH 7.4

125mM Na2HPO4

20mM KH2PO4

1.4M NaCl

Blocking buffer

0.05% fish skin / PBS

## 6.3. Transfection experiments

### 6.3.1. Plasmid preparation

The luciferase plasmids 4xwtCBFLuc-pGL2-Promoter (Fig. 32A) and 4xmtCBFLuc (Fig. 32B) und the eukaryotic transient expression plasmid pSG5-Flag-NICD (Fig. 32C) were kind gifts of S. Diane Hayward. Both, the wild type and mutant CBF1 binding sites were cloned into pGL2-Promoter Vector via Bgl-II, respectively. The NICD domain was cloned into the eukaryotic expression vector pSG5 (Stratagene). Two negative controls for the NICD vector were obtained. First, the NICD domain was removed by digestion with BglII and BamH1. Second, the whole fragment including the Flag and polyA tail were excised using EcoR1 and Xba1. Since both controls showed similar effects in Notch signaling pathway stimulation in transfection studies, the EcoR1/XbaI generated negative control was used for all further studies.

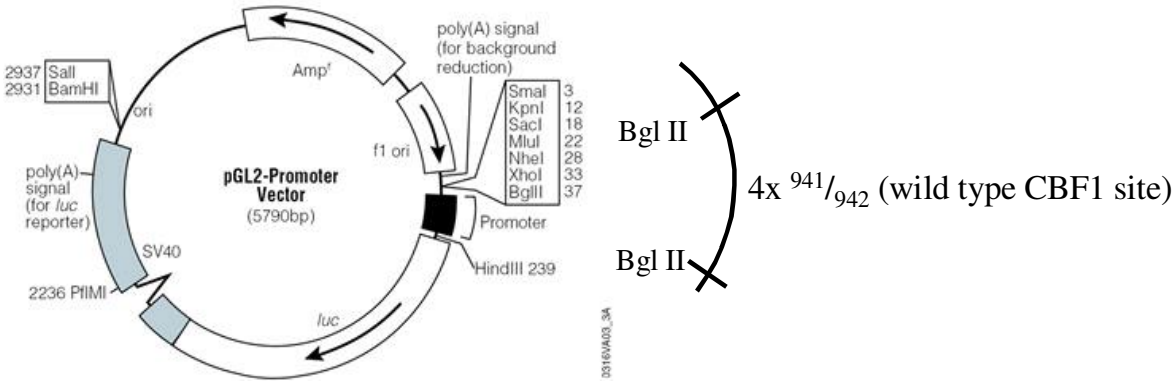


**Fig. 36A: 4xwtCBFLuc**

Four copies of the wild type CBF1 binding site are cloned into a pGL2 promoter vector via the Bgl-II restriction site.

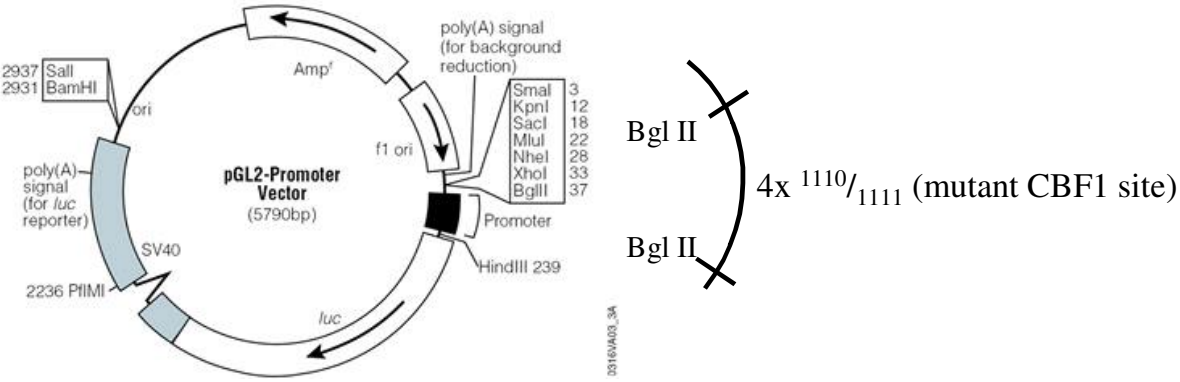


**Fig. 36B: 4xmtCBFLuc**

Four copies of a mutated variant of the CBF1 binding site are cloned into the pGL-2 promoter vector via the Bgl-II restriction site

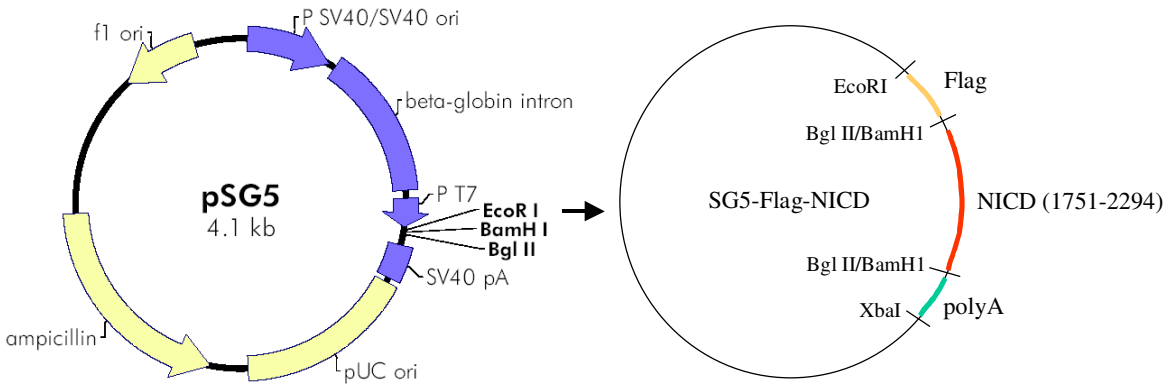


**Fig. 36C: SG5-Flag-NICD**

The notch 1 intracellular domain is cloned into the eukaryotic transient expression vector pSG5 via Bgl-II/BamH1; the mock control is generated excising the whole insert via EcoRI and XbaI.

## 6.3.2. Transfection of SGHPL-5

For reporter studies, SGHPL-5 cells were transiently transfected using Lipofectamine (Stratagene; 2µl each well) as described by the supplier. 50.000 cells were seeded into each well of a 24well plate. After 24h, the culture medium was changed and the cells were transfected. The concentrations of the plasmids, different combinations and stimulations are listed in table 5. Two parallel transfections per condition were performed. The transfection mixture was added to the cells and left for 6h under standardized culture conditions. After the incubation period, the medium was replaced with fresh, prewarmed culture medium with or without 10µM DAPT was added. After another hour, further stimulating agents were substituted (see table 5).

For stimulation with decidual fibroblasts (Fig. 33), I used SGHPL-5 (passage 7-9) and isolated decidual fibroblasts (passage 2-3). First, I prepared the transient transfection with the SGH-PL-5 cells according to the protocol described above. After 6h, I washed the cells and added the fibroblasts in two-fold excess to the SGHPL-5 cells.

After 18h of incubation the cells were either treated with Tri-Fast reagent for RNA preparation, protein lyses buffer for western blot analyses or 5xreporter lyses buffer (promega) for β-Galactosidase assay and luciferase activity assay.

| **Plasmids** | **concentrations** | **stimulations** | **analyses** |
|---|---|---|---|
| 4xwtCBF1Luc<br>pCMV-β-Gal | 0.75µg/well<br>0.25µg/well | +/- 10µM DAPT<br>+/- DMSO<br>+/- decidual fibroblasts/<br>+/- conditioned medium | β-Gal assay<br>Luciferase assay |
| 4xmtCBF1Luc<br>pCMV-β-Gal | 0.75µg/well<br>0.25µg/well | +/- 10µM DAPT<br>+/ DMSO<br>+/- decidual fibroblasts/<br>conditioned medium | β-Gal assay<br>Luciferase assay |
| 4xwtCBF1Luc<br>pSG5-Flag-NICD<br>pCMV-β-Gal | 0.75µg/well<br>0.75µg/well<br>0.25µg/well | | β-Gal assay<br>Luciferase assay |
| 4xwtCBF1Luc<br>pSG5-Flag-mock<br>pCMV-β-Gal | 0.75µg/well<br>0.75µg/well<br>0.25µg/well | | β-Gal assay<br>Luciferase assay |

| Plasmids | concentrations | stimulations | analyses |
|---|---|---|---|
| 4xmtCBF1Luc<br>pSG5-Flag-NICD<br>pCMV-β-Gal | 0.75µg/well<br>0.75µg/well<br>0.25µg/well | | β-Gal assay<br>Luciferase assay |
| 4xmtCBF1Luc<br>pSG5-Flag-mock<br>pCMV-β-Gal | 0.75µg/well<br>0.75µg/well<br>0.25µg/well | | β-Gal assay<br>Luciferase assay |
| pSG5-Flag-NICD<br>pCMV-β-Gal | 0.75µg/well<br>0.25µg/well | | RT-PCR<br>Western blot |
| pSG5-Flag-mock<br>pCMV-β-Gal | 0.75µg/well<br>0.25µg/well | | RT-PCR<br>Western blot |

**Table 3: different approaches used in transfection experiments**
The combinations and concentrations and the following analysis applications are given in table 4

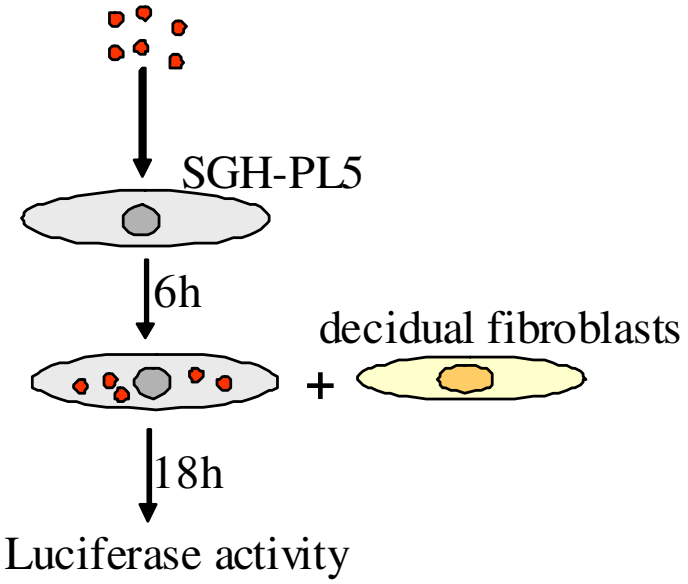


**Fig. 37: Transient transfection of SGHPL-5 following stimulation with decidual fibroblasts**
The SGHPL-5 were transiently transfected with either 4wtCBFLuc of 4mtCBFLuc for 6h. Then, the cells were washed and decidual fibroblasts were added in a ration of 1:2; the coculture was left for another 18h. Finally the cells were harvested and the protein lysates were prepared for the measurement of luciferase activity displaying only the activity in SGHPL-5 cells.

### 6.3.3. β-Galactosidase and Luciferase Activity Assay

At the end of transfection, the cells were lysed using 100µl reporter lyses buffer (Promega) according to the manufacturer's instructions. For β-Gal activity measurement, 20µl of the protein sample were mixed with 200 µl chromogenic substrate chlorophenol

red-β-D-galactopyranoside (CPRG) buffer and incubated until a clear colour change from yellow to orange was visible indicating the conversion of the CPRG reagent. The reaction was stopped with 1ml A.d. and the optical density was photometrically determined at 570nm.

The luciferase activity was determined on a luminometer using Luciferase Assay System (Promega) and 10µl protein extract. Both activity assays, β-Gal and luciferase, were measured in duplicates.

**Solutions:** CPRG buffer

80mM $NaPO_4$

18mM $MgCl_2$

200mM β-Mercaptoethanol

16mM CPRG

| Name (anti-) | Species | Cat.# | Company | Dilution | Application |
|---|---|---|---|---|---|
| Cytokeratin 7 | Mouse | | Dako | 1:100 | IHC |
| Vimentin | Rabbit | | Abcam | 1:100 | IHC |
| KI67 | Mouse | | Chemicon | 1:100 | IHC |
| Kip2p57 | Rabbit | | Santa Cruz | 1:200 | IHC |
| Notch1 | Rabbit | 3608 | Cell Signaling | 1:150 | IHC |
| Notch2 | Rabbit | PAB1123 | Abnova | 1:150 | IHC |
| Notch3 | Rabbit | Sc-5593 | Santa Cruz | 1:150 | IHC |
| Notch4 | Rabbit | Sc-5594 | Santa Cruz | 1:40 | IHC |
| Jagged1 | Rabbit | Sc-8303 | Santa Cruz | 1:100 | IHC |
| Jagged2 | Rabbit | 2210 | Cell Signaling | 1:150 | IHC |
| DLL1 | Rabbit | Ab76655 | Abcam | 1:150 | IHC |
| DLL3 | Rabbit | Sc-67269 | Santa Cruz | 1:150 | IHC |
| DLL4 | Rabbit | PAB10200 | Abnova | 1:200 | IHC |
| NICD cleaved | Rabbit | | Cell signaling | 1:1000 | WB |
| Flag | Rabbit | | Cell signaling | 1:1000 | WB |
| GapDH | Rabbit | | Cell signaling | 1:1000 | WB |

**Table 4: list of primary antibodies**
Immunohistochemistry (IHC), Western blot (WB)

| Name (anti-) | Species | Cat.# | Company | Dilution | Application |
|---|---|---|---|---|---|
| Alexa flour 488 | Goat-anti-mouse | | Mol. probes | 1:1000 | IHC |
| Alexa fluor 488 | Goat-anti-rabbit | | Mol. probes | 1:1000 | IHC |
| Alexa flour 568 | Goat-anti-rabbit | | Mol. probes | 1:1000 | IHC |

| Name (anti-) | Species | Cat.# | Company | Dilution | Application |
|---|---|---|---|---|---|
| HRP | Goat-anti-rabbit | | | 1:50000 | WB |
| HRP | Goat-anti-mouse | | | 1:50000 | WB |

**Table 5: list of secondary antibodies**
Immunohistochemistry (IHC), Western blot (WB)

## 6.4. Statistical analyses

Statistical analyses were performed with Student's *t* test. A $P$ value $< 0.05$ was considered statistically significant

# 7. References

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# CURRICULUM VITAE


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## BILDUNG

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TNFα-mediated induction of Pai-1 restricts invasion of HTR-8/SVneo trophoblast cells, **S. Bauer**, P. Husslein, JD Aplin, M. Knöfler; 2005 EPG/IFPA Tagung, Glasgow

TNFα inhibits trophoblast migration through induction of plasminogen activator inhibitor-1,
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Tumour necrosis factor α inhibits migration/invasion of extravillous trophoblasts in first trimester villous explant cultures
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