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1. Introduction

1.1. The human placenta

The placenta connects the growing fetus with the mother and is responsible for oxygen- and nutrient supply to the embryo as well as clearance of waste products. Secretion of hormones, maintaining pregnancy and controlling the mother's immune system are other important functions of the placenta.

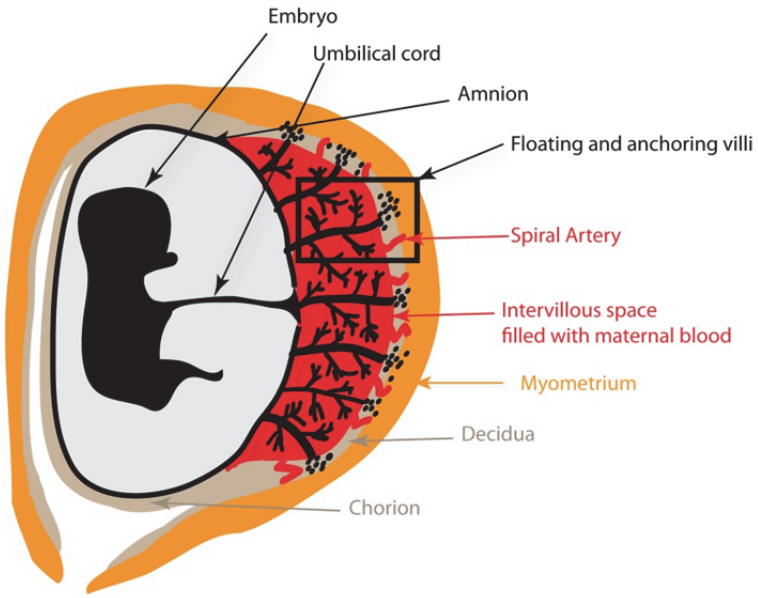
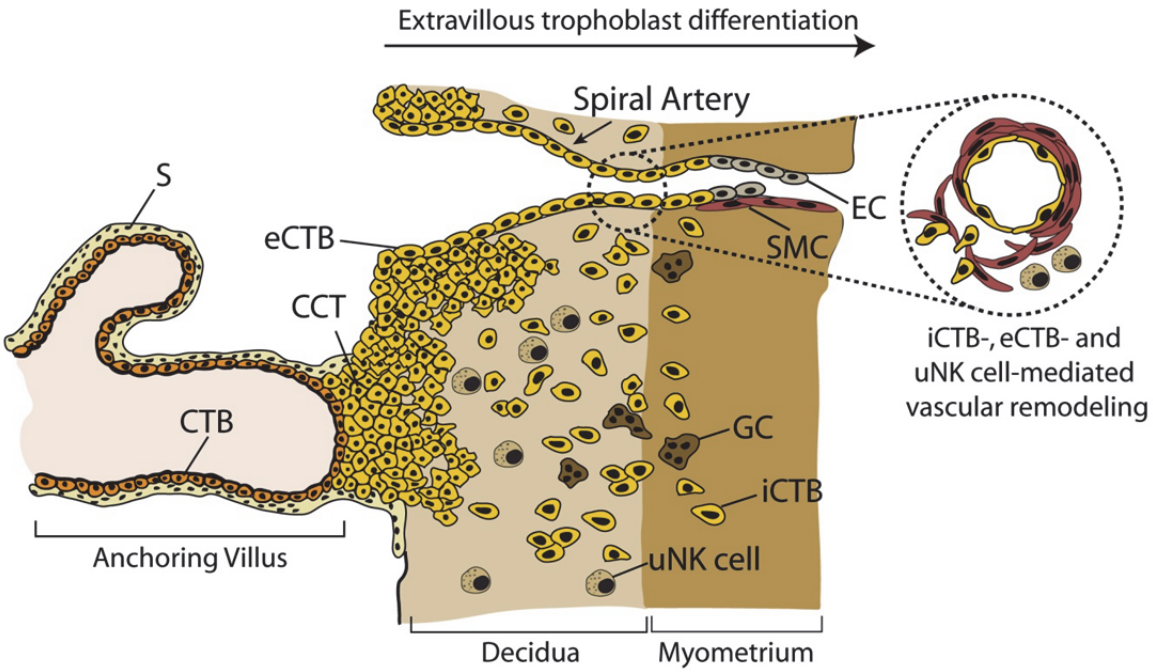


Figure 1:
Schematic drawing showing the fetal-maternal interface in the first trimester of gestation
Components depicted in black are of embryonic origin, colored ones are derived from maternal tissues. The rectangle shows floating and anchoring villi, as detailed in Figure 2.

The placenta covers the maternal decidua. Maternal blood floods placental floating villi. The tree-like structure of placental villi gives rise to a huge area (approx. 15m^2) where gas and nutrient exchange takes place. Anchoring villi, on the other hand, are in direct contact with the maternal decidua and fix and stabilize the organ. The placenta is covered by amniotic membranes and the umbilical cord connects the fetus with the placenta.

1.1.1. Cell types in the human placenta

In the basement membrane of placental villi, trophoblastic progenitor cells are the source of different epithelial cell types that fulfill diverse functions in the growing tissue. The surface of floating villi, the so-called multinucleated syncytium, is formed by asymmetric division of villous cytotrophoblasts (CTB) and cell fusion.



	Embryonal		Maternal
S	syncytium	**SMC**	smooth muscle cell
CTB	cytotrophoblast	**EC**	endothelial cell
CCT	cell column trophoblast	**uNK**	uterine natural killer cell
iCTB	interstitial trophoblast	**Decidua**	rebuilt maternal endometrium
eCTB	endovascular trophoblast		
GC	giant cell		

Figure 2:
Schematic drawing of an anchoring villus of a first trimester placenta
Proliferative villous CTBs of anchoring villi that attach to the uterine epithelium form cell columns (CCT) and differentiate into extravillous trophoblasts (EVT), that invade the maternal uterine epithelium and one third of the myometrium and remodel spiral arterioles. The table lists origin of cell types. [Pollheimer, 2012]

The syncytium, which is in contact with the maternal blood, is mainly responsible for transport of nutrients and waste products as well as hormone production. Proliferative villous CTBs of anchoring villi that attach to the uterine epithelium form cell columns (CCT) and differentiate into extravillous trophoblasts (EVT). Two types of invasive trophoblasts arise from cell columns. On the one hand, distally located CTBs exit the cell cycle and differentiate into interstitial CTBs (iCTBs) that invade the decidual stroma. The switch of proliferation to invasion is regulated by an intrinsic differentiation program and contact to extracellular matrix and decidua, as well as oxygen concentration. Interstitial CTBs produce pregnancy hormones, such as human chorionic gonadotropin (hCG) and placental lactogen (hPL). Additional functions of iCTBs are differentiation into placental bed giant cells and anchorage of the growing placenta to the decidua. Furthermore, iCTBs invade maternal spiral arteries, transform the vessel endothelium and differentiate to

endovascular CTBs (eCTBs). In addition, CTBs originating from proliferating cell columns likely migrate on the surface of the decidua and invade and remodel maternal spiral arteries by displacing the endovascular endothelium. Both processes result in an enlargement of vessel diameter and regulation of the blood flow into the intervillous space [Knöfler, 2010]. The invasion process of trophoblasts into the dezidualized endometrium and the inner third of the myometrium is a process involving many genes important for epithelial-to-mesenchymal transition (EMT), underlying a strict regulation [Weier at al., 2005] which is not fully characterized.

1.1.2. Origin of the placenta and early placental development

The placenta consists of embryonic (trophoblasts) and maternal parts (decidua) and forms the contact surface between mother and child. Trophoblasts are exposed to the maternal immune system and are able to avoid maternal allorecognition by expressing human leukocyte antigens such as HLA-G, HLA-C and HLA-E. Furthermore, they show a highly invasive phenotype.

Fetal development starts after ovulation when the secondary oocyte enters the tube/oviduct and is fertilized by a sperm (Figure 3).

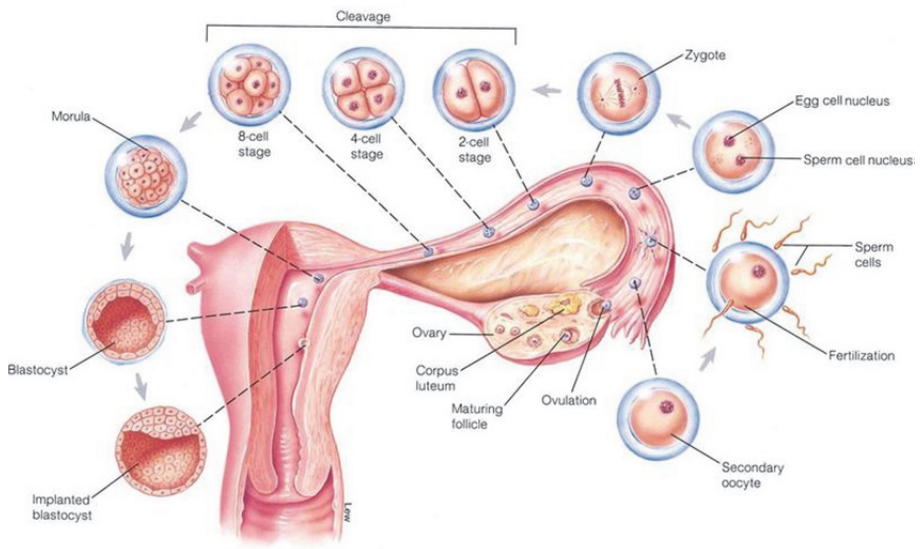


Figure 3:
Illustration showing fetal development from ovulation to implantation of the blastocyst
The secondary oocyte enters the tube/oviduct and is fertilized by a sperm. 3 mitotic cell divisions proceed until morula stage and entry into the uterus lumen is reached. Now the blastocyst differentiates into inner cell mass and trophectoderm and implantation goes on. [http://humanphysiology2011.wikispaces.com/15.+Reproductive+Physiology]

After the first three mitotic cell divisions (2-cell, 4-cell and 8-cell state), the morula enters the uterus. The morula matures to the blastocyst, which is built up by the zona pellucida, a sheath surrounding a cave that contains two cell populations, the inner cell mass (ICM) and the trophectoderm that gives rise to extraembryonic tissues, the fetal part of the placenta. The ICM is an accumulation of stem cells and the origin of all embryonic tissues. Just before implantation, the blastocyst peels off the zona pellucida and differentiates to the epiblast and the primitive endoderm (progenitors of the embryo). The trophectoderm adheres to the maternal endometrium and invasion starts: a primitive trophblast syncytium forms that infiltrates utrine epithelial cells and breaks into the basal lamina. Trophoblasts start to invade the maternal interface [Wang et al, 2006]. A concerted interplay between blastocyst and maternal endometrium is necessary for a successful implantation. Ovarian steroid hormones (progesteron and oestrogen) prepare the endometrium for attachment of the blastocyst. Cytokines including leukemia inhibitory factor-1 (LIF-1), epidermal growth factor (EGF) and interleukin-1 (IL-1) as well as expression of specific integrin patterns in the receptive endometrium and blastocyst are necessary for attachment. Local depletion of MUC1, an uterine cell surface glycoprotein, by the blastocyst controls location and time point of implantation.

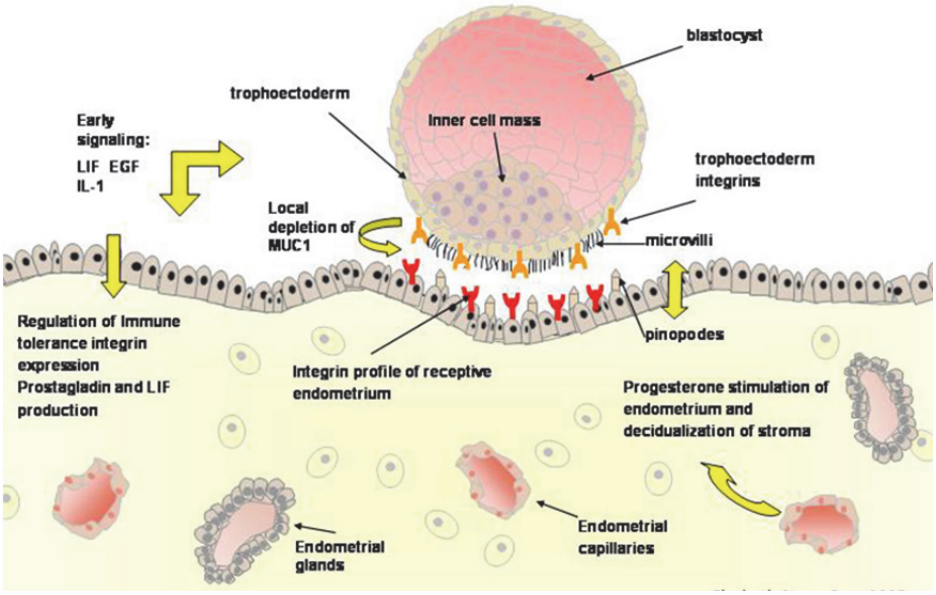


Figure 4:
Illustration showing implantation of the blastocyst into the maternal endometrium
Attachment of the blastocyst is controlled by interplay of extracellular matrix, integrin patterns, hormones, cytokines, inflammatory factors and extracellular matrix degrading proteins. [Staun-Ram E, 2005]

After blastocyst attachment, the trophoblast starts to invade the maternal endometrial epithelium under strict control of extracellular matrix (ECM) molecules including collagens,

fibronectin, laminin and vitronectin and hormones such as human chorionic gonadotropin and progesterone, as well as growth factors (EGF, heparin-binding (HB)-EGF, transforming growth factor beta (TGF-β), insulin-like growth factor binding protein (IGFBP-1). Furthermore, cytokines (LIF-1 and IL-1), inflammatory factors (cortico-releasing hormone, tumor necrosis factor alpha (TNFα)), prostaglandins and extracellular matrix degrading proteinases (matrix metalloproteinase (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs) and the serine proteases plasminogen activator inhibitor/urkinase plasminogen activator (PAI/uPA) system) are involved.

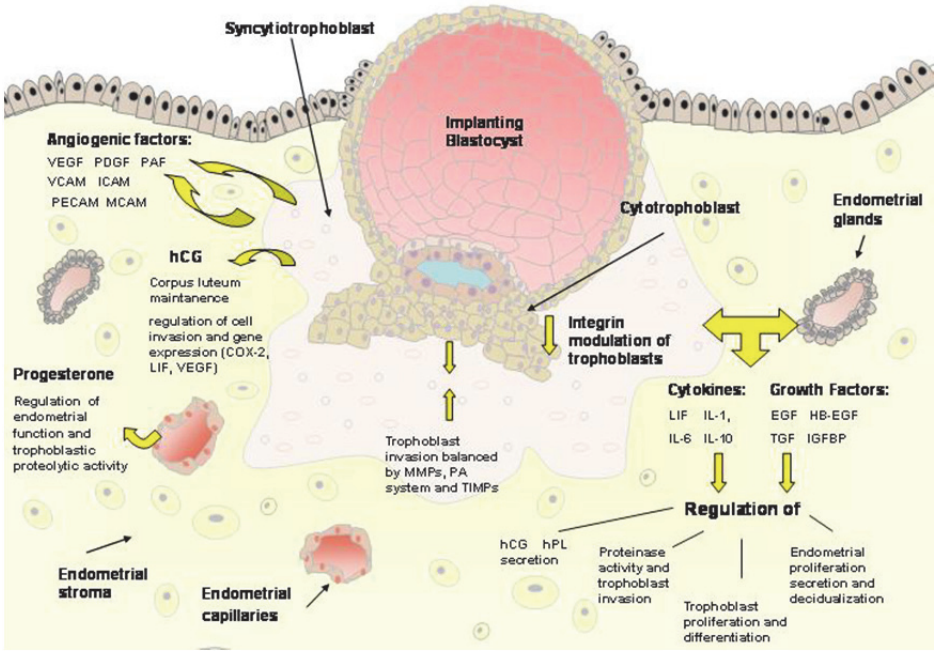


Figure 5:
Summary of factors controlling early trophoblast invasion and placental development
Cooperation of fetal and maternal tissue via integrin modulation, cytokines, growth factors, hormones, angiogenic factors and extracellular matrix degradation is essential for the implantation process. [Staun-Ram E, 2005]

All mentioned factors activate various signaling cascades for the regulation of target gene expression, as reviewed by Knöfler, 2010. Among them are the Mitogen activated protein kinases (MAPK), Phosphoinositide 3-kinase (PI3K)-AKT, Focal adhesion kinase (FAK), Notch and last but not least the Wnt signaling pathway.

1.2. Wnt Signaling

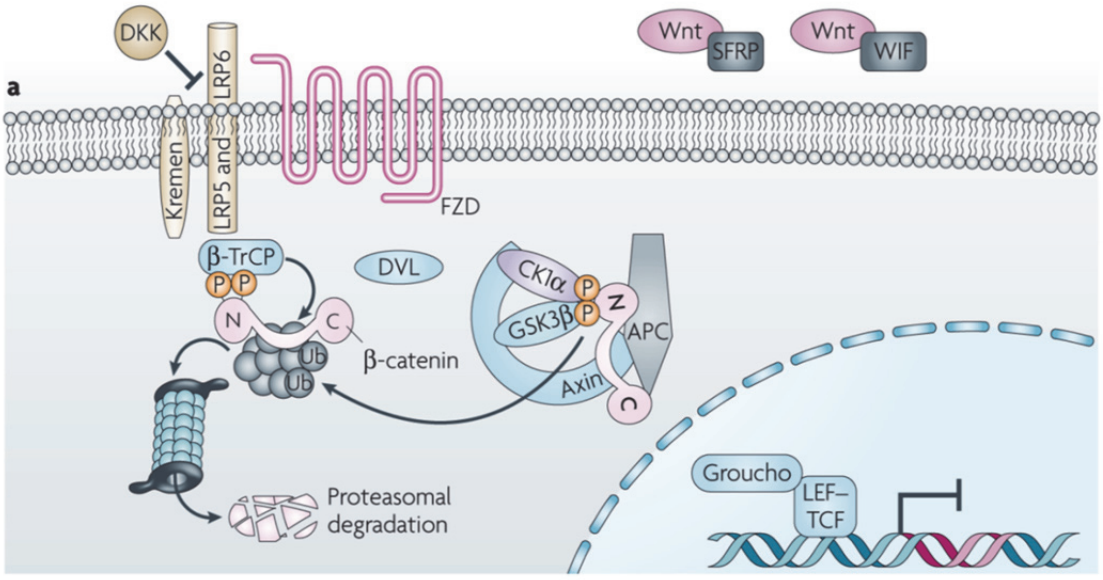
In 1982, Nusse and Varmus reported the discovery of a tumourvirus that induced mouse mammary gland tumours by activating the expression of the *Int1* gene. Already in 1973, a drosophila mutant lacking wings was reported by Sharma et al. and in 1987, Rijsewijk et

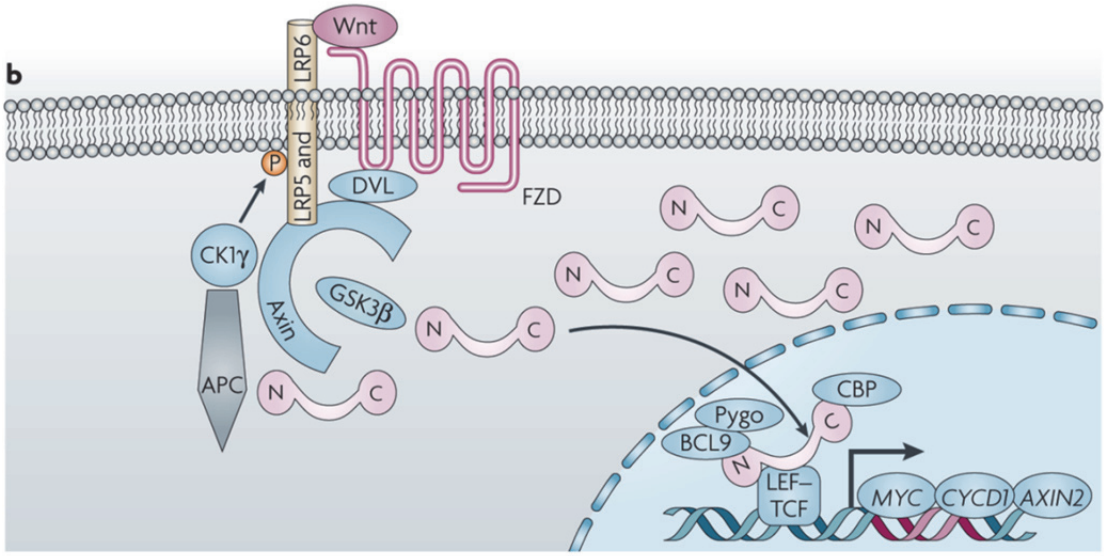
al. showed that the mutated wingless gene (*Wg*) is the homolog of the mammalian *Int1*. A fusion of both genes named the pathway *Wnt* signaling.

This evolutionary highly conserved signaling pathway is involved in embryonic development, organogenesis and regulation of proliferation, differentiation, stem cell maintenance and apoptosis. Mutations or deregulated expression of components can cause disease and hyperactivity and defect regulation of the pathway is found in many types of cancer.

β-catenin is the key mediator of the canonical Wnt signaling pathway. Located at adherent junctions in epithelial cells, it connects E-cadherin to the filaments of the cytoskeleton. When β-catenin is released from the cell membrane to the cytoplasm and Wnt signaling is not active, cytoplasmatic β-catenin is bound by a multi-protein destruction complex (MDC) consisting of Axin, APC (adenomatous polyposis coli), GSK3-β (glycogen synthase kinase-3 beta) and CK1α (casein kinase 1 alpha). GSK3-β and CK1α phosphorylate β-catenin at the N-terminus. Phosphorylated β-catenin is targeted by β-TrCP (β-transducin repeat-containing protein), forming part of the E3 ubiquitin ligase complex, and is degraded by proteasomes. In the nucleus, members of the T-cell factor family (TCF-1, -3 -4 and LEF-1, lymphoid enhancer factor 1) form complexes with the co-repressors of the Groucho/Transducin enhancer of split family (TLE-1, -2, -3, -4 and -5) upstream of target genes and transcription is repressed (Figure 6a).

Figure 6:
Illustration of the Wnt signaling cascade in the inactive (a) and active state (b) [Klaus A and Birchmeier W, 2008]





a: Devoid of Wnt ligand activation cytosolic ß-catenin is targeted by an destruction complex phosphorylated and degraded. Inhibitory DNA complexes block target gene transcription.
b: Extracellular Wnt ligands destabilize the destruction complex upon receptor binding. Hypophosphorylated ß-catenin shuttles to the nucleus and target gene transcription is activated.

Upon activation, extracellular Wnt ligands bind to LRP5/6 and Frizzled receptors and the MDC is destabilized, which results in hypophosphorylation of β-catenin. Therefore, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it displaces the Groucho/TLE co-repressors from the LEF-TCF binding site and target gene transcription is induced. Pygopus (1 and 2) and Bcl9 (B-cell CLL/lymphoma 9/ legless) also form part of the transcriptional complex. Bcl-9 acts as an adaptor between Pygopus and TCF-bound β-catenin and is important for the transcriptional activation of target genes (Figure 6b). [Klaus, 2008] In the nucleus, gene transcription is regulated through chromatin remodeling and regulation of RNA polymerase II.

The complex pathway can be manipulated in various forms: e.g. the Wnt signaling inhibitor DKK (Dickkopf) (Fig. 6a) antagonizes Wnt signaling during head formation in mice [Mukhopadhyay et al., 2001]. Kremen is a DKK receptor that inhibits Wnt signals by inducing rapid endocytosis of the LRP receptors [Mao et al., 2002]. SFRPs (secreted Frizzled-related proteins) and WIF (Wnt inhibitory factor) also have an inhibitory function on Wnt signaling. [Klaus et al., 2008].

19 secreted Wnt ligands were described to induce Wnt signaling in the human system. These hydrophobic Wnt ligands are found in association with cell membranes or extracellular matrix [Reichsmann et al., 1996]. In Wnt secreting cells, the Wnt ligands are modified (palmitoylated) in the ER by porcupine acyltransferase. Wntless/Evi, located at the golgi and the cell membrane, controls the transport and secretion of the Wnt ligands using secretory vesicles. Furthermore, a retromer complex is involved in the production of Wnt ligands that are packed in lipoprotein particles and are secreted in this form [Mikels and Nusse, 2006].

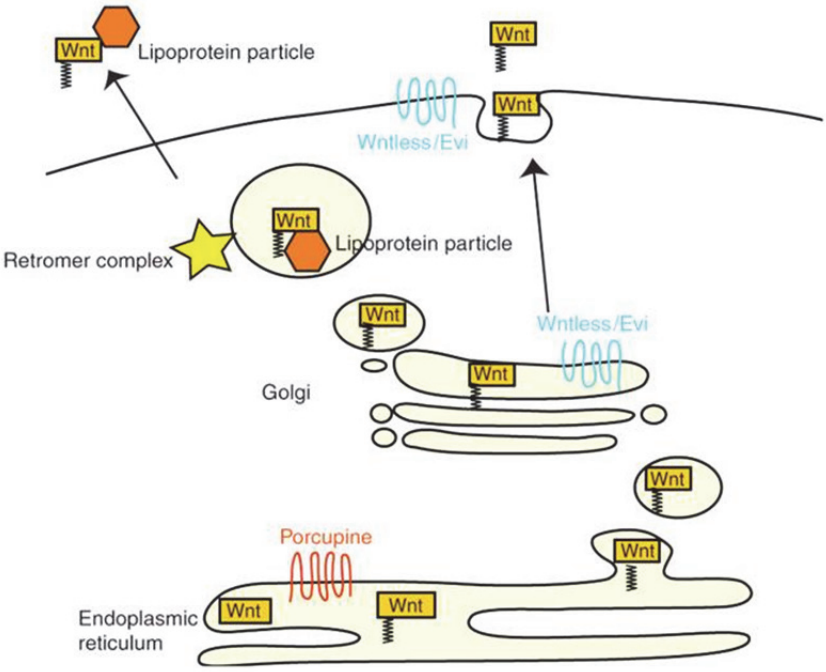
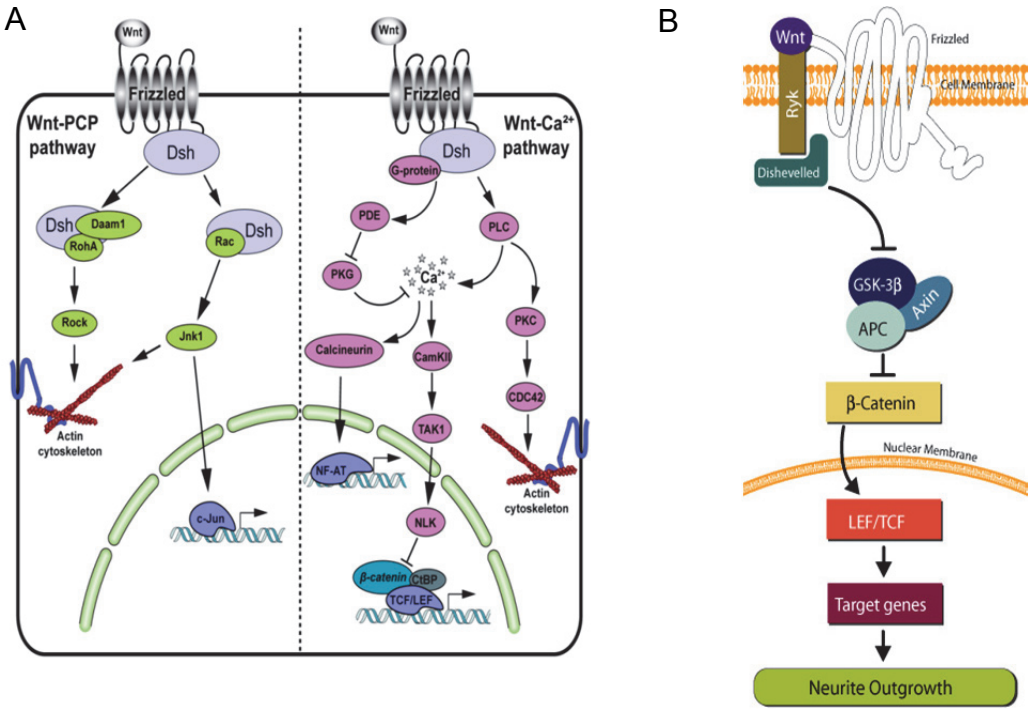


Figure 7:
Schematic illustration of Wnt ligands
Wnt ligands are modified (palmitoylated) in the ER. Wntless/Evi, located at the golgi and the cell membrane, controls the transport and secretion of the Wnt ligands using secretory vesicles. Furthermore, a retromer complex is involved in the production of Wnt ligands that are packed in lipoprotein particles and are secreted in this form. [Mikles and Nusse, 2006]

Furthermore norrin or R-spondin interact with frizzeled receptors and act as activators of Wnt signaling [Klaus et al., 2006].

Besides the canonical Wnt signaling cascade, some players can regulate target genes in β-catenin-independent non-canonical signaling cascades. These include theWnt/PCP (planar cell polarity) and Wnt-Ca^{2+}-pathways as well as the ROR/Ryk pathway.

Figure 8: Illustration of the non-canonical Wnt signaling cascades (A) Wnt/PCP and Wnt-Ca^{2+} [Sonderegger, 2010] and (B) ROR/Ryk pathway [R&D]



1.2.1. Wnt signaling in the human placenta

Sondereggeret al., 2010 reviewed extensively that Wnt signaling is important for murine uterine development, pre-implantation and decidualization, but not for blastocyst development. Furthermore, Wnt signaling controls extra-embryonic development: placental vascularization and chorion allantois formation. Fusion and labyrinth function are regulated by the canonical as well as non-canonical Wnt pathways. In the human system, Wnt ligands and frizzeled receptors were identified in endometrial cell types and it was also shown that during the menstrual cycle some players are hormonally regulated. Using trophoblast cell models it was shown that, similar to mouse blastocysts, adhesion to the endometrium is influenced by Dickkopf. Further, trophoblast migration and differentiation is also regulated by Wnt signaling. Pollheimer, 2006 showed that TCF-3 and TCF-4 expression is restricted to extravillous trophoblasts (EVTs) in the human placenta. Dropping of β-catenin from the cell membranes in the proliferative cell column trophoblasts (CCTs) and detection of nucler β-catenin during EVT differentition in a small percentage of trophoblasts suggests a tight regulation of Wnt signaling in this process. Elevated nuclear β-catenin found in complete hydatidiform moles (CHM) suggests that Wnt signaling could be an important regulator of the switch from proliferation to migration and /or differentiation and also to malignant transformation of trophoblasts. Stimulation of a trophoblastic cell line and primary cytotrophoblasts (CTBs) with the Wnt ligand Wnt3a

showed elevated migration as well as induction of the Wnt target Cyclin D1. Sonderegger et al., 2007 identified several members of the Wnt signaling cascade in the human placenta. Among the detected Wnt ligands, Wnt 1, 2b, 4, 5a/b and Wnt11 showed the highest expression in EVTs. The frizzeled receptors FZD 1, 3, 5, 6, 7, and 10 were detectable in placental trophoblasts (CTBs and EVTs). Elevated outgrowth under Wnt3a induction of placental explant cultures and blocking of the induced outgrowth using DKK1 shown by Sonderegger et al., 2010 emphasizes the importance of the pathway during migration/differentiation of trophoblasts. The authors could further show a Wnt3a-dependent MMP2 induction in the trophoblastic cell line SGHPL-5 and in EVTs, which explains the elevated migration potential upon Wnt3a activation. Wnt3a-induced β-catenin accumulation was blocked by Dickkopf. Furthermore, the cross talk between canonical Wnt signaling and PI3K/AKT activation was found to contribute to increased trophoblast mobility. Wnt3a-induced AKT phosphorylation in SGHPL-5 cells and EVTs could be blocked by the AKT inhibitor LY294002. However, Dickkopf1 was not able to block AKT phosphorylation suggesting that a non-canonical receptor pathway induces PI3K/AKT signaling. One important metalloproteinase, namely MMP2, was identified to be upregulated upon Wnt3a stimulation. Blocking with DKK1 or LY294002 inhibited MMP2 induction but failed to show a direct MMP2 mRNA regulation. These data suggest that another unknown Wnt target activates MMP2. They concluded that activation of AKT signaling by Wnt3a must use an non-canonical receptor patway because Dickkopf that blocks Wnt ligand activation over LRP5/6 failed to block AKT phosphorylation. Besides this, we know from expression studies (Sonderegger 2007) that Wnt3a is not detectable in the placenta suggesting that other Wnt ligands are regulating placental development.

1.2.2. The LEF/TCF-family

T-cell factors (TCF-1,-3,-4) and lymphoid enhancer factor (LEF)-1 are a group of transcription factors containing a high mobility group (HMG-box).They are highly evolutionary conserved and play important roles during development such as correct embryonic body plan formation, specification of the cell fate and regulation of cell survival and proliferation. In the adult organism, LEF/TCF-family members regulate the survival, proliferation as well as differentiation of self-renewing tissues in the intestinal mucosa, the skin or the lymphoid compartment [Hurlstone and Clevers, 2002].

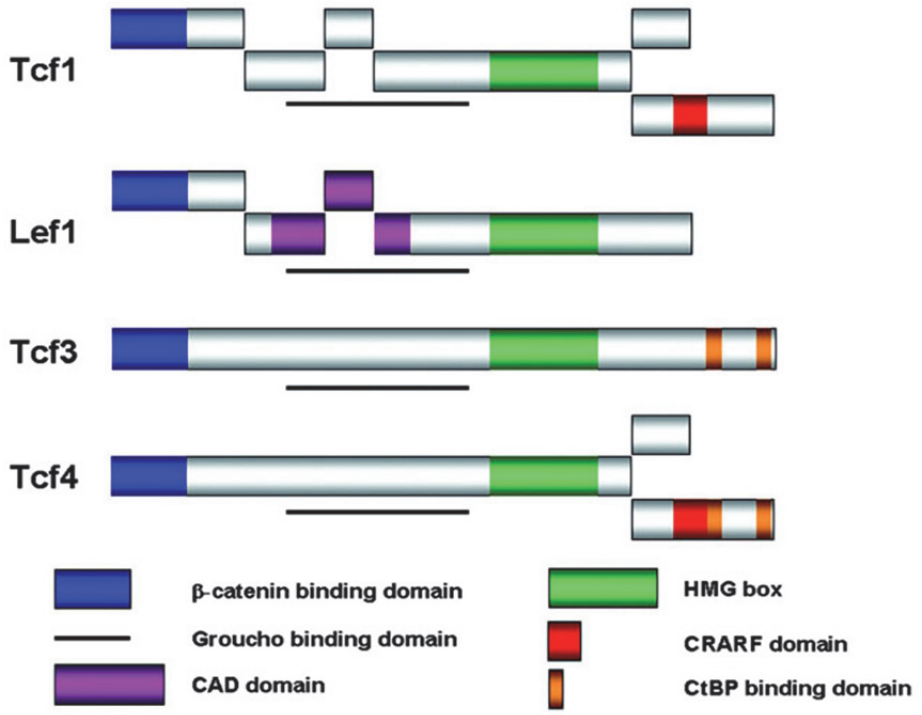


Figure 9: Illustration of the most conserved domains of the LEF/TCF family. [*Hurlstone and Clevers, 2002]*

The HMG-box (green) mediates target site recognition in promoters of Wnt signaling-regulated genes. LEF/TCFs bind as monomers to the core sequence AG **A/T TCAAAG**GG [Pantelis Hatzis et al., 2008 and Hurlstone and Clevers, 2002] and induce DNA bends and the recruitment of transcriptional complexes or chromatin modifiers to promoters. They themselves have no transcriptional activity but modulate transcription by the formation of complexes consisting of auxiliary factors (inducers or inhibitors) shown in fig. 10. The key transcriptional activating partner of the LEF/TCFs is β-catenin and a highly conserved 50 amino acid-comprising N-terminal region (blue box, fig. 9) of the proteins is used for binding to β-catenin. The inhibitory function of LEF/TCFs is triggered by binding (black line) a family of inhibitory proteins, termed Transducin-like Enhancer of Split (TLE)/ grouchos, and recruitment of silencing chromatin modifiers (fig. 10 OFF) to promoter regions.

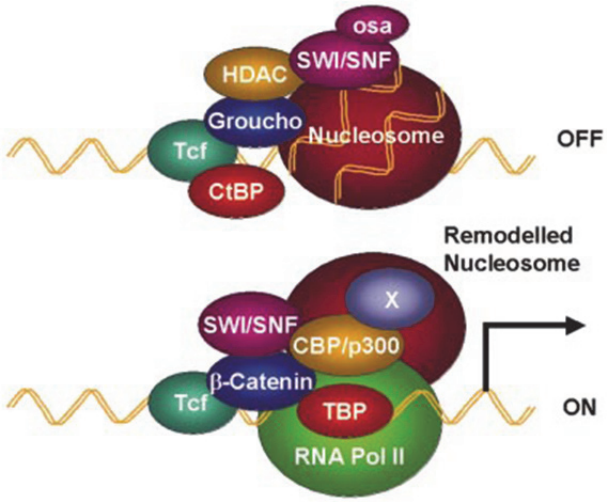


Figure 10:
Illustration of the transcriptional complex of canonical Wnt signaling in on and off state
A complex of DNA inactivating proteins is formed at target sites in the off state. Upon activation RNA polymerase and activating factors are recruited to target sites. **[*Hurlstone and Clevers, 2002]***

1.2.3. TCF-4

Korinek et al. 1998 first described the role of TCF-4 in the intestinal epithelium. They found that TCF-4 knock out mice lack proliferative stem cells in the intestinal epithelium. Furthermore, they showed that TCF-4 and Wnt signaling are important regulators of tissue homeostasis in this compartment. In the human placenta, TCF-4 is one of the most upregulated genes in EVTs found by comparative gene chip analyses of cytotrophoblasts and EVT [Bilban et al., 2009], suggesting that TCF-4 is an important factor promoting EVT invasion and/or differentiation. Besides the recruitment of inhibitory or activating complexes to promoters, the transcription-regulating activity of TCF-4 is also influenced by post transcriptional modifications. Waltzer et al., 1998 described that acetylation in the Armadillo/ β-catenin binding domain of TCF-4 by CREB-binding protein (CBP) reduced the binding affinity to Armadillo/ β-catenin in Drosophila. Moreover, NEMO-like kinase (NLK) binding to phosphorylated TCFs was reported to reduce the binding affinity of TCFs to β-catenin and DNA [Ishitani et al., 1999]. Furthermore, sumoylation which is a reversible modification that can change the subcellular localization, transcriptional activity or protein stability is known to influence TCF-4 activity. Yamamoto et al., 2003 found that the small ubiquitin-related modifier SUMO-1 sumoylates TCF-4 in HEK 293 cells by showing a mobility shift of TCF-4 in SDS-PAGE. IP with SUMO-1 and TCF-4 antibodies was able to precipitate the proteins and confirmed the finding. Additionally they showed that the sumoylation state of TCF-4 is controlled by PIAS, an enhancer of sumoylation and Axam (SENP), a sumo specific protease in HeLa cells: down regulation of Axam

enhanced the sumoylated form of TCF-4. The binding ability of TCF-4 to β-catenin or DNA was not changed by the sumo modification but sumoylation enhances the transcriptional activity of TCF-4 shown by TOP reporter assays. They identified Lys297 (K297) being a sumoylation site in TCF-4.

1.2.4. TLE/groucho Family (TLE-1,-2,-3,-4, AES/TLE-5,-6)

Members of the TLE /groucho/grg family are transcriptional co-repressors and act in key developmental signaling pathways like Notch, Wnt, Hedgehog and Dpp/BMP. In vertebrates, they play important roles during neuronal development, somitogenesis, establishment of left right asymmetry, osteogenesis and haematopoiesis. Alteration of TLE expression is found in several tumours. Five regions characterize the members of the TLE family.

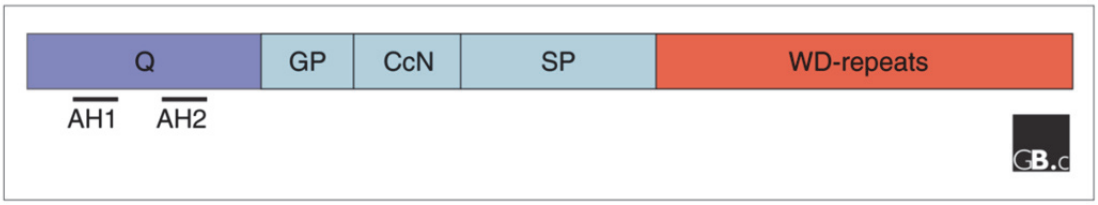


Figure 11: Schematic of the conserved domains of the TLE/groucho family. [Jennings BH, Ish-Horowicz D, 2008]

The N-terminal Q-domain and the WD-domain at the C-terminus are the highly conserved and best characterized domains of the family. The Q-domain favours oligomerization into tertamers and binding to transcription factors like LEF/TCFs, FoxA, or cMyc. Tetramerization is necessary for the repressive function as well as the interaction with transcription factors. The GP region triggers the recruitment of HDACs and the CcN is a nuclear localization signal and harbours regulatory phosphorylation sites. The C-terminal WD-repeats interact with transcription factors. The AES/Grg5 protein is a truncated version consisting of only a Q and GP domain and acts as negative regulator of TLE/Groucho. TLE6/Grg6 has a divergent N-terminal region but the WD-domain is very similar to the other family members and competes with the binding of TLE1 to FoxG1. It antagonizes TLE1 repression in neuronal differentiation. The mechanism how TLE/grouchos repress transcription has not been elucidated. They have no repressing potential but they recruit chromatin modifying complexes or directly interact with the transcriptional machinery. Furthermore, posttranscriptional modifications influence the repressive activity of the TLE/grouchos [Jennings and Ish-Horowicz, 2008]. The Aes/grg5

proteins are able to multimerize with other TLEs or interact with transcription factors like LEF/TCFs [Brantjes et al., 2001].

1.2.6. Inhibition of Wnt Signaling

There are diverse possibilities to inhibit a signaling pathway, including small interfering (si) RNA-mediated silencing or treatment with chemical inhibitors.

In this work ON Target plus siRNA technology was used to silence TCF-4. A pool of 4 optimized siRNAs were used to target TCF-4 mRNA and to induce mRNA cleavage as well as translational repression. Optimized protocols promise high specifity with no side effects (Dharamcon). For chemical inhibition PKF115-584 was tested first in trophoblasts.

Lepourcelet et al., 2004 identified PKF115-584, a compound of microbial origin, in a screen of small molecules as an inhibitor of TCF-4/β catenin complex formation.

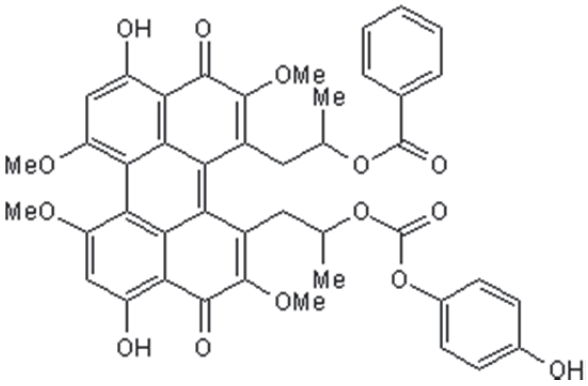


Figure 12: Chemical structure of PKF115-584

Using ELISA, they showed that binding of GST-TCF-4 fusion proteins to immobilized β-catenin was disrupted by PKF115-584 in a dose-dependent manner. In IP experiments, PKF115-584 abolished GST-TCF-4 precipitation of cellular β-catenin. In electrophoretic mobility shift assay (EMSAs) the inhibitor disrupted the TCF-4/β-catenin complex but not a pRb/E2F complex in control cells. Further, they showed that PKF115-584 dose-dependently inhibits transactivation of the canonical Wnt reporter TOPflash as well as the expression of known Wnt target genes including Cyclin D1 [Tetsu O et al., 1999] and cMyc [He TC et al., 1998]. In contrast, expression of Cyclin E was not inhibited. PKF115-584 was able to prevent the formation of an additional body axis due to ectopic expression of β-catenin in xenopus embryos. However, it was not able to circumvent the formation of an additional axis due to ectopic Siamois expression, a downstream target of TCF-4/β-catenin. Finally, IP experiments showed that PKF115-584 also interferes with β-catenin/APC binding but not with β-catenin/E-cadherin binding.

The chemical structure of PKF115-584 is identical to the Proteinkinase C (PKC) inhibitor calphostin C whose effects on the Wnt signaling pathway were described by Chen et al., 2000. They showed that serum/growth factors were able to induce the Lithium-dependent accumulation of β-catenin due to inhibition of GSK3β in mammary epithelial cells. Wnt-1 induced accumulation was not enhanced by serum/growth factors. They found that calphostin C/PKF115-584 reduced Lithium- as well as Wnt-1 -induced accumulation of cytosolic β-catenin in a dose dependent manner as well as the activity of the canonical Wnt reporter TOP-flash. Furthermore, they showed the inhibition of a dominant negative TCF-4 on luciferase activity of the TOP-reporter. They concluded that two interactive processes, regulation of GSK3β and CK1α, that are induced by serum/growth factors influence cytosolic and further nuclear β-catenin levels.

Therefore, the work from Lepourcelet et al. probably can change the interpretation by Chen et al. in the way that the effect of PKF115-584 is a result of disruption of the TCF-4/β-catenin complex and/or APC-β-catenin complex or both as well as inhibition of β-catenin phosphorylation. All effects of the compound taken together may explain the high potency in some assays and might contribute to its cytotoxic effect after 72 hours. Doghman et al*, 2008* showed that PKF115-584 reduced the proliferation rate of adrenocortical carcinoma cells (H295R) harboring a β-catenin Wnt signaling activation mutation but not of HeLa cells where the β-catenin pathway is inactive. Sinnberg et al., 2011 showed that PKF115-584-mediated downregulation of β-catenin signaling strongly inhibits primary and metastatic melanoma cells in migration and matrigel invasion assays and in physiological skin equivalents, but normal human melanocytes were not affected by the inhibitor. Further, it inhibits the tumor-inducing ability of metastatic and non-metastatic melanoma cell lines. Also, they showed that PKF115-584 reduced Wnt targets Cyclin D1 and cMyc as well as anti-apoptotic genes like Bcl-xl and Bcl-2 in their cell system. Here, inhibition of β-catenin signaling triggers the cells into an apoptotic pathway.

2. Goal of the study

Regulation of growth and function of the developing human placenta is a multi-factor event requiring the interplay of diverse pathways in order to form a functional organ that satisfies all the needs of a growing embryo and a successful pregnancy outcome. Wnt signaling, an evolutionary highly conserved pathway, plays a major role during embryonic and placental development. Aim of this work is the identification of players of the transcriptional complex of the canonical Wnt signaling cascade contributing to the formation of a functional organ. In particular, the main focus of this work was to gain more insights into the role of T-cell factor 4, a highly-induced transcription factor during extravillous trophoblast differentiation and invasion, in order to contribute to a better understanding how a physiological invasion process is controlled.

3. Results

3.1. Description of the canonical transcriptional complex in the human first trimester placenta

To figure out the distribution of important players of the canonical Wnt signaling cascade in the human placenta, immunofluorescence stainings of placental tissues of the first trimester of gestation (8-12th week) were performed. Double stainings were done to show co-expression of proteins of interest with cell-type specific markers. As shown in panel 1 in figures 13-16, DAPI staining (blue) was performed to visualize cell nuclei.

To get an overview of placental tissue sections, common markers characterizing individual cell types in the placenta are shown in figure 13. Markers which discriminate cells of mesenchymal or epithelial origin are visualized in panel A, while panel B identifies the cell cycle state of placental cells in a first trimester placenta.

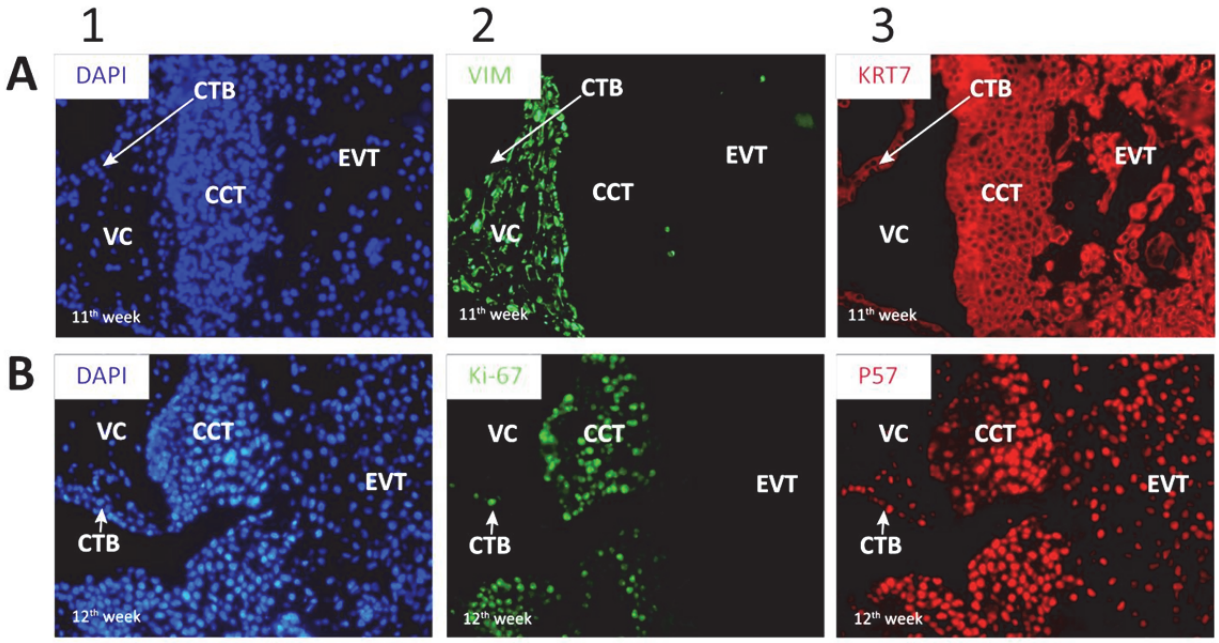


Figure 13: Immunofluorescence characterizing cell types in anchoring villi of first trimester placentae

VIM and KRT7 are markers for stromal and trophoblastic cells, respectively. DAPI staining was performed to visualize cell nuclei. Ki-67 and P57 characterize proliferative and resting cells. VC = villous core, CCT = cell column trophoblast , CTB = cytotrophoblast, EVT= extravillous trophoblast.

Vimentin (VIM), an intermediate filament protein expressed in mesenchymal cells was detectable in the placental villous core (VC) , as well as in decidual cells (Fig.13 A2, Fig. 16 B2 and B3) and was completely absent from trophoblastic cells. Keratin 7 (KRT7), a member of the keratin family, which is expressed in simple epithelia of internal organs,

was found to stain positively for trophoblasts and negatively for mesenchymal cells (Fig.13 A3).

Ki-67 (Schwarting et al, 1993), a marker for mitotic cell cycle active cells, was detectable in cytotrophoblasts (CTBs) located on a basement membrane (indicated by the arrow) as well as in cell column trophoblasts (CCTs) (Fig. 13 B2). P57/kip-2, the cyclin dependent kinase inhibitor C1, is a negative regulator of proliferation that inhibits several G1 cycline/cdk complexes and detects non-proliferative cells [Lee, 1995] stained positively for cells located in the villous core and for CTB, CCT and EVT (Fig. 13 B3). Here, the switch from downregulation of proliferation and exit from the cell cycle to non-growing cells, that will differentiate and undergo a controlled invasion process is illustrated.

Figures 14-16 describe the localization of proteins involved in canonical Wnt signaling in the human placenta. Stainings of TCF-3,-4 and LEF-1 are shown. TCF-1 was not detectable (data not shown). Further, the co-expression of β-catenin and TCF-4 is visualized.

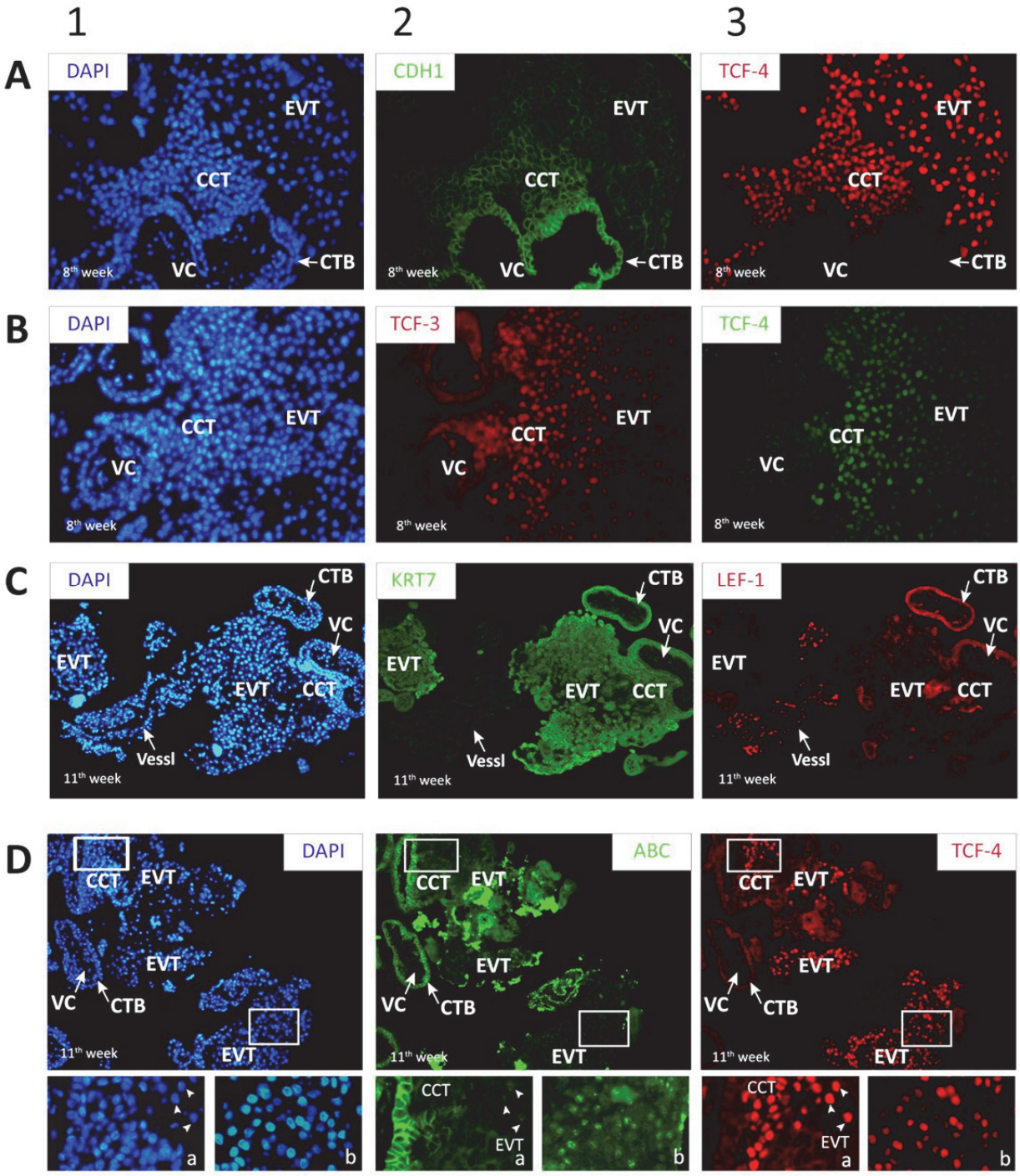


Figure 14: Immunofluorescence stainings of first trimester placental tissue showing members of the LEF/TCF-family, E-cadherin and ß-catenin

Sections are showing the co-expression of CDH1 (E-cadherin), hypophosphorylated ß-catenin (ABC) and KRT7 with the transcription factors TCF-3, 4 and LEF-1. DAPI staining was performed to visualize cell nuclei. VC = villous core, CCT = cell column trophoblast , CTB = cytotrophoblast, EVT= extravillous. White rectangles in D2, 3 are digitally zoomed to stress interesting regions shown in D2, 3a-b where translocation of ß-catenin to the nucleus (indicated by arrowheads) and co-expression with TCF-4 is depicted.

The tumor suppressor gene E-cadherin (CDH1), a cell-cell adhesion molecule in epithelia [Takeichi et al, 1988], was detectable on cell membranes of CTBs located on the trophoblastic basement membrane. CCTs and EVTs also showed a clear membrane staining and a decrease of the signal was noticed in more distally located cells (Fig 14 A2).

TCF-4 was found to be expressed in the nuclei of CCTs and EVTs, and upregulated upon differentiation to EVTs (Fig. 14 A3 and B3). TCF-3 was detectable in CCTs and EVTs (Fig. 14 B2). Co-staining shows the co-expression of TCF-3 and TCF-4 in trophoblast cells (Fig. 14 B2 and B3).

LEF-1 showed no nuclear staining in cells of the villous core or in trophoblastic cell types but was detectable in KRT7 negative nuclei of cells which are probably endothelial cells of a placental vessel, but were not further characterized here (Fig.14 C3).

Fig 14 D2 and D3 showed an anchoring villus at 100-fold magnification and distally located EVTs. White rectangles are digitally zoomed to stress interesting regions shown in D2, 3a-b. Hypophosphorylated active β-catenin (ABC), located at adherent junctions or translocated into the nucleus of Wnt signaling-active cells, could be observed at the membranes of CTBs located on the trophoblastic basement membrane and in nuclei of EVTs (Fig 14 D2a, b). Dropping of β-catenin from the membranes and translocation into the nucleus (indicated by arrowheads) is clearly shown (Fig 14 D2a, b). Further co-staining of TCF-4 and β-catenin in nuclei of EVT and absence of TCF-4 in cells with membrane bound β-catenin visualized that the decrease of β-catenin at the cell membrane in CCTs goes hand in hand with the appearance of TCF-4 (Fig. 14 D3a,b).

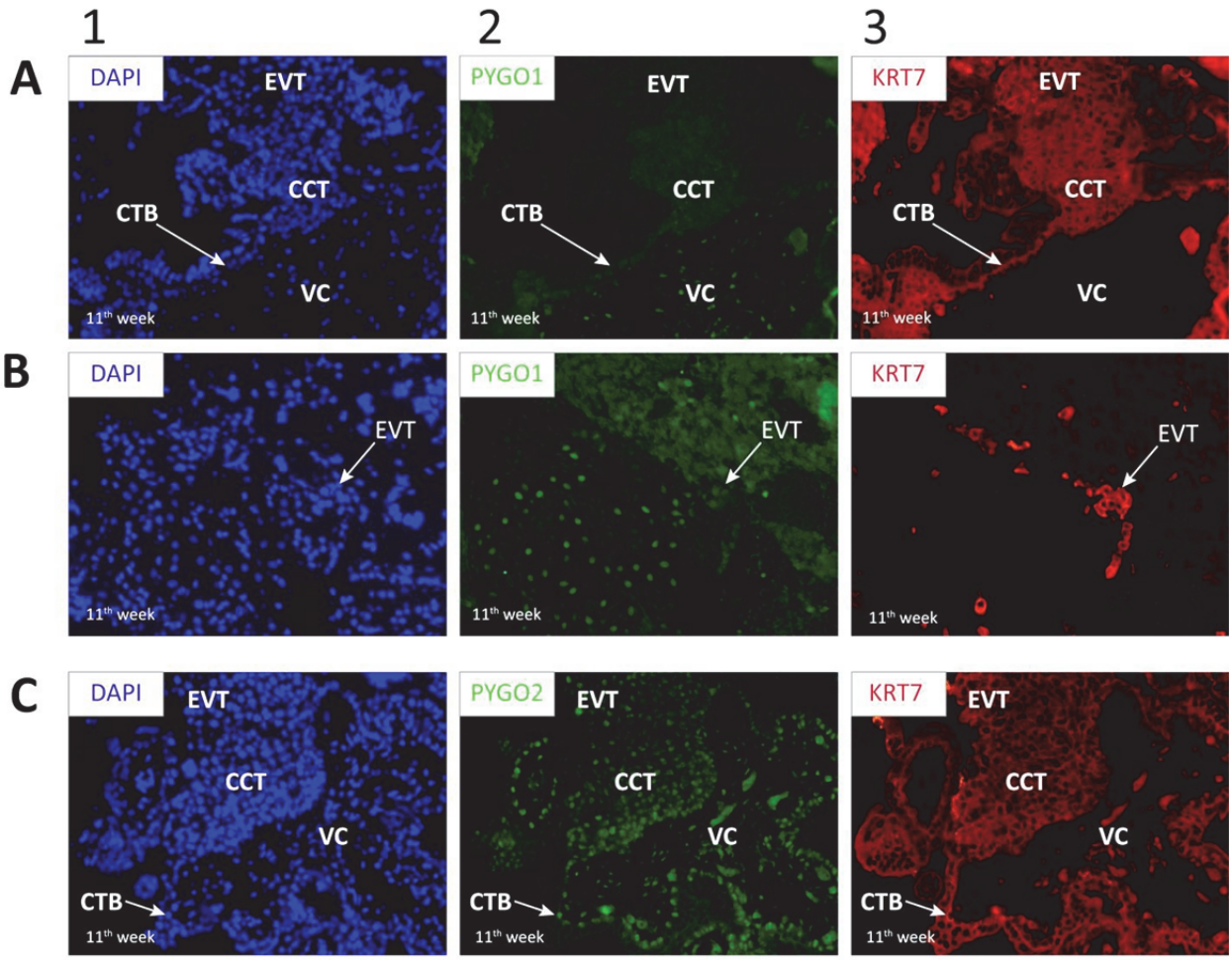


Figure 15: Localization of Pygopus1 and 2 in the human placenta using immunofluorescence
Immunofluorescence co-stainings of first trimester placental tissue sections detecting KRT7 and PYGO1, PYGO2 in placental cells. VC = villous core, CCT = cell column trophoblast , CTB = cytotrophoblast, EVT= extravillous trophoblast.

Pygopus 1 (PYGO1) was detectable in nuclei of cells of the villous core but was absent from trophoblastic cells (Fig.15 A2,3). KRT7-negative cells in the decidua stained positively for PYGO1 (Fig. 15 B 2 and 3).

Pygopus 2 (PYGO2) showed a positive nuclear staining in cells of the villous core and in KRT7 positive CTB, CCT and EVT (Fig 15 C2, 3).

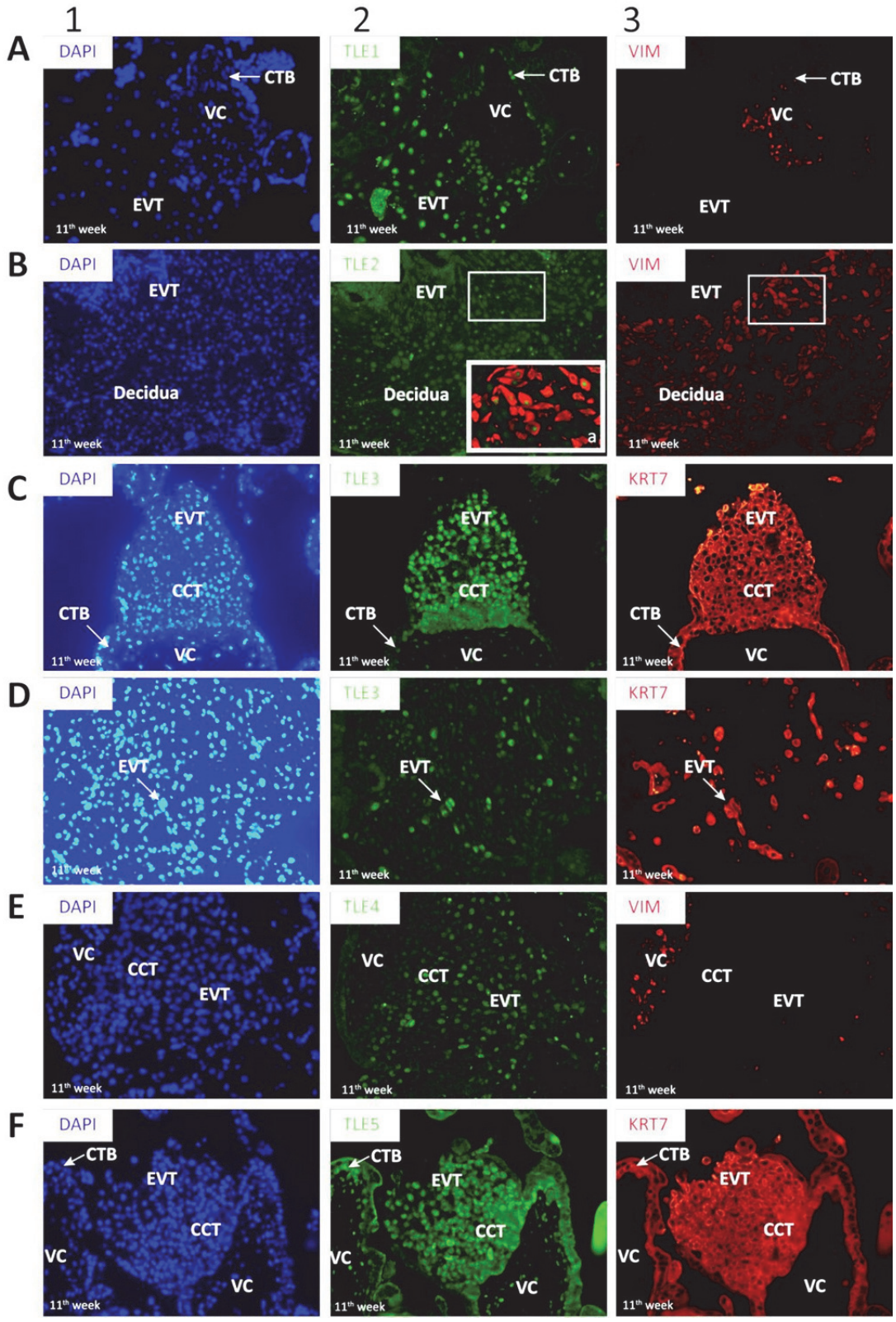


Figure 16: Detection of members of the TLE-family in the human placenta shown by immunofluorescence

Co-stainings of tissue sections detecting KRT7 or VIM and members of the TLE-family in placental cells. VC = villous core, CCT = cell column trophoblast , CTB = cytotrophoblast, EVT= extravillous trophoblast.

Nuclear staining of TLE1 (shown in green) was detectable in nuclei of vimentin (red)-negative CTBs, CCTs and EVTs (Fig. 16 A2-3). TLE2 was absent from trophoblastic cells in anchoring or floating villi (data not shown) but detectable in nuclei of vimentin positive cells in the decidua (Fig 16 B2, 3 and 2a). TLE3 (green) showed a nuclear staining in KRT7 (red) positive CCTs and EVTs and a weak staining in CTBs. Cells of the villous core were also positive for TLE3 (Fig. 16 C2-3). TLE3 was detectable in KRT7 positive cells in the decidua (Fig. 16 D2-3). Nuclear staining of TLE4 in CCTs and EVTs but not in vimentin positive cells of the villous core was shown (Fig. 16 E2-3). TLE5 protein is detectable in nuclei of KRT7-positive CTBs, CCTs and EVTs and also in KRT7 negative cells of the villous core (Fig. 16 F2-3).

3.2. Functional analyses of TCF-4 in SGHPL-5 cells

3.2.1 siRNA-mediated silencing of TCF-4 in SGHPL-5 cells

In order to learn more about the particular role of the EVT-specific transcription factor TCF-4, siRNA-mediated silencing of its gene was performed. For these experiments the trophoblastic cell line SGHPL-5 was used as an invasive model system. Cells treated with a pool of 4 siRNAs targeting TCF-4 showed a clear downregulation of the protein when compared to cells treated with non-targeting control siRNAs, which do not recognize any known sequence, and are therefore refered to as ntc siRNA (Fig. 17).

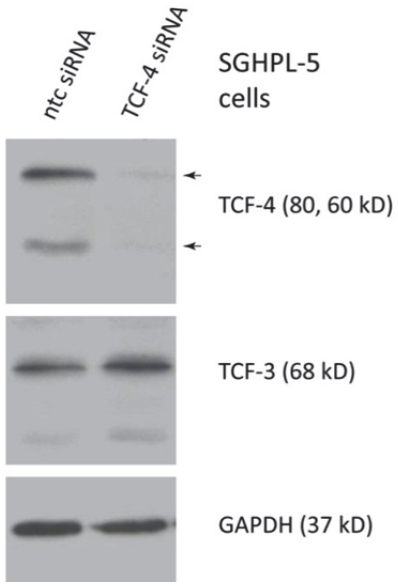


Figure 17: Western blot detecting TCF-4 silencing upon siRNA treatment in SGHPL-5 cells
Expression of TCF-4, TCF-3 and GAPDH protein in SGHPL-5 cells treated with ntc (non targeting control) or TCF-4 siRNAs for 72h are shown.

Figure 17 shows a Western blot analysis of TCF-4, TCF-3 and GAPDH, used as loading control, in cells that were exposed to siRNA for 72h. In ntc siRNA treated cells, two TCF-4 bands at 80 and 60 kD were detectable. The 80 kD band represents the sumoylated form of TCF-4 [Yamamoto et al., 2003]. In the TCF-4 siRNA treated sample, a clear downregulation was observed. In contrast, no difference in TCF-3 expression in both samples was observed, proving the specificity of the siRNA pools. To determine average TCF-4 down regulation levels, the 80 kD band of TCF-4 and the GAPDH band were quantified using ImageJ software in four independent silencing experiments. A ratio of TCF-4/GAPDH was calculated to compensate differences in loaded protein amounts. The mean TCF-4 level in ntc siRNA treated cells was set to 100 percent. Results were shown in the bar graph of Figure 18.

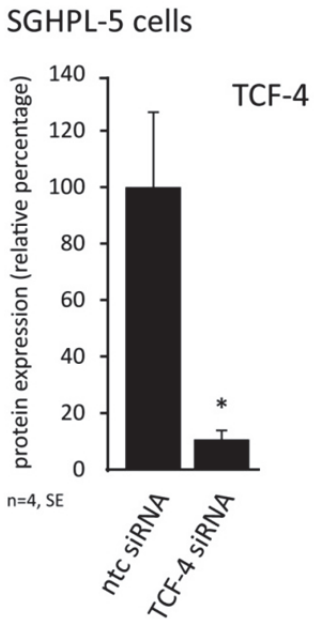


Figure 18 Quantification of the 80 kD band of TCF-4 in siRNA treated SGHPL-5 cells
Silencing efficiency was determined by quantification of the 80 kD band of TCF-4 and the GAPDH band using ImageJ software. The calculated ratio of TCF-4/GAPDH in ntc siRNA treated cells was set to 100 percent. TCF-4 was significantly downregulated to 10.6 % in TCF-4 siRNA treated cells compared to control cells. Mean values of 4 individual experiments were shown and deviations from the mean were displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and is indicated by an asterisk.

TCF-4 was significantly downregulated to 10.6 % in TCF-4 siRNA treated cells compared to control cells. TCF-4 silencing was also detectable on mRNA levels, as measured by quantitative real time PCR. RNA was extracted from siRNA treated cells after 72h. TBP levels were measured as house keeping control. TCF-4 and TBP signals were detectable between 20 and 30 performed cycles. TCF-4 levels were compensated by TBP levels and

the amounts of TCF-4 in ntc siRNA treated cells was set to 1. Results of 5 individual silencing experiments are shown in Figure 19.

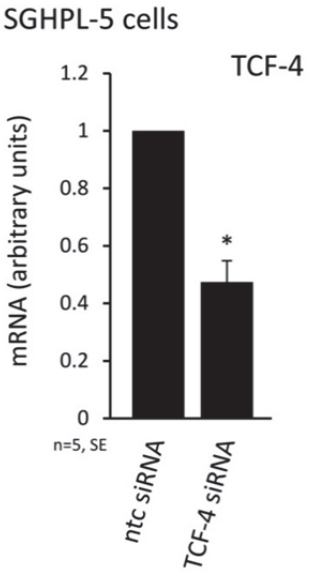


Figure 19: Quantification of the TCF-4 mRNA in siRNA treated SGHPL-5 cells using RT-qPCR
TCF-4 mRNA levels compensated to the housekeeping gene TBP are displayed and TCF-4 amounts in ntc siRNA treated cells were set to 1. TCF-4 mRNA was 2.1-fold downregulated in TCF-4 siRNA treated cells compared to ntc siRNA treated cells. Mean values of 5 individual silencing experiments were shown and deviations from the mean are displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

TCF-4 mRNA was 2.1-fold downregulated in TCF-4 siRNA treated cells showing that the siRNA pools directly target and degrade TCF-4 mRNA.

3.2.2. Activity of a canonical Wnt signaling reporter in TCF-4-silenced SGHPL-5 cells

As described by Pollheimer et al., 2006 and Sonderegger et al., 2006, the canonical Wnt reporter TOP flash was active in SGHPL-5 cells and inducible by treatment with recombinant Wnt3a. Figure 20 shows the activity of the reporter in TCF-4-silenced SGHPL-5 cells upon activation with the Wnt ligand Wnt3a and the global GSK-β inhibitor LiCl. 72 h after treatment with TCF-4 and ntc siRNA pools, cells were transfected with superTOPflash and CMV-β Gal plasmids, constitutively expressing ß-Gal, for normalization of the transfection efficiency. 3h post-transfection, reporter activity was activated by Wnt3a or LiCl and after 24h, the activity of β-galactosidase and luciferase was determined.

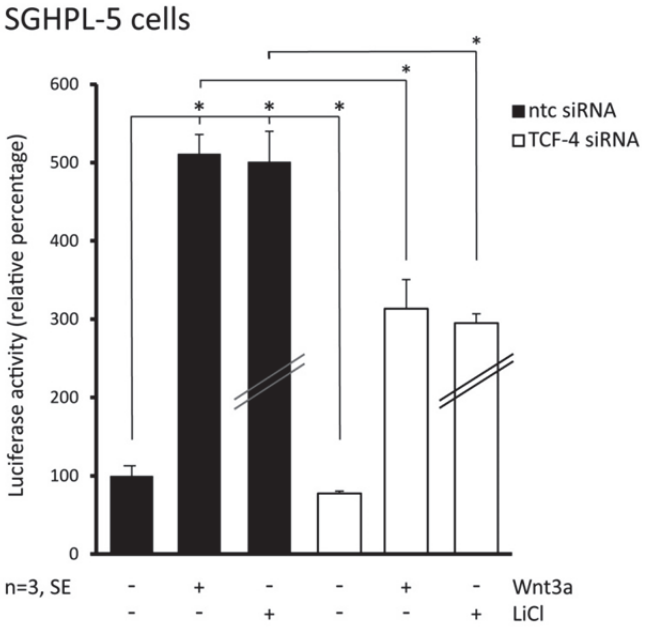


Figure 20: Activity of the canonical Wnt signaling reporter Super TOPflash in TCF-4 treated SGHPL-5 cells upon treatment with Wnt signaling inducers
TCF-4 and ntc siRNA treated cells were transfected with superTOPflash and CMV-ß Gal plasmids, reporter activity was induced by Wnt3a or LiCl, the activity of ß-galactosidase and luciferase was determined. Luciferase activity was normalized to ß-galactosidase and ntc siRNA treated SGHPL-5 cells were set to 100 %. All samples were set into relation to this value and results of 3 experiments performed in duplicates for each condition are shown. For a better graphical presentation, LiCl-induced values were divided by 100 and marked with diagonal double lines in the diagram. Mean values were shown and deviations from the mean are displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

The activity of the canonical Wnt reporter superTOP flash was significantly induced by recombinant Wnt3a (5-fold) and LiCl (500-fold) in ntc siRNA treated cells. The activity of the reporter in the TCF-4 siRNA treated cells compared to control cells was significantly reduced to 77.5 %. Furthermore, TCF-4 siRNA-treated cells showed a significantly reduced ability to be activated by recombinant Wnt3a (3-fold) and LiCl (300-fold).

3.2.3. Migratory properties of TCF-4-silenced SGHPL-5 cells

To test whether TCF-4 influences the migratory behavior of SGHPL-5 cells, ntc siRNA and TCF-4-siRNA treated cells were seeded onto fibronectin-coated transwells. As shown by Pollheimer et al., 2006, recombinat Wnt3a induced migration in SGHPL-5 cells. Therefore, activation of Wnt signaling using recombinant Wnt3a was performed as control in this assay. Invaded cells on the lower side of each transwell were stained after 24h and digitally counted. The quantification is shown in figure 21.

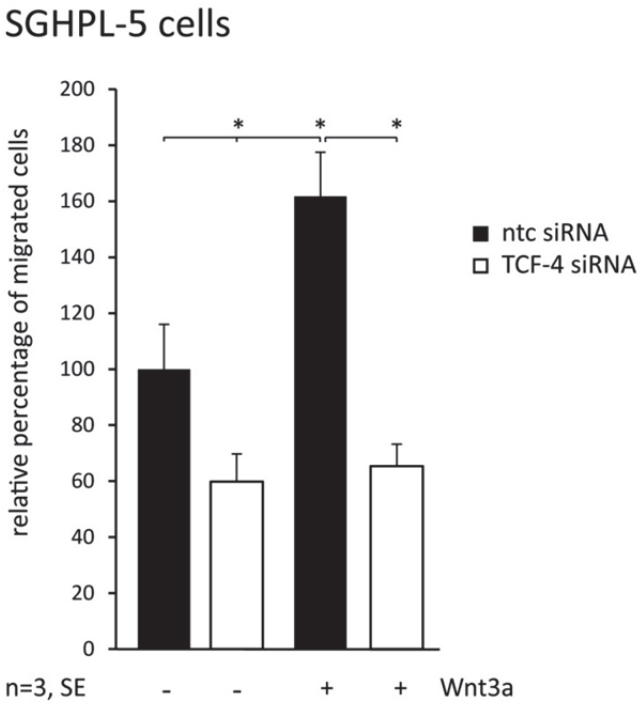


Figure 21: Migration assay comparing ntc and TCF-4 siRNA-treated SGHPL-5 cells under the influence of Wnt3a

Ntc siRNA and TCF-4-siRNA treated cells were seeded onto fibronectin coated transwells and incubated with or without Wnt3a, migrated cells were determined after 24h. Ntc siRNA-treated SGHPL-5 cells were set to 100 % and all other conditions were correlated to this value. Means of three independent experiments were shown, deviations from the mean are displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

The ability to migrate through fibronectin was significantly reduced to 60 % in TCF-4 siRNA-treated cells compared to control cells. Wnt3a induced migration in control cells 1.62-fold and TCF-4 siRNA-treated cells showed reduced migration to 65% upon activation with Wnt3a compared to control cells. These data suggest that TCF-4 is important for the migratory potential of SGHPL-5 cells.

3.2.4. Proliferative properties of TCF-4 silenced SGHPL-5 cells

TCF-4 controls proliferation in the stem cell compartment of the small intestine [Korinek et al., 1998]. Therefore, we evaluated whether silencing of TCF-4 and/or induction of Wnt signaling using recombinant Wnt3a alters the proliferation rate of SGHPL-5 cells. After treatment of SGHPL-5 cells with ntc and TCF-4 siRNA, cumulative cell numbers of both cell pools were measured in the absence or presence of recombinant Wn3a after 24, 48 and 72h. The mean of four independent experiments performed in triplicates are shown in figure 22.

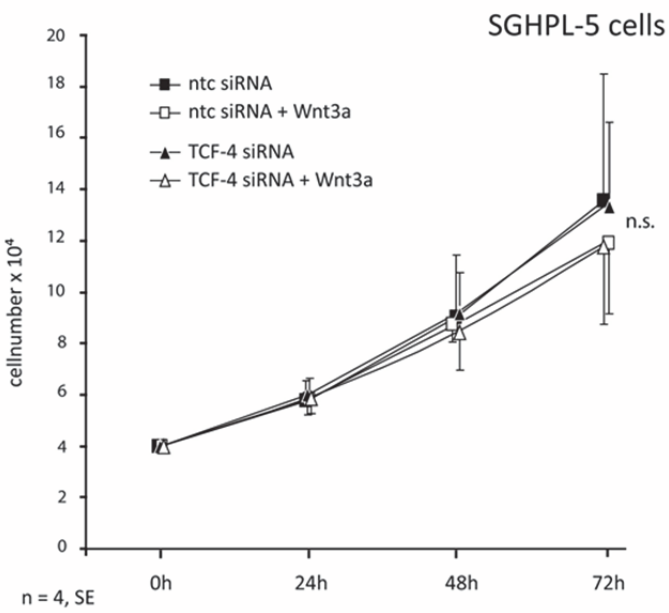


Figure 22: Proliferation assay showing the influence of TCF-4 and Wnt3a on siRNA-treated SGHPL-5 cells
Cumulative cell numbers of both siRNA treated cell pools were measured in the absence or presence of recombinant Wn3a after 24, 48 and 72h. The mean of four independent experiments performed in triplicates are displayed and deviations from the mean are indicated by the SE. Decrease of proliferation under Wnt3a stimulation at 72h of both cell pools did not reach statistically significance indicated by n.s..

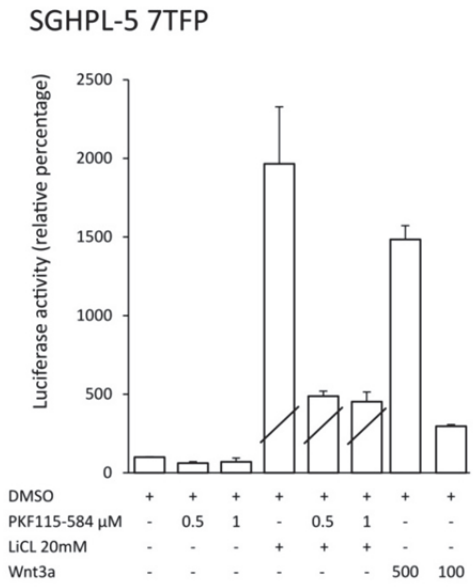
Interestingly, no significant alteration of the proliferation rate was detectable under the indicated conditions.

3.2.5. Inhibition of canonical Wnt signaling in SGHPL-5 cells

The Wnt signaling inhibitor PKF115-584, which directly interferes with TCF-4/β-catenin, was tested in SGHPL-5 cells in order to establish an additional tool to inhibit Wnt signaling besides siRNA treatment. For this purpose, the lentiviral canonical Wnt reporter cassette 7TFP was stably introduced into SGHPL-5 cells and tested for its activity. SGHPL-5-7TFP cells were established by lentiviral transduction and puromycin selection as described in

materials and methods. For functional testing of the reporter cell line, induction with recombinant Wnt3a and LiCl as well as treatment with PKF118-584 was performed.
SGHPL-5-7TFP cells were treated with PKF115-584, recombinant Wnt3a and LiCl for 24h. (figure 23 A and B).

A



B

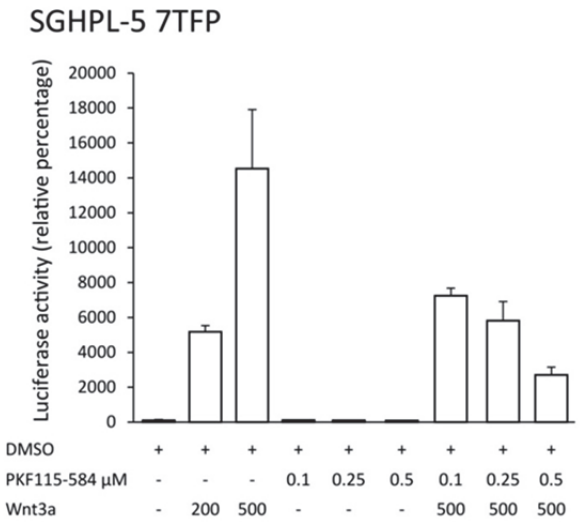


Figure 23: Reporteractivity of stable SGHPL-5 7TFP cells upon inhibition or activation of canonical Wnt signaling
Cells were treated with PKF115-584, recombinant Wnt3a and LiCl for 24h. Activity of luciferase was measured and correlated to the whole protein amount of each sample, measured by Bradford assay. DMSO-treated cells were set to 100 % and all conditions were related to this value. All conditions were performed in duplicates. For a better graphical presentation, LiCl-induced valus were divided by 5 as indicated by diagonal lines.

As shown in figure 23A, the Wnt signaling inhibitor PKF115-584 reduced the basal activity of the reporter to 62% and 69% at a concentration of 0.5 µM and 1 µM, respectively. Another titration in figure 23B showed that 0.1 or 0.25µM did not influence the activity of the reporter, but 0.5 µM PKF115-584 reduced the reporter activity to 93%. LiCl activated the reporter 100-fold, Wnt3a 14-fold at 500 ng/ml and 3-fold at 100 ng/ml concentration. The LiCl induction of the reporter activity was 24-fold reduced by PKF115-584 at a concentration of 0.5 µM and 22-fold reduced at 1 µM, showing a clear repression of activation. In figure 23B, Wnt3a induced reporter activity 50-fold at a concentration of 200 ng/ml and 145-fold at a concentration of 500 ng/ml. The induction of the reporter with 500 ng/ml Wnt3a was repressed by PKF115-584 in a dose-dependent manner at a concentration of 0.1, 0.25 and 0.5 µM to 72, 58 and 27 fold. The repressive effect on basal activity was not compelling and needs further verification, however these results are in accordance with published data in other cell systems. For further experiments, PKF115-584 was used at a concentration of 0.5µM.

Proliferation studies using 0.5 µM PKF115-584 showed no significant alterations in proliferation after 24h or 48h. Toxic effects of the substance were observed after 72 hours (data not shown) as published in other cell systems.

3.2.6. Influence of PKF115-584 on migrating SGHPL-5 cells

Fibronectin or growth factor-reduced matrigel coated and uncoated transwells were used to test the ability of SGHPL-5 cells to migrate under Wnt-inhibited conditions. Cells were seeded on transwells and incubated in the absence or presence of 0.5µM PKF115-584 for 24 hours. For fibronectin-coated transwells, two individual experiments performed in duplicates and for matrigel and plastic one experiment in duplicates are shown. Control cells were treated with DMSO, the solvent of the inhibitor, in equal amounts.

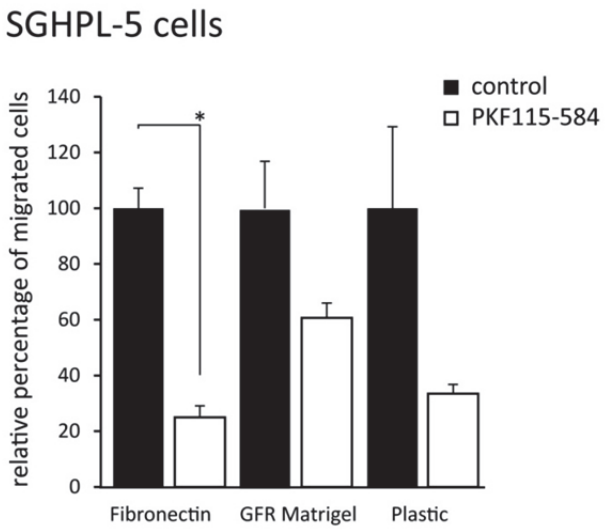


Figure 24: Migration assay showing the effects of PKF115-584 on SGHPL-5 cells
Cells were seeded on fibronectin-coated, growthfactor-reduced matrigel or uncoated transwells and incubated in the absence or presence of 0.5µM PKF115-584 for 24 hours. For fibronectin-coated transwells, two individual experiments performed in duplicates and for matrigel and plastic one experiment in duplicates is shown. Control cells were treated with DMSO, the solvent of the inhibitor, in equal amounts and set to 100%, all other samples were related to this value. Means ± SE are shown and for fibronectin samples a T-test p-value <0.5 was regarded as statistically significant and indicated by asterisk.

Migration of SGHPL-5 cells was reduced under the influence of the Wnt inhibitor PKF115-584 on fibronectin to 25%, on growth factor-reduced matrigel to 60% and on plastic to 34% compared to control cells. Taken together, these data suggest that inhibition of canonical Wnt signaling reduces migration properties of the trophoblastic cell line, which confirms the results with TCF-4-silenced SGHPL-5 cells.

3.2.7. Putative TCF-4 target genes in SGHPL-5 cells

To learn more about genes that are directly controlled by the transcription factor TCF-4 and canonical Wnt signaling, genes that influence migration were selected. To investigate whether TCF-4 directly targets the matrix metalloproteinases MMP2, MMP9,the PAI/ uPA-system (Plasminogen activator inhibitor / human u-Plasminogen Activator/Urokinase-system), Cyclin D1 or the fibronectin receptor integrin α5 (ITGA5). SGHPL-5 cells were treated with ntc and TCF-4 siRNAs. The next day, medium was changed to serum-free growth medium and supernatants as well as total protein extracts from the cell pools were collected 96h after siRNA treatment. The Western blots from cell extracts and concentrated supernatants are shown in Figure 25.

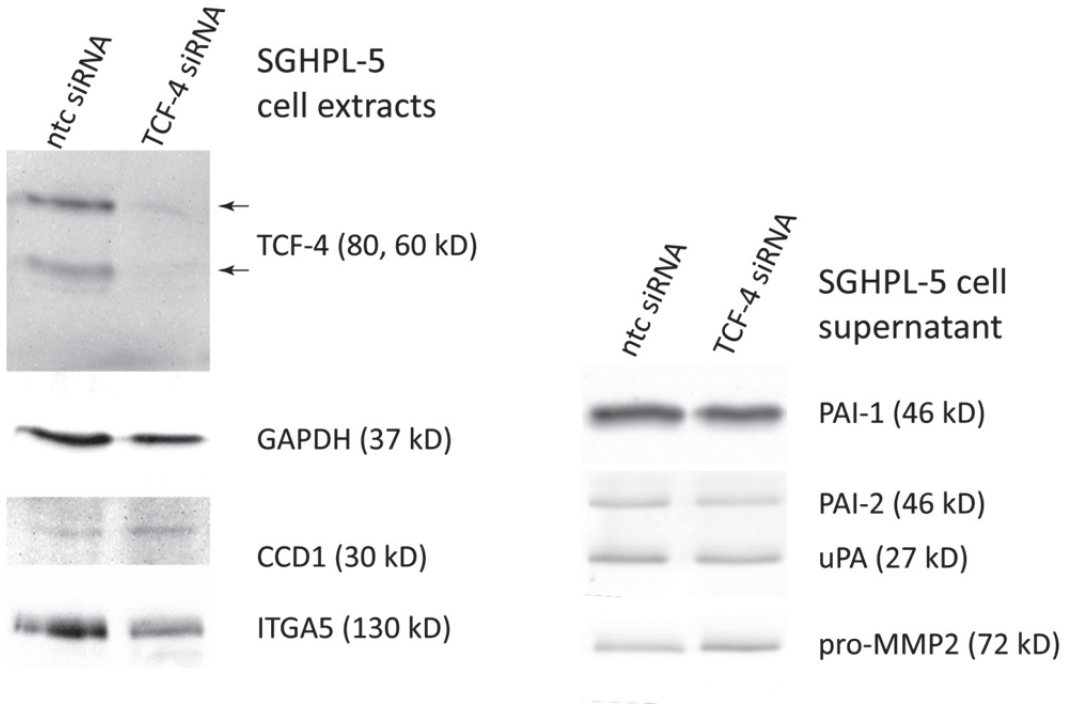


Figure 25: Western blot of siRNA-treated SGHPL-5 cells detecting putative target genes
Cells were treated with ntc and TCF-4 siRNAs. The next day, medium was changed to serum-free growth medium and supernatants as well as total protein extracts from the cell pools were collected 96h after siRNA treatment. TCF-4 protein expression was shown to prove silencing efficiency; GAPDH was shown as loading control. CyclinD1 and ITGA5 were detected in cell extracts, furthermore secreted PAI1 and PAI2, uPA and pro-MMP-2.

In the prepared cell extracts, a strong downregulation of TCF- 4 in the TCF-4 siRNA treated cells was detectable compared to ntc siRNA treated cells. GAPDH was used as loading control. Furthermore, no change in the expression of CyclinD1, a known Wnt target, was detectable. In contrast, expression of ITGA5, the fibronectin receptor was reduced in the TCF-4-silenced population. Western blots from supernatants showed no changes in the amounts of sectreted PAI-1 and PAI-2 or uPA. Further, no changes in MMP-2 or MMP-9 (data not shown because of weak signals) expression were detectable.

Working with cell lines as model system has a lot of advantages but still, such cell systems differ from the in vivo situation. Isolation of primary EVTs and the villous explant culture system and manipulation of these cells/cultures is a big challange but opens the door to answer a lot of questions regarding the in vivo situation. To figure out if findings from SGHPL-5 cells can be confirmed in isolated and in vitro cultivated EVTs and in placental explants, canonical Wnt reporter assays were performed.

3.3. Functional analyses of TCF-4 in primary trophoblasts

3.3.1 Activity of canonical Wnt signaling in EVTs

Isolated EVTs are slowly dividing cells and lose their proliferation activity within 24h in culture. Since plasmid transfection of these cells is challenging, electroporation was used to deliver the superTOP/FOP reporter and the CMV-β Gal plasmids into primary trophoblasts. After 24h of transfection, recombinant Wnt3a was added to the cells and luciferase and β-galactosidase activity was measured 24h later (Figure 26).

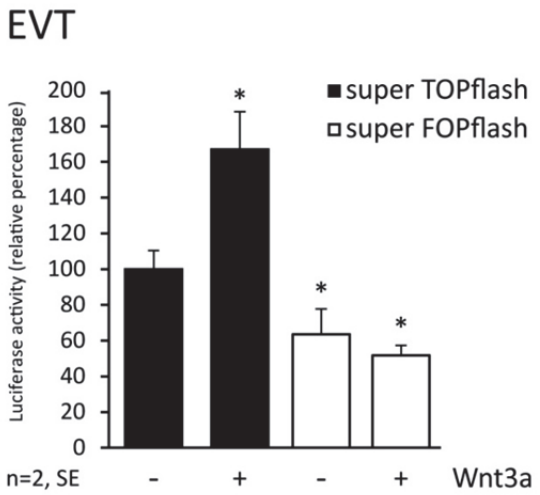


Figure 26: Activity of the canonical Wnt signaling reporter Super TOP/FOPflash in primary EVTs upon Wnt3a induction
Electroporation was used to deliver the superTOP/FOP reporter and the CMV-ß Gal plasmids into primary trophoblasts. After 24h of transfection, recombinant Wnt3a was added to the cells and luciferase and ß-galactosidase activity was measured 24h later. Luciferase activity was normalized to ß-galactosidase activity. TOP activity was set to 100% and all conditions were correlated to this value. 2 independent experiments were performed in duplicates. Means ± standard error (SE) were shown, a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

The basal activity of the TOP reporter was found to be significantly higher (40%) than the activity of the mutated FOP reporter. The TOP reporter was inducible 1.67-fold in EVTs upon Wnt3a treatment, but the activity of the FOP reporter was unchanged. To identify specific cells in the growing placenta showing active canonical Wnt signaling, placental explant culteres were transduced with the lentiviral canonical Wnt reporter 7TFP as mentioned above. Explants showing outgrowth after 24h, were incubated with lentiviral particles diluted in polybren-containing growth medium. After 48h, explants were fixed with parafromaldehyde and stained with an antibody detecting produced luciferase indicating active canonical Wnt signaling. Non-transduced explants were used as control (Fig. 27 A). Representative pictures are shown in figure 27.

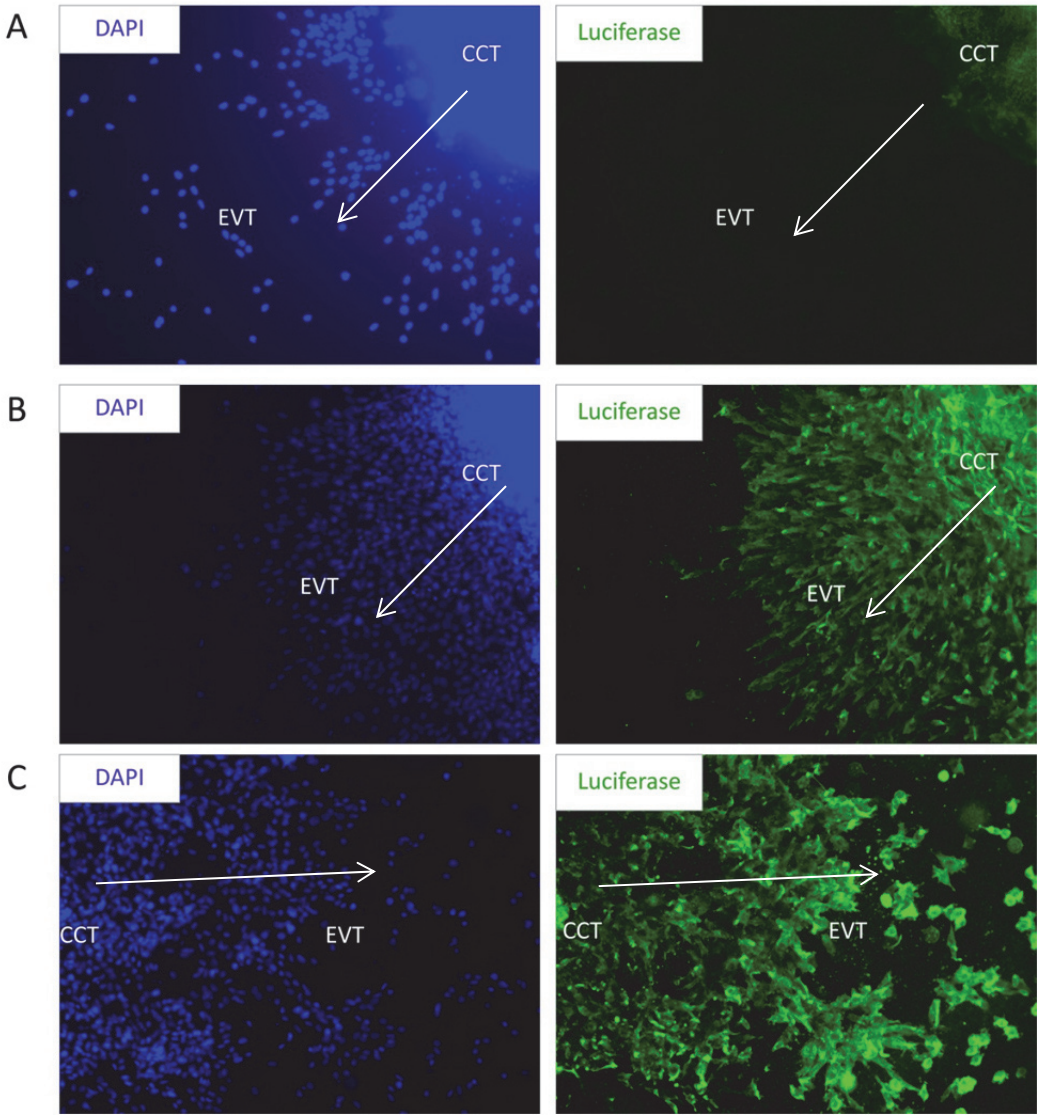


Figure 27: Activity of the canonical Wnt signaling reporter 7TFP in placental explants
Placental explant cultures were transduced with the lentiviral canonical Wnt reporter 7TFP fixed with paraformaldehyde and stained with an antibody detecting produced luciferase indicating active canonical Wnt signaling. Non-transduced explants were used as control. Panel A showed a fixed and and luciferase stained control explant. Panel B and C showed reporter casette treated explants. Localication of nuclei was shown by DAPI staining and direction of outgrowth was indicated by the arrows.

In panel A representing a control explant no luciferase was detectable. As shown in panel B, luciferase was detectable in proliferative CCTs as well as in migrating EVTs of the growing explant. Panel C showed a different explant with a larger outgrowth area and EVTs which migrated more distally. In these explants an inrease of luciferase in more distally located EVTs was noticed.

3.3.2. siRNA-mediated silencing of TCF-4 in isolated EVTs

To learn more about the role of TCF-4 in EVT migration and/or differentiation, TCF-4 silencing was performed in isolated EVTs using electroporation directly after isolation. The Western blot in figure 28 shows the downregulatin of TCF-4 in TCF-4 siRNA-treated cells compared to control cells. GAPDH served as loading control.

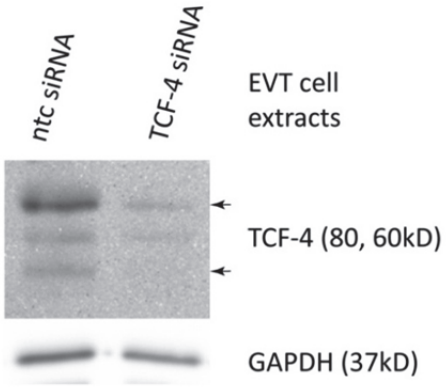


Figure 28: Western blot of siRNA-treated EVTs
TCF-4 and GAPDH protein levels of siRNA treated primary trophoblasts were shown to confirm TCF-4 silencing in TCF-4 siRNA treated cells.

Efficient downregulation of TCF-4 was observed in cell extracts of isolated EVTs 72 hours after treatment with siRNA. To quantify this effect, the 80kD band of TCF-4 was normalized to GAPDH in 5 individual silencing experiments and results are shown as bar graphs in figure 29.

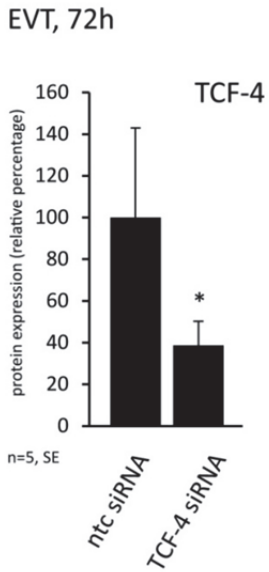


Figure 29: Quantification of the 80 kD band of TCF-4 in siRNA-treated EVTs
Silencing efficiency was determined by quantification of the 80 kD band of TCF-4 and the GAPDH band using ImageJ software. The calculated ratio of TCF-4/GAPDH in ntc siRNA treated cells was set to 100 percent. Mean values of 5 individual experiments were shown and deviations from the mean were displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

TCF-4 was downregulated in isolated EVTs to 38.7%. Compared to the data on SGHPL-5 cells, downregulation was less efficient in primary cells. To analyse whether the siRNAs directly target TCF-4 mRNA in EVTs, total RNA was extracted from 6 individual silencing experiments after 24h and 48h and TCF-4 mRNA was quantified and normalized to TBP (Figure 30).

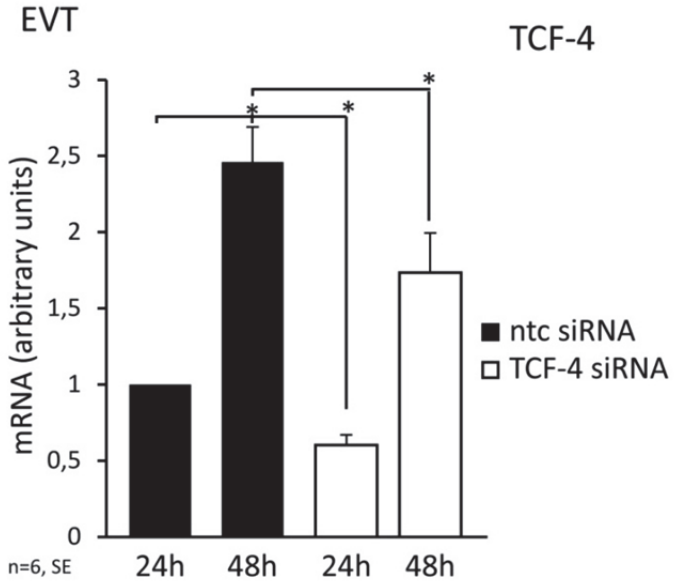


Figure 30: Quantification of the TCF-4 mRNA in siRNA-treated EVTs using RT-qPCR
Total RNA was extracted from 6 individual silencing experiments after 24h and 48h and TCF-4 mRNA was quantified and normalized to TBP. The normalized TCF-4 value at 24h in the ntc siRNA treated samples were set to 1 and all other conditions were related to this value, fold changes were shown. Mean values of 6 individual experiments were shown and deviations from the mean were displayed by the standard error (SE), a T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk.

TCF-4 mRNA was found to be 2.45-fold upregulated in ntc siRNA-treated EVTs after 48 hours compared to control, measured 24 hours after seeding. A 60% downregulation of TCF-4 in TCF-4 siRNA-treated cell extracts was observed after 24h, reaching 68% after 48h. These results show that the used siRNAs directly target TCF-4 mRNA. Downregulation of TCF-4 protein after 24h and 48h was also detectable on the protein level (data not shown) but was most efficient after 72h.

3.3.3. Putative TCF-4 target genes in EVTs

Immunofluorescence co-stainings of ITGA5/TCF-4 and ITGA1/P57 were performed on TCF-4-silenced EVTs after 24h and 48h to assess whether TCF-4 downregulation and changes in the differentition process can be observed.

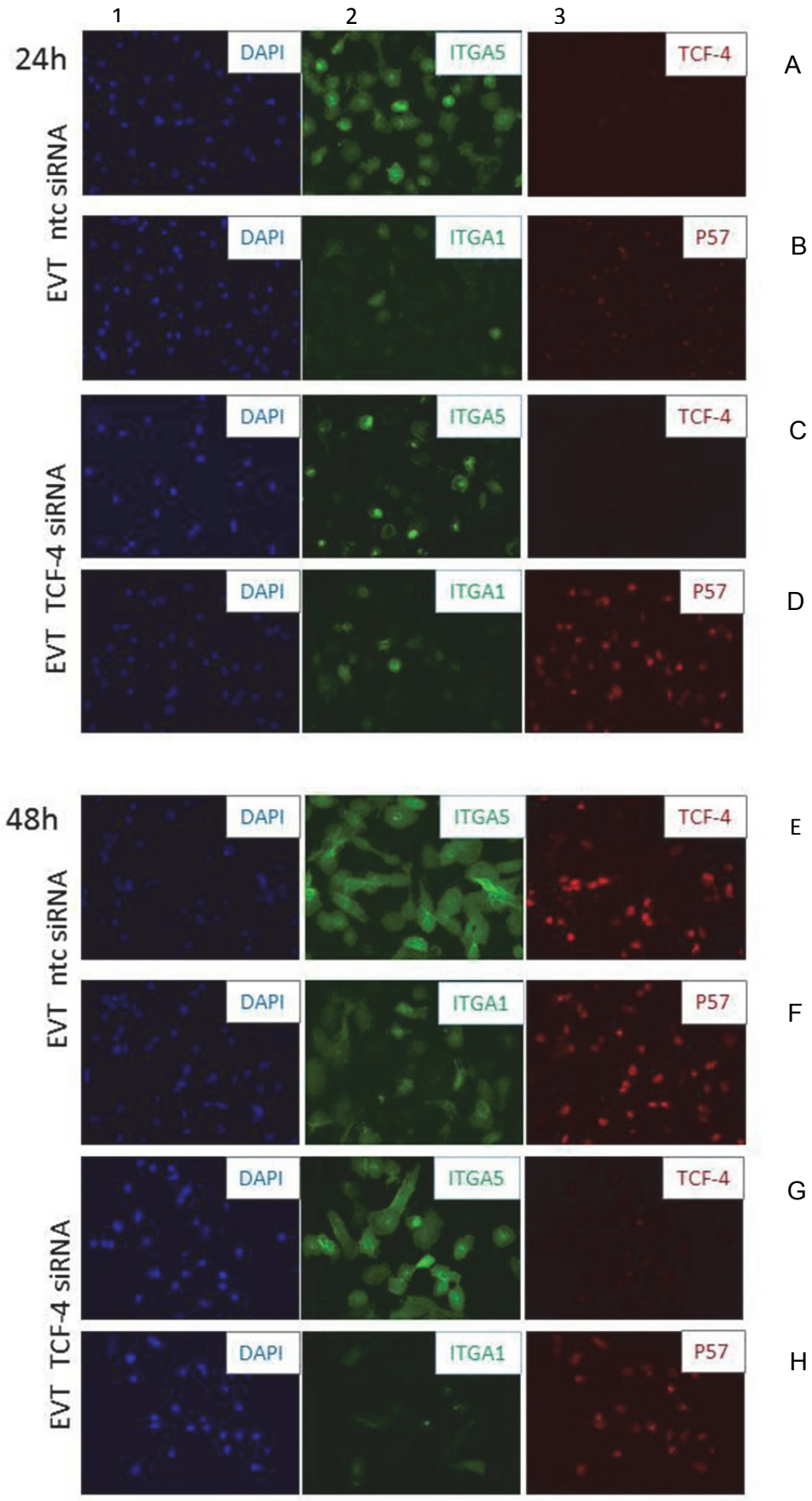


Figure 31: Immunofluorescence of siRNA-treated EVTs 24h and 48h after isolation
Co-stainings of ITGA5/TCF-4 and ITGA1/P57 were performed on TCF-4-silenced EVTs after 24h and 48h compared to controls. Panel 1A-H shows DAPI staining to localize cell nuclei in EVTs.

After 24 hours of cultivation, ntc and TCF-4 siRNA-treated EVTs showed membrane staining of ITGA5 (2A, C), ITGA1 (2B, D) and nuclear staining of P57 (3B, D). Nuclear staining of TCF-4 (3A and 3D) was found to be very weak. After 48h, ITGA5 (2E) and ITGA1 (2F) expression increased clearly as well as nuclear TCF-4 (3E) staining in ntc siRNA-treated EVTs. Changes in P57 (3F and 3H) staining were not observed. In TCF-4-treated EVTs, expression of TCF-4 (3G) was considerably weaker when compared to the ntc siRNA-treated sample (3E). Reduced ITGA5 (G2) and ITGA1 (H2) expression in the TCF-4-silenced cells was ambiguous and therefore requires further verification. However, it seems that downregulation of TCF-4 decelerated EVT differentiation. This assumption is underlined by treatment of EVTs with the Wnt inhibitor PKF115-584 for 24h, which resulted in morphological changes of the cells as shown in figure 32 and 33.

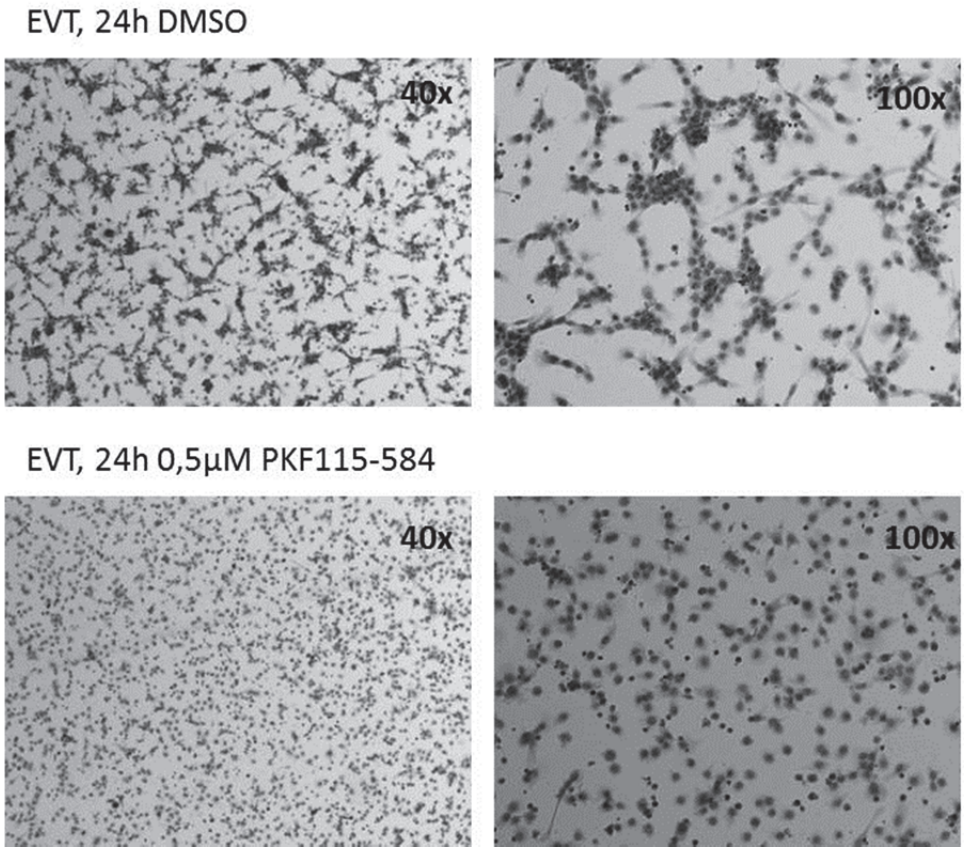


Figure 32: Hematoxyline staining of EVTs in the absence or presence of PKF115-584
Isolated trophoblasts were seeded on fibronectin-coated chamberslides and incubated for 24h with or without the Wnt inhibitor PKF115-584 (0.5 µM). After staining pictures were taken at 40-fold and 400-fold magnification.

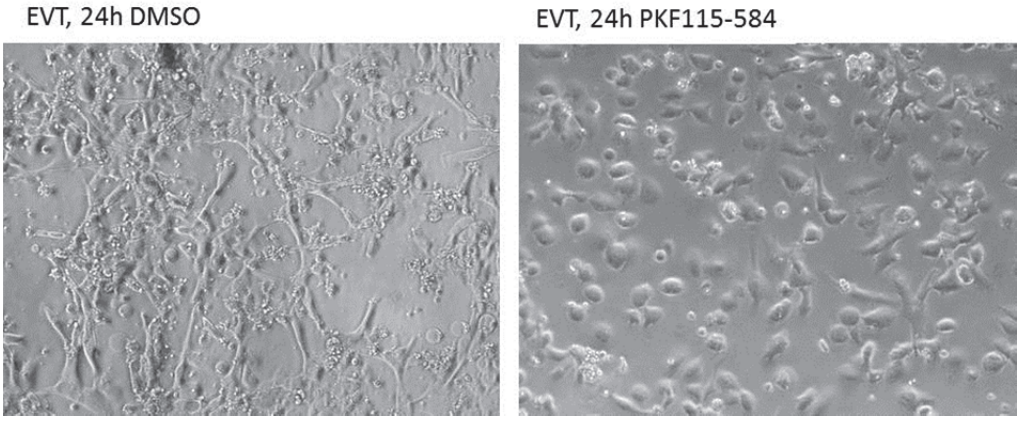


Figure 33: In vivo photographed EVTs +/- PKF115-584 treatment

3.3.4. Migratory properties of Wnt signaling-inhibited EVTs

To investigate whether reduced Wnt signaling affects the migratory ability of EVT migration, motility assays using fibronectin-coated transwells were performed (Figure 34).

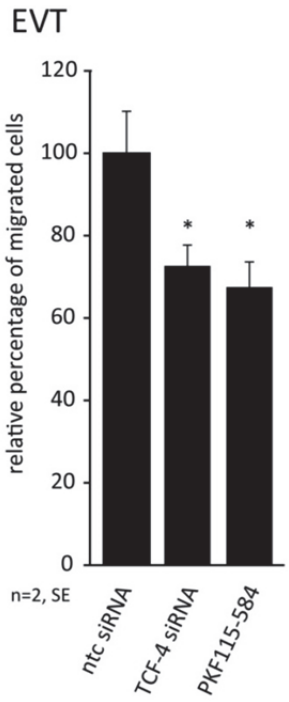


Figure 34: Migration assay of TCF-4-silenced EVTs +/-PKF115-584 treatment
TCF-4 silencing was performed in isolated EVTs using electroporation directly after isolation. siRNA treated trophoblasts and untreated trophoblasts were seeded on fibronectin-coated transwells an treated with DMSO and PKF115-584 (0.5µM) containing medium, repectively. Migrated cells were counted after 72h. Ntc siRNA-treated EVTs were set to 100 % and all conditions were correlated to this value. Means ±SE of 2 individual experiments performed in dublicates are displayed and a T-test p-values <0.05 was regarded as statistically significant and indicated by asterisk.

Upon silencing of TCF-4, migration of EVTs through fibronectin-coated transwells was significantly reduced to 72.7% in TCF-4 siRNA-treated cells and to 67.5 % in PKF115-584-treated cells.

The placental villous explant system allows to test effects of specific substances on trophoblast proliferation, migration and differentiation. This model system has the advantage that the anatomical structure of the placenta is preserved. Therefore, villous explants were used to study the effects of Wnt inhibition where the cell-cell interactions are more similar to the in vivo situation as compared to isolated cells. Villous explants were seeded on collagen and submerged with medium containing DMSO as control or 0.5 µM PKF115-584 (Figure 35).

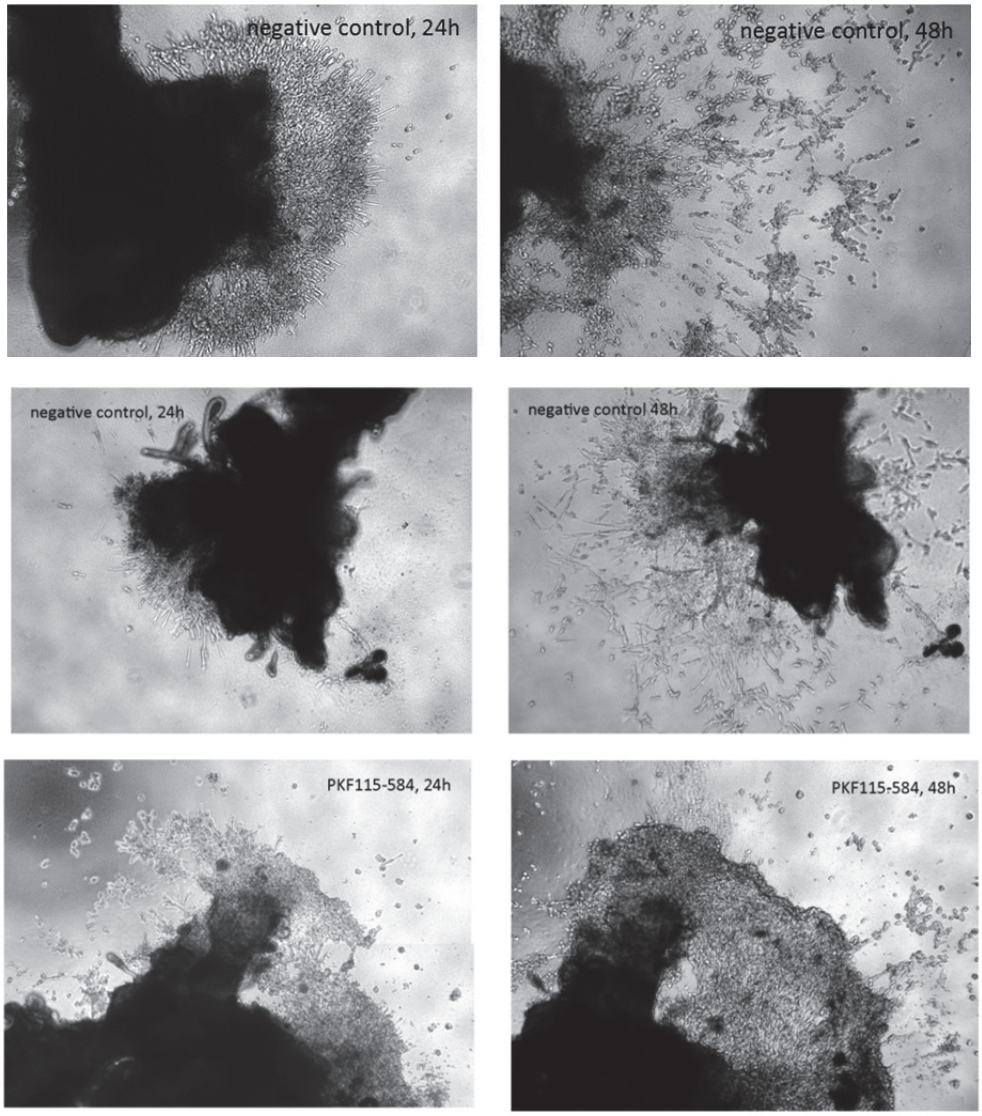


Figure 35: Placental explants +/- PKF115-584 treatment
Villous explants were seeded on collagen and submerged with medium containing DMSO as control or 0.5 µM PKF115-584. 16 explants per condition in two individual experiments were performed and representative pictures were taken after 24 and 48h at a 40-fold magnification.

The pictures marked with negative control show 2 examples of growing explants 24 and 48h after seeding on collagen drops. Cells proliferate at the villous tips, differentiate and migrate on the surface of the collagen and spread over the drop. In the PKF115-584-treated explants, the proliferative part of the explant seemed to expand and the ability of EVTs to migrate away from the villous tip was reduced. Here, EVTs stayed in the tightly packed environment of the cell column rather than spreading away.

To investigate whether treatment with TCF-4 si RNAs shows similar effects, one experiment using 10 explants per condition was performed. Due to poor outgrowth, pictures shown in Figure 36 are preliminary and not statistically significant.

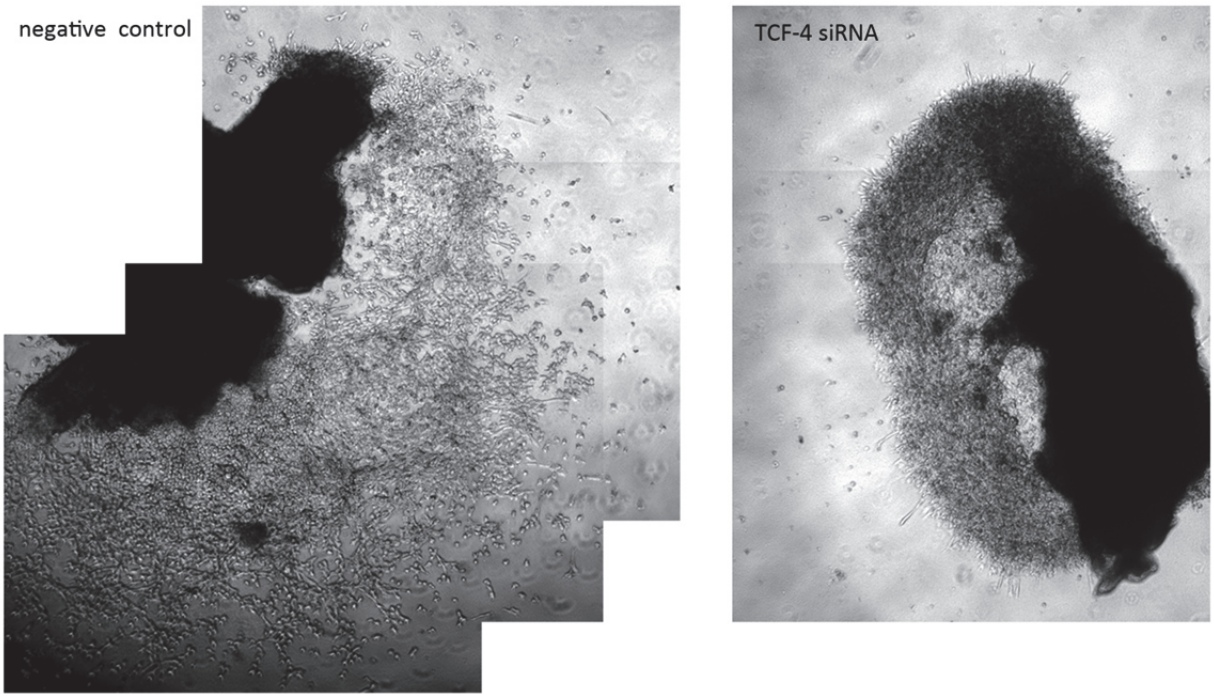


Figure 36: Placental explants treated with TCF-4 siRNA after 48h in culture
An untreated explant with a large outgrowth aera is shown as control. The TCF-4 siRNA-treated explant on the other hand showed a tightly-packed proximal outgrowth area and the ability of the cells to detach and migrate from the explant was reduced.

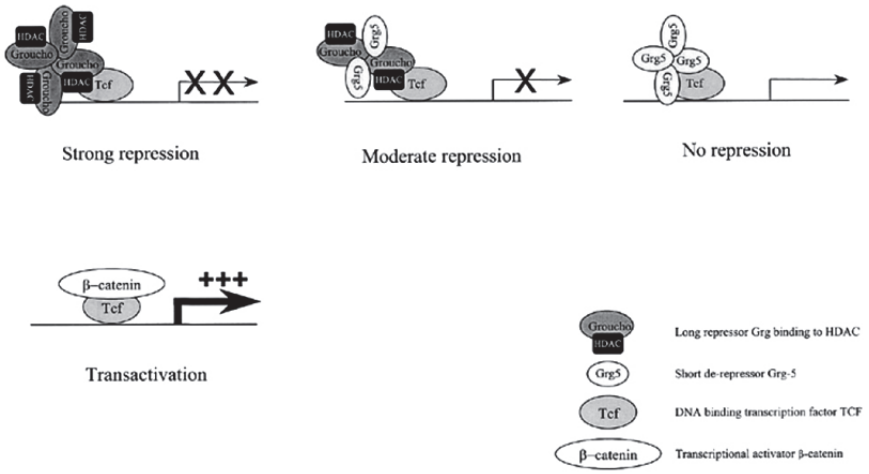
4. Discussion and future aspects

Invasion of EVTs into maternal tissue is a remarkable process showing similarities to tumor cell invasion and endothelial to mesenchymal transition (EMT). However, in contrast to tumour cell invasion invasiveness and deepness of EVT invasion are tightly regulated processes controlling development of a functional placenta that will satisfy the requirements of the growing fetus. Among others, Wnt signaling is one of the pathways contributing to this process. The current work focuses on the description of the transcriptional complex of canonical Wnt signaling in trophoblasts. Activity studies in an invasive cell model system under activating or inhibitory stimuli, and in isolated primary cells as well as villous explant cultures showed the functionality of the cascade. siRNA-mediated downregulation of TCF-4 and chemical inhibition of the transcriptional complex were used to explain downstream effects of TCF-4 and canonical Wnt signaling on migration and differentiation of trophoblasts.

Composition of the transcriptional complex of canonical Wnt signaling in EVTs of the human placenta

Immunofluorescence stainings of placentae of the first trimester of gestation (Fig. 13-16) revealed that the transcription factors TCF-4 and TCF-3 but not TCF-1 or LEF1 are expressed in CCTs and EVTs (Fig 14). In the *off state* of Wnt signaling, where transcriptional repression happens at target gene promoters, TCF-4 and TCF-3 are able to form complexes with transcriptional inhibitors TLE1, TLE3 or TLE4 and the truncated TLE5, which are all detectable in EVTs. TLE2 was only found in nuclei of vimentin positive cells in the decidua. As shown by Brantjes et al. 2001, all these proteins can interact with each other, which opens a wide field of regulation illustrated by the cartoon in figure 37.

Figure 37: Schematic of a putative inhibitory mechanism of the TLE family [Brantjes, 2001]



All illustrated scenarios of regulation are possible in trophoblasts and perhaps different modes of inhibition occur at individual target sites in just one cell.

In the *on state,* TCF-4/TCF-3 form a complex with β-catenin and use Pygopus2 as adaptor protein which is detectable in CCTs and EVTs but also in the villous core and in CTBs. Pygopus 1 expression was found to be restricted to KRT7-negative cells in the decidua. Membrane staining of β-catenin was detectable in CTBs (Fig14 B) and it was shown that β-catenin dropps off the membranes of CCTs and translocates to the nucleus in individual cells (Fig. 14 D2a,b). Distally located nuclei showed an increase of nuclear β-catenin (Fig 14 D2b). However, nuclear expression is transient and therefore hard to visualize. The translocation of β-catenin from the cell membrane is accompanied with the appearance of nuclear TCF-4 and TCF-3. Both proteins are co-expressed in CCTs and EVTs. E-cadherin decreases at the cell membranes as the cells leave the cell columns. The TCF-4/3 complex was detectable in proliferactive Ki-67 positive CCTs but mainly in cells that already left the cell cycle (P57 positive) and the expression was maintained in differentiated EVTs. Here, exit from the cell cycle and differentiation/invasivness goes hand in hand with the upregulation of TCF-4/3. Therefore, we postulate these transcription factors as differentiation markers of trophoblasts.

TCF-4 modulates canonical Wnt reporter activity in SGHPL-5 cells

As already shown by Pollheimer and Sonderegger, the canonical Wnt reporter assay TOP/FOP is active and inducible in SGHPL-5 cells, which were used as invasive model system in this study. To study the effect of TCF-4 on reporter activity, siRNA-mediated silencing of TCF-4 was used to develop cells in a Wnt signaling-repressed state. Downregulation of TCF-4 was confirmed by Western blot (Fig 17) and RT-qPCR (Fig 18)

which revealed a reduced basal as well as LiCl- or Wnt3a-induced activity of a the canonical Wnt signaling reporter in TCF-4-silenced cells compared to control cells transfected with non targeting siRNAs (Fig. 20). Although TCF-4 silencing was very efficient in the cell system, the effects were significant but poorer than expected. Here, we hypothesize that that TCF-3, whose expression was not affected by the siRNAs (Fig. 17), has the ability to overcome the loss of TCF-4. Furthermore, we tested whether the small molecule inhibitor PKF115-584, published to interfere with TCF-4-β-catenin binding, shows similar effects on the activity of the reporter. A stable canonical Wnt reporter cell line was therefore established by lentiviral transduction of a luciferase reporter cassette habouring 7 LEF/TCF sites into trophoblasts and selection with puromycin. The inhibitor was able to suppress the LiCl- or Wnt3a-induced reporter activity in a dose-dependent manner as published for other cell systems and thus represents another powerful tool for the manipulation of primary cells and explant cultures. Canonical Wnt signaling was found to be reduced upon TCF-4 depletion as well as upon interference with TCF-4-β-catenin binding.

Primary trophoblasts exhibit canonical Wnt reporter activity

In primary EVTs, which were isolated from first trimester placentae and transfected with the TOP/FOP system, reporter activity was detectable and inducible by the addition of recombinant Wnt3a after 48h in culture. These data prove that EVTs exhibit an active and inducible canonical Wnt signaling cascade in vitro. Further, in villous explant cultures, which are hard to transfect, a lentiviral shuttle was used to introduce the reporter cassette and to verify if signaling is restricted to distinct cell types. Figure 27 shows that canonical Wnt signaling is active in proximal as well as in distally located outgrowing trophoblasts. Interestingly, an increase of the reporter activity was observed in distally located cells, suggesting that the Wnt signaling cascade could regulate migration and differentition in trophoblasts.

Trophoblast motility is TCF-4 dependent

The motility of SGHPL-5 cells was tested under Wnt signaling-inhibited conditions. In TCF-4-silenced SGHPL-5 cells, the basal as well as Wnt3a-induced migration was found to be reduced (Fig 21). The chemical inhibitor PKF115-584 showed the same effect on the basal migration but an even stronger inhibition (Fig 24).

TCF-4 expression arises in cells, which are at the boundary between proliferation and differentiation. In this study it was shown that TCF-4 regulates migration in SGHPL-5 cells and it is known that Wnt signaling regulates the cell cycle by directly targeting Cyclin D1 or cMyc. Furthermore, TCF-4 is a well-known regulator of proliferation and stemmness in the

small intestine. The questions asked in this study were: Is TCF-4 an inhibitor of proliferation in trophoblasts? Does less TCF-4 trigger the cells into a proliferative state?

TCF-4 silencing does not affect proliferation in SGHPL-5 cells

Since no Wnt3a-induced changes in the proliferative behaviour or in cyclin D1 (Figure 25) expression were observed in SGHPL-5 cells upon TCF-4 silencing, we conclude that TCF-4 does not regulate proliferation in these cells. These observations could be explained by Large T transformation, which overcomes direct cell cycle regulators. This makes the cell line useless for proliferation studies. Another explanation might be that TCF-3 has the ability to counterbalance TCF-4, which might be supported by the finding that blocking of Wnt signaling by the chemical inhibitor PKF115-584 resulted in cell death after 48-72h. However, this cytotoxic effect might also be independent of Wnt signaling and is also observed in other cell systems.

TCF-4 loss does not alter pro-migratory factors but a differentition marker in SGHPL-5 cells

To explain the reduced migratory ability of TCF-4-silenced SGHPL-5 cells and to find putative direct effectors and target genes, pro-migratory factors like MMPs and the PAI-uPA system were investigated. In siRNA-mediated TCF-4-silenced SGHPL-5 cells no effects on protein expression or activation of MMP2 and 9 or PAI1, 2 or uPA were monitored (Fig.25). Interestingly, the fibronectin receptor ITGA5, a differentiation marker in EVTs, was reduced in TCF-4 silenced cells compared to control.This supports the assumption that TCF-4 is an important factor for trophoblast differentiation. The intergrin patterns of SGHPL-5 cells are not in accordance with those of EVTs since for example membranous expression of ITGA5, as seen in EVTs, is not detectable in SGHPL-5 cells. This could be explained by incorrect processing of the receptor in the cell line. Due to this discrepancy, the effects of TCF-4 were studied in primary trophoblasts.

Inhibition of Wnt signaling in primary EVTs affects differentiation and migration

siRNA-mediated silencing of TCF-4 in isolated EVT was performed and repression was confirmed on the protein level by Western blot (Fig. 28) and on the RNA level by RT-qPCR (Fig. 29). Our data showed that downregulation at the protein level was less efficient in EVTs when compared to SGHPL-5 cells (Fig 18 and Fig. 25). The same was observed at the RNA level (Fig 19 and Fig 30). The differentiation-mediated increase of TCF-4 mRNA was significantly repressed in TCF-4 siRNA-treated cells.

Immunofluorescence staining of siRNA treated cells confirmed these observations (Fig 31). Furthermore, ITGA5 and the collagen receptor ITGA1 expression was increased in control cells between 24 and 48h of cultivation. In TCF-4-silenced cells, there was no increase of TCF-4 and also the upregulation of the above-mentioned integrins was slowed down. These findings confirm the assumption that TCF-4 is a differentiation factor in EVTs. Treatment of cells with the inhibitor PKF115-584 resulted in morphological changes (Fig 32 and 33). For example, cells did not form cell clusters as seen in the negative controls. Furthermore, the migratory ability of EVTs was reduced upon siRNA-mediated TCF-4 silencing as well as PKF115-584 (Fig 34) treatment similar to the effects observed in SGHPL-5 cells. Since villous explant cultures are hard to transfect, they were first treated with the inhibitor PKF115-584 to test putative effects of Wnt signaling inhibition. The outgrowth of villous explants (Fig 35) was not changed by the inhibitor but the ability of the cells to dissociate from the cell column and to migrate on the surface was clearly affected. Moreover, the cells remained longer in the tight proximity and failed to break out. After treatment with TCF-4 siRNAs, a similar picture was observed, as shown in figure 36. Notably, these experiments are preliminary and need repetition to reach statistical significance.

Wnt signaling and especially TCF-4 are important regulators of EVT differentiation. Whether the expression of TCF-4 is a sign of cell cycle exit and whether the repression of TCF-4 is able to bring cells back into a proliferative state needs further investigation. Therefore, future plans include BrdU incorporation assays and Ki-67 stainings of TCF-4-silenced primary cells and villous explant cultures, since these model systems seem more appropriate than SGHPL-5 cells. The fact that inhibitory transcription factors (TLEs) are widely expressed in trophoblasts suggests that suppression and activation of target genes happens within one cell. The production of diverse Wnt ligands by trophoblasts, having the ability to signal in low distances (cell to cell, polarity induction, gradient) amplifies the potentials of regulation which is very hard to retrace in vitro. For such studies, the explant model system may probably be the best choice.

In summary, all these findings emphasize the requirement of proteins known to be important for EMT, including E-cadherin, nuclear β-catenin, LEF/TCF and snail, for trophoblast differentiation and invasion.

It is tempting to speculate that TCF-4 has additional functions in distally located EVTs, since TCF-4 and β-catenin signaling in astrocytes was reported to repress HIV replication [Narasipura et al., 2012 and Henderson et al., 2012]. This function might be also important in the placenta to protect the growing fetus and might be an interesting field for further investigations.

5. Materials and methods

Tissue Collection

First trimester human placental tissues (6-12th week of gestation, n=55) were obtained from elective pregnancy terminations. Use of tissues was approved by the local ethical committee and required informed consent of patients.

Cell culture

SGHPL-5 cells, a trophoblastic cell line showing features of invasive trophoblasts such as Keratin-7 (KRT7), human leukocyte antigen G (HLA-G) and T-cell factor 4 (TCF-4) expression were used as a trophoblast model system. The cell line was generated from first trimester placentae [*Choy et al 2000*]. Briefly, placental villi were minced and digested with trypsin to collect cells from the basal membrane. Trophoblasts were separated by gradient centrifugation and isolated cytotrophoblasts (CTBs) were SV40 large T Antigen-transformed. Cells were cultivated in a mixture of Dulbecco´s Modified Eagles Medium (DMEM)/Ham´s F12 (1:1) (PAA) supplemented with 0.05 mg/ml gentamicin (Gibco) and 10% fetal calf serum (FCS, Biochrom).

HEK293T cells are derived from Adenovirus-transformed human embryonic kidney (HEK) cells, which were additionally SV40 large T Antigen-transformed. These cells are able to replicate episomal plasmids with an SV40 origin of replication (copynumber 400-1000) and were therefore used for lentivirus production. Cells were cultivated in DMEM high glucose medium supplemented with 10% FCS and gentamicin (0.05 mg/ml).

Isolation and cell culture of primary trophoblasts

Isolation of primary trophoblasts was performed according to Tarrade et al., 2001. Briefly, pooled first trimester placentae (6-9th week of gestation, n=52) were washed with ice-cold PBS and kept overnight in a 1:1 mixture of DMEM/Ham's F-12 supplemented with 0.05 mg/ml gentamicin (Gibco), 0.5 µg/ml fungizone (Gibco) and 100 Units/ml penicillin / 100µg/ml streptavidine (Gibco). Villous tips were scraped with a scalpel blade and digested for 30min in 0.125% trypsin (Gibco) and 12.5 mg/ml deoxyribonuclease I (Sigma) in 1x Mg/Ca-free Hank's balanced salt solution (Sigma) at 37°C. After filtration through a 70-100µm cell strainer (Greiner Bio-One GmbH), activity of trypsin was stopped with 10% FCS. Tissue was digested a second time for 30 minutes in fresh trypsin solution. Cells from both digestions were pooled and after a washing step by centrifugation (1500 rpm, 10 min), cells were filtered again and separated by Percoll gradient centrifugation (10-70%

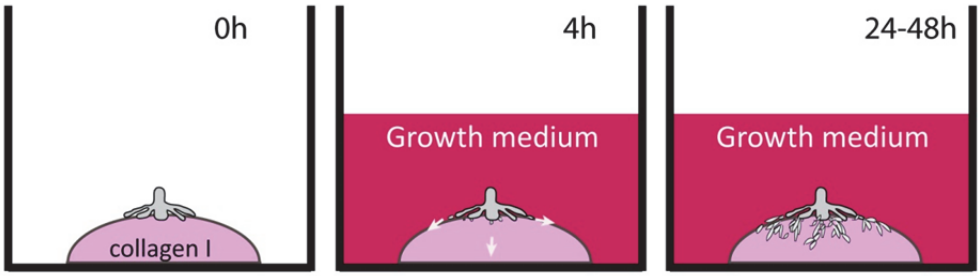
Percoll Pharmacia). Purified cells from 35-50% Percoll layer were washed 3 times, counted and seeded on non-coated culture dishes (BioLite, Thermofisher Scientific) for 30min to remove contaminating non-trophoblastic cells. Remaining trophoblasts were cultivated on fibronectin (B/D 2.5µg/cm^2) -coated dishes at a density of 5x10^5/ 24 well, 1.25x10^5 per chambeslide or transwell and cultivated in DMEM/Ham's F12, supplemented with 10% FCS , 0.05 mg/ml gentamicin and 0.5µg/ml fungizone.

Human first trimester villous explant cultures

First trimester (7-9th week of gestation) villous explant cultures were performed as described by Bauer et al., 2004.

Small pieces (2x2 mm) of villous tissue were dissected under the microscope and cultivated in DMEM/Ham's F12 (1:1) supplemented with 0.05 mg/ml gentamicin overnight at 37°C. Explants were seeded on collagen-I drops (BD Biosciences, Franklin Lakes, NJ) for 4 hours to allow anchorage, and then incubated with serum-free growth medium containing DMSO, 0.5 µM PKF115-584 or siRNAs (described later). Outgrowth of EVTs on collagen-I was microscopically monitored every day, and representative samples were photographed (Olympus IX71 and Cell^D software) after 24 and 48 hours.

Figure 38: Illustration showing outgrowth of villous explant culture



Immunofluorescence of tissues

Human first trimester placentae (6-12th week of gestation) were fixed in 4.5% paraformaldehyde for 24h, dehydrated and embedded in paraffin (Merck, Darmstadt, Germany). Serial sections were prepared (3µm) on a microtome as described (Knofler M et al., 2004). After deparaffinization, antigen retrieval was performed in a KOS Microwave HistoSTATION (Milestone Medical Technologies, Kalamazoo, MI, USA) using PT Module Buffer 1, citrate buffer, pH=6 (Thermo Scientific Fremont, CA, USA). Blocking was performed for 30 min at room temperature using 0.05% fish skin gelatin (Sigma, G7041) followed by overnight incubation with primary antibodies at 4°C diluted in blocking buffer. The following primary antibodies were used: Cytokeratin 7 (clone OV-TL 12/13, Dako

M7018, 1.96 µg/ml) Vimentin (GeneTex gtx100619, 1.44 µg/ml), Vimentin (clone Vim3B4, Dako M7020 0.51 µg/ml), Ki-67 (Chemicon, mab4190, 1:100), P57/kip-2 (clone C-20, Santa Cruz sc-1040, 1µg/ml), TCF-4 (clone C9B9, Cell signaling 2565, 1:100) TCF-4 (clone 6H5-3, Millipore 05-511, 10 µg/ml) TCF-3 (Proteintech Group 14519-1-AP, 3.6 µg/ml) LEF-1 (clone C12A5, Cell Signaling 2230, 1:100), TLE1 (clone M-101, Santa Cruz sc-9121, 2 µg/ml), TLE2 (clone H-191, Santa Cruz sc-9122, 2 µg/ml), TLE3 (clone M-201, Santa Cruz sc-9124, 2 µg/ml), TLE4 (clone M-200, Santa Cruz sc-9125, 2 µg/ml), TLE5 (Novus Biologicals NBP1-02957, 5 µg/ml), PYGO1 (Sigma Prestige Antibodies HPA042248, 1 µg/ml), PYGO2 (Sigma Prestige Antibodies HPA023689, 1 µg/ml), active β-catenin (ABC) (clone 8E7, Millipore 058-665, 5 µg/ml), E-cadherin (clone N-20, BD Transduction laboratories 610181, 1.25 µg/ml). Subsequently, samples were incubated with 4 µg/ml secondary antibodies Alexa Fluor 488 f(ab')2 fragment of goat anti-mouse IgG (H+L), Alexa Fluor 546 f(ab')2 fragment of goat anti-mouse IgG (H+L), Alexa Fluor 488 f(ab')2 fragment of goat anti-rabbit IgG (H+L) and Alexa Fluor 568 f(ab')2 fragment of goat anti-rabbit IgG (H+L). Nuclei were stained by intercalation of DAPI (4',6'-diamino-2-phenylindole 1µg/ml, Roche) into the DNA, slides were embedded using Fluoromount-G (Soubio) and analyzed by fluorescence microscopy (Olympus BX50, Cell^P software) and digitally photographed at a 200-fold magnification. Interesting image sections were digitally zoomed using Adobe Photoshop CS5 software.

Immunofluorescence of cells

Primary trophoblasts grown on fibronectin-coated chamber slides (Thermo Scientific, 177445) were fixed in 4% PFA for 10 minutes at 4°C and washed carefully with PBS. Cells were treated with 0.1% Triton X 100 for 5 minutes at 4°C, washed again and unspecific binding of antibodies was blocked by incubation with 0.05% fish skin gelatin (Sigma). Cells were stained as described above. Additionally, antibodies against integrin alpha 5 (ITGA5) (Chemicon mAB 1986, 1 µg/ml) and ITGA1 (Millipore MAB1973, 10 µg/ml) were used.

Hematoxyline staining of cells

Trophoblasts which were cultivated on fibronectin-coated chamberslides were fixed as described above and stained for 5 minutes with Papanicolaou´s 1a Harris´ haematoxyline solution (Merck 1.09253.05000) at room temperature. After washing with Aqua dest., cells were incubated with Scott´s solution (Morphisto 11192.01000) for 2 minutes, washed several times and mounted in Aquatex (Merck 1.08562.0050) mounting medium. Nuclei are stained in dark violet and cytoplasm in light grey. Pictures were taken on an Olympus microscope (BX50, Cell^P software) at a 40- and 100-fold magnification.

Light microscopy of living cells

Trophoblasts which were cultivated on fibronectin-coated chamberslides were photographed at a 10-fold magnification using an Olympus IX71 microscope and cell^D software.

Plasmid isolation

Bacteria carrying plasmids containing the Wnt reporter cassette 7TFP [Fuerer et al., 2010] in the pCF826 vector, Super8xTOPflash and Super8xFOPflash and pCMV-βGAL were cultivated overnight in 250 ml LB-broth, containing 100 µg/ml ampicillin. Plasmid preparation was performed using StrataPrep EF Plasmid Midiprep Kit (Argilent Technologies #400721) according to the manufacturer's instructions. Concentration of plasmids was measured photometrically and quality was checked by agarose gel electrophoresis.

Production of lentiviral particles (TransLenti Viral GIPZ Packaging System # TLP4615,Thermo Scientific Open Biosystems)

$5.5x10^6$ HEK293T cells were plated per 100 mm petri dish in DMEM high glucose medium, supplemented with.10% FCS and 0.05mg/ml gentamycin. The next day, a transfection mix was prepared by diluting 37.5 µg DNA (28.5 µg Expression arrest TransLenti Viral Packaging System stock + 9 µg 7TFP) in DMEM high glucose medium. 187.5 µl Arrest-In was diluted in 1ml DMEM high glucose. Both, DNA and transfection reagent were mixed and incubated for 20 minutes at room temperature. Subsequently, growth medium was removed from the cells. Transfection complexes were diluted in 3 ml serum-free growth medium without antibiotics and cells were carefully covered with the solution. After 6 hours, transfection medium was replaced by 12 ml fresh standard culture medium. Virus-containing supernatants were collected after 48 hours and stored at 4°C. 12 ml fresh standard culture medium was added to the cells and harvested after 72h. Supernatants of 3 100 mm dishes were pooled and centrifuged for 5 minutes at 4500 rpm to remove cell debris. To purify the virions, supernatants were centrifuged through a 20% sucrose cushion. 33 ml Beckman conical tubes were filled with the supernatants and underlayed with 4 ml 20% sucrose and centrifuged for 2h at 25000 rpm and 4°C in a Beckman SW28 swingle bucket rotor. After centrifugation, supernatant and sucrose were discarded and virions were suspended in 2 ml serum-free medium. Virions were aliquoted and stored at -80°C.

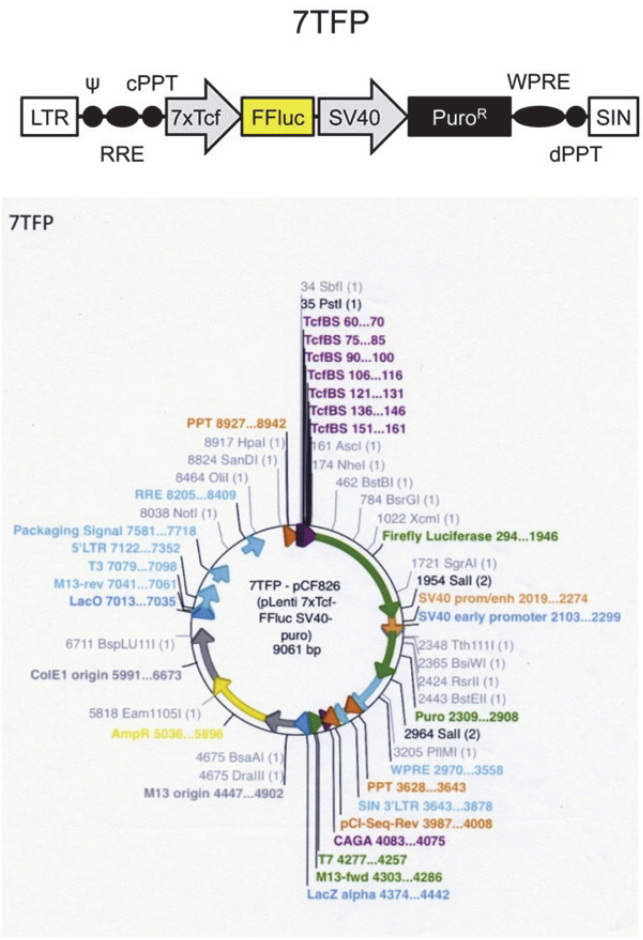
Determination of puromycin-sensitivity of SGHPL-5 cells for selection

$7.5x10^4$ SGHPL-5 cells were plated per 24 well. The next day, complete culture medium containing 1, 5 or 10 µg puromycin was added to the cells. In the well containing 5 µg/ml puromycin, cells died within 24h. Therefore, this concentration was chosen for selection.

Establishment of a stable Wnt reporter cell line "SGHPL-5 7TFP" using lentiviral transduction

The used cassette was constructed as described by Fuerer C and Nusse R, 2010.

Figure 39: Schematic of the used Wnt reporter cassette and plasmid map [Fuerer and Nusse, 2010]



The day before transduction, 2 wells of a 12 well culture dish per condition and 1 well for selection control were seeded with $1.5x10^5$ SGHPL-5 cells. 10 µl virus particle stocks were diluted with 1 ml DMEM/Ham's F12 medium, containing 5 ng/ml Polybrene. Medium was removed from the cells and they were washed with PBS. 0.5 ml transduction mix was added per well. After 6 hours, 0.5 ml DMEM/Ham's F12 medium, supplemented with 20% FCS and 0.05 mg/ml gentamicin was added. Selection started after 48 hours. Cells were split 1:2 into standard growth medium containing 5 µg/ml puromycin. During selection,

medium was changed every day to remove dead cells. After 4 passages, puromycin concentration was reduced to 2.5 µg/ml for further cultivation.

RNA Isolation

For RNA isolation, PeqGold Trifast (Peqlab) was used for direct lysis in the culture dish according to manufacturer's instructions. The amount of prepared RNA was determined with a NanoDrop (Peqlab) spectrophotometer.

cDNA Synthesis

For reverse transcription, 0.5 µg RNA, 1.5 µg hexanucleotide-mix (Roche), 0.25 mM dNTPs (peqGOLD dNTP-Set) and 100 units M-MuLV Reverse Transcriptase (Fermentas) in a reaction volume of 20 µl were used according to manufacturer's instructions.

Real time quantitative PCR

The 7500 Fast Real-time PCR system (Applied Biosytems) and TaqMan Gene Expression Assays (Applied Biosystems) TCF-4 (Hs001814036_m1) was used for quantitative real time PCR. All measurements were done in duplicates. 1 µl of cDNA, 0.5 µl primers and 5µl Fast 2x PCR-Mix (TaqMan Universal PCR Master Mix, Applied Biosystems) were used per sample and 45 cycles were performed. For normalization, TATA-box binding protein, TBP (ABI # 4333769F) was amplified as mentioned by Saleh, 2011. Evaluation of the signals was done according to manufacturer's instructions (PE Biosystems Sequence Detector User Bulletin). Cycle threshold (Ct) value was defined as the first cycle where the fluorescence was significantly above the background. Ct values of the individual samples were normalized to the Cts of the housekeeping gene TBP by calculating the differences of the Ct (ΔCt). Relative expression levels were calculated by using ntc siRNA-treated cells as calibrator (ΔΔCt).

Western immunoblotting

Protein extracts were prepared after washing the cells with ice-cold PBS by adding hot lysis buffer containing 30 mM Tris, 1% SDS, 5% glycerol, 2.5% β-mercaptoethanol and bromphenolblue directly into the culture wells. Lysates were transferred into a vial and boiled for 5 minutes.

Proteins were separated using 10% SDS PAGE and transferred onto Amersham Hybond-P PVDF membranes (GE Healthcare) using a Millipore transfer Unit and a transfer buffer containing 20% methanol. Membranes were blocked in 5% non-fat dried milk/ 1xTBS (Tris-buffered saline)-0.1% Tween 20 for 30 minutes and incubated with primary

antibodies (see below), diluted in 5% BSA (bovine serum albumin)/1xTBS-Tween 20 overnight : TCF-4 (clone C9B9, Cell Signaling 2565, 1:1000), TCF-3 (Proteintech Group 14519-1-AP, 0.36 µg/ml) GAPDH (clone 14C10, Cell Signaling 2118, 1:5000) ITGA5 (Abcam ab93943, 1:2000), MMP2 (Cell Signaling 4022, 1:1000), CyclinD1 (clone 92G2, Cell signaling 2978, 1:1000), PAI 1 (Oncogene IML29L, 1 µg/ml), PAI-2 (American Diagnostic, ABIN124916 1 µg/ml), uPA (clone 204212, R&D MAB1310, 1µg/ml, diluted in non-fat dried milk). After 3 times washing in 1xTBS-0,1% Tween 20 for 5 minutes, membranes were incubated in HRP-labeled anti-mouse IgG (Fab)-specific peroxidase antibody produced in goat (Sigma A2304-1ML, 1:50000) and ECL Anti-rabbit IgG, Horseradish Peroxidase-linked species specific whole Antibody from donkey (GE healthcare NA934, 1:50000), diluted in blocking buffer, for 1 hour. After 3 washing steps for 5 minutes chemiluminescence signal development was performed using ECL prime detection kit (GE Healthcare). Signals were detected in a FluorChemQ imaging system (Alpha Innotech, San Leandro, USA) Alpha View Version 3.1.1.0 software (Alpha Innotech, San Leandro, USA).

TCF-4 gene silencing using siRNAs in SGHPL-5 cells and primary trophoblasts

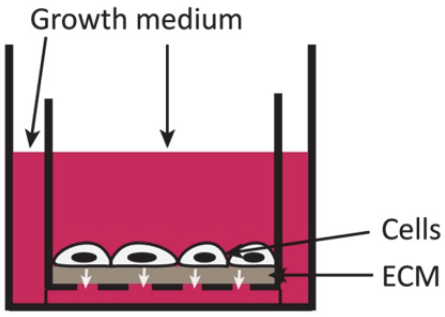
According to Rosner M. et al. 2010, SGHPL-5 cells were seeded in 6 well plates ($2.5x10^5$ cells) to reach about 40% confluency. After 6h, culture medium was changed to 1.5 ml complete antibiotics-free medium. A mixture of 4 different siRNAs targeting TCF-4 (Dharmacon-Thermo Fisher Scientific, ON- TARGETplus SMARTpool L-003816-00-0005) and a non-targeting control pool hereinafter referred to as ntc siRNA (Dharmacon-ThermoFisherSientific D-001810-10-20) were added to 245µl OPTI-MEM I (Gibco). 5µl Lipofectamin RNAiMAX (Invitrogen) were mixed with 245µl OPTI-MEM I and incubated at room temperature for 5 minutes. siRNA pools and lipid solution were mixed and incubated at room temperature for another 20 minutes and subsequently added to the cells to reach a final concentration of 50 nM during transfection. Knockdown efficiency was determined using RT-qPCR and Western immunoblotting. TCF-silenced cells were used for further experiments 72h post siRNA delivery. For production of serum-free cell supernatants, medium was changed 24h after siRNA transfection and harvested after 96h. Supernatants were collected and concentrated 10-fold by centrifugation for 45min at 3500rpm at room temperature using Amicon Ultra-4 3K (Millipore) centrifugal filters.

As described by Forbes et al., 2009, primary trophoblasts were transfected with the siRNA pools using Amaxa Basic Nucleofector Kit for primary mammalian epithelial cells (VPI-1005, Lonza, Germany). Briefly, $1.5x10^6$ isolated primary trophoblasts were resuspended in nucleofector solution mixed with siRNA (400nM) and program W-001 was used. After

electroporation, cells were diluted with complete medium and seeded on fibronectin-coated transwells or tissue culture dishes.

Migration studies in Wnt signaling repressed SGHPL-5 cells and primary trophoblastst

Figure 40: Schematic drawing of a transwell



Cells are seeded on top of the matrix. To pass the matrix and to migrate through pores smaller than the cell diameter, cells are forced to produce matrix degrading enzymes (e.g. matrix metalloproteinases) and must be able to change their shape (e.g. rebuilding of cytoskeleton).

For testing the migratory properties of TCF-4 siRNA and control cells in the absence or presence of Wnt3a, as well as of wilde type SGHPL-5 cells and trophoblasts in the absence or presence of PKF115-584 (Tocris 1626, 0.5µM), fibronectin coated transwells (13µg/cm^2) with a pore size of 8.0 µm (Millipore PI8P01250) for SGHPL-5 cells or 12 µm (Millipore PIXP01250) for primary trophoblasts were used. Further, SGHPL-5 cells +/- PKF115-584 were also seeded on uncoated and growth factor-reduced Matrigel (B/D, 1:50)-coated transwells. Cells were counted using a Casy cell counting system (150 µm capillary, 0-40µm, calibration file K000_040.150, cursor set 8-23µm) prior to seeding. SGHPL-5 cells were washed once and 2.5x10^4 cells were seeded per transwell in a volume of 200 µl. 1.25x10^5 trophoblasts were seeded immediately after electroporation. 24h or 72h after seeding of SGHPL-5 cells or trophoblasts, respectively, transwells were washed with PBS and cells were fixed with ice-cold 4% paraformaldehyde for 10 min at 4°C. After washing with PBS and a 5 minute treatment with 0.1% TritonX 100, cells were washed again with PBS and unspecific binding was blocked using 0.05% fish skin gelatin (Sigma) for 30 minutes. Not-migrated cells from the upper side of each transwell were mechanically removed using a cotton swab. Polycarbonate membranes were cut out with a scalpel. Tophoblasts were co-stained using KRT7 (Dako M7018, 1.96 µg/ml) and Vimentin (GeneTex gtx100619, 1.44µg/ml) antibodies for 1h at 37°C. After incubation of another hour with secondary antibodies Alexa Fluor 488 f(ab')2 fragment of goat anti-mouse IgG (H+L) and Alexa Fluor 568 f(ab')2 fragment of goat anti-rabbit IgG (H+L),

4µg/ml each, at room temperature, nuclei were stained with DAPI (1 µg/ml 4′, 6-diamidine-2′-phenylindole dihydrochloride, Roche Diagnostics, Mannheim, Germany) for 10 minutes. For SGHPL-5 cells solely nuclear staining was performed. Samples were mounted upside down on glass slides in Fluoromount G. Migrated cells were photographed (Olympus BX50, Cell^P software) and 5 non-overlapping photos per transwell, representing 40-50% of the total area were taken at a 40-fold magnification. The number of migrated cells was determined by counting the nuclei of trophoblasts and SGHPL-5 cells using ImageJ software.

Proliferation assay

To test whether TCF-4 silencing changes the proliferation rate of SGHPL-5 cells, $4x10^4$ cellswere seeded in triplicates into 24 wells and the cell number was counted after 24, 48, and 72 hours using a Casy cell counting system (Casy1 Model TTC, Schärfe System, 150 µm capillary, 0-40µm, calibration file K000_040.150, cursor set 8-23 µm). For induction of canonical Wnt signaling, 200 ng/ml recombinant human Wnt3a (R&D) was added every day.

Testing the activity of canonical Wnt signaling using the SuperTOP reporter system

The idea for the construction of a M50 Super 8xTOPFlash (Addgene plasmid 12456) and M51 Super 8x FOPFlash (Addgene plasmid 12457) (Veeman et al., 2003) used for detecting the activity of canonical Wnt signaling is from Hans Clevers lab who designed the original TOPflash plasmid. SuperTOPFlash carries 7 LEF/TCF binding sites upstream of a minimal TK promoter and a firefly luciferase reporter (backbone: pTK-Luc Clontech). SuperFOPFlash has 6 mutated LEF-TCF sites. If Wnt- signaling is induced, β-catenin binds to LEF/TCFs and firefly luciferase transcription is activated. To correct transfection efficiency, pCMV-β-Gal plasmids were co-transfected.

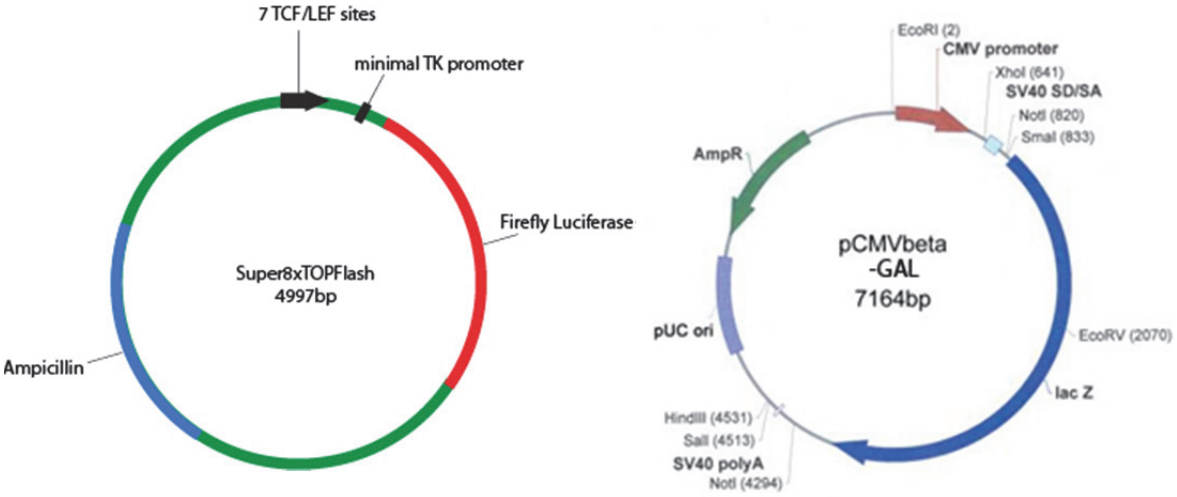


Figure 41: Plasmid maps of SuperTOPFlash and pCMVbeta-Gal used for transfection

Lipofectamine 2000 (Invitrogen) was used for transfection. One day before transfection, cells were seeded in duplicates into 24 wells ($1x10^5$ cells/well). For transfection, 0.75 µg SuperTOPFlash plasmid and 0.2 µg pCMV-β-Galacosidase plasmid were added to 50 µl OPTI-MEM and incubated at room temperature for 5 minutes. 1.5 µl Lipofectamin 2000 was added to 48.5 µl OPTI-MEM and incubated at room temperature for 5 minutes. DNA and lipid solution were mixed and incubated at room temperature for another 20 minutes to form lipid-DNA particles. In the meantime, medium was replaced by 300 µl fresh growth medium (DMEM Ham´s F12/ 10% FCS/ gentamicin). Packed DNA was slowly added dropwise to cells. After gentle mixing, cells were transferred to the incubator. After 3 hours, medium was removed and fresh medium +/- recombinant Wnt3a (R&D 200 ng/ml,) and LiCl (20mM) was added to induce the activity of the Wnt reporter. LiCl inhibits the activity of GSK-3β independently of active Wnt-signalling [Hedgepeth et al., 1997] and was used as positive control for the reporter assay as well as recombinant Wnt3a, a canonical Wnt ligand not expressed in the human placenta. 24h later, cells were washed once with PBS and then lysed using 100 µl 1x Cell Culture Lysis Reagent (Promega E153A) per well. After 10 minutes on a vertical shaker, protein solution was harvested and tranferred to 1.5 ml tubes. After 2 freeze and thaw cycles, the protein solution was centrifuged for 10 min at 10,000 rpm to remove cell debris. Luciferase activity of 10 µl protein solution was measured in duplicates using 50 µl Luciferase Assay Substrate (Promega #E151A) and a Lumat LB 9507; EG&G Berthold (measurment: 5 seconds after injection for 10 seconds). To correct the transfection efficiency, the β-Glactosidase activity of the protein solution was determined by using the chromogenic substrate chlorophenol red-β-d-galactopyranoside (CPRG, Roche Diagnostics). 10 µl of protein-solution were mixed with 100 µl of 80mM Na_2PO_4 pH 7.3, 102mM β-Mercaptoethanol, 9mM $MgCl_2$ and

8mM CPRG in plastic cuvettes. After 3-5 min incubation at room temperature, samples were diluted with 1 ml water and OD_{570} was immediately quantified using a photometer (Eppendorf), as described (Sonderegger et al., 2010). Whole protein amounts of SGHPL-5 7TFP cells were determined by Bradford assay according to the manufaturers´ instructions (Biorad). 2-5 µl of protein lysates were incubated for 10 minutes in 1ml 1x Bradford reagens and the OD was determined at 590 nm using a photometer. Calculated means of luciferase values were normalized to β-Galactosidase values to correct transfection variations or total protein values in the stable cell line.

Reporter analyses in primary trophoblasts using electroporation

$1.5x10^6$ isolated primary tophoblasts were resuspended in a mixture of 3.8 µg SuperTOPFlash or SuperFOPFlash and 0.8 µg pCMV-β-Galactosidase plasmid and nucleofection buffer (100µl) provided in the Amaxa Basic Nucleofector Kit for primary mammalian epithelial cells (VPI-1005, Lonza, Germany). Nucleofector program W-001 was used for electroporation and cells were plated on fibronectin-coated wells. The next day, growth medium was replaced by medium containing 200 ng/ml Wnt3a and luciferase and β-galactosidase activity were determined after 24h as described above. Due to poor transfection efficiency, luciferase activity was determined from 20 µl protein extracts and β-gal activity from 40µl and incubation times up to 30 minutes. Three independent experiments were performed in duplicates.

Lentiviral transduction of the Wnt reporter cassette 7TFP into human first trimester villous explant cultures and detection of luciferase activity

First trimester (9^{th} week of gestation) villous explant cultures were performed as described above. Outgrowth of EVTs on collagen-I was monitored microscopically and representative samples (4 per condition) were selected. Medium was replaced carefully by warm growth medium containing 5 ng/ml Polybrene and lentiviral particles containing the 7TFP cassette; for control explants the same medium was used omitting the virions. 24h later, explants were fixed using 4% paraformaldehyde for 15 minutes at 4°C. Samples were washed carefully with ice-cold PBS and treated for 5 minutes with 0.1 Triton X 100. After another washing step, unspecific binding was blocked by treatment with 0.05% fish skin gelatin. All explants were incubated with Anti-firefly luciferase antibody (Abcam ab498, 5µg/ml) diluted in 0.05% fish skin gelatin for 2 days followed by Alexa Fluor 488 Donkey Anti-Goat IgG (H+L) secondary antibody (Molecular Probes, A11055, 4 µg/ml) for 1h and DAPI for 10 minutes at room temperature. After washing in PBS, collagen drops

with explants were transferred to glass slides and mounted in Fluoromount G. Explants were digitally photographed at a 200-fold magnification (Olympus BX50, Cell^P software).

Statistics

To compare samples, statistics was calculated using Student's paired T-test (Microsoft Exel). A T-test p-value < 0.05 was regarded as statistically significant and indicated by asterisk. Deviations from the mean are displayed by the standard error (SE).

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechet ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit einzuholen. Sollte dennoch eine Urheberrechts-verletzung bekannt werden ersuche ich um Nachsicht.

7. ADDENDUM

7.1. Zusammenfassung

Wie Migrations- und Differenzierungsprozesse von Trophoblastenzellen der humanen Plazenta kontrolliert werden, ist nach wie vor nicht ausreichend geklärt. In einem Transkriptom-Vergleich zwischen villösen Cytotrophoblasten und extravillösen Trophoblasten wurde der „T-cell factor -4 (TCF-4)“ als eines der am stärksten regulierten Gene identifiziert. TCF-4 ist einer der Schlüsselfaktoren des transkriptionellen Komplexes der kanonischen Wnt Signalkaskade mit sowohl inhibierendem als auch aktivierendem Potential. Im Fokus dieser Arbeit liegt die Funktion von TCF-4 in extravillösen Trophoblasten.

Mittels Immunfluoreszenzfärbungen wurden Mitglieder der LEF-TCF-Familie, sowie der TLE-Familie und PYGOPUS in den unterschiedlichen Zelltypen der humanen Plazenta nachgewiesen, um die mögliche Zusammensetzung des transkriptionellen Komplexes der kanonischen Wnt Signalkaskade zu beschreiben. Durch siRNA verursachte reduzierte Genexpression von TCF-4 und die chemische Inhibierung der TCF-4-β-Catenin Komplexbildung wurde die Rolle von TCF-4 in Trophoblasten untersucht. Kanonische Wnt-Reporter Experimente in SGHPL-5 Zellen, einer invasiven Trophoblastenzelllinie, zeigte die Abhängigkeit der Reporteraktivität von TCF-4. Sowohl die basale, als auch induzierte Aktivität des Reportergenes war unter Inhibierung der Wnt Signalkaskade vermindert. Reduktion von Wnt-Aktivität führt zu verminderten Migrationsfähigkeit der Zelllinie, jedoch konnte keine Veränderung in der Proliferationsrate nachgewiesen werden. Expressionsveränderungen von Faktoren die Migration steuern könnten (MMP, PAI/uPA-System), wurden aufgrund verminderter TCF-4 Expression in SGHPL-5 Zellen nicht beobachtet. Jedoch war eine verminderte Expression des Fibronectin Rezeptors Integrin α5, nachweisbar.

In isolierten Trophoblasten konnte die Aktivität und Induzierbakeit des kanonischen Wnt Reporters in vitro gezeigt werden. In villösen plazentaren Explant-Kulturen wurde die Aktivität der Kaskade in proliferativen und differenzierten Trophoblasten nachgewiesen; eine Zunahme wurde in weiter migrierten, differenzierten Zellen sichtbar. Die Hemmung der Wnt Signalkaskade bewirkte in isolierten Trophoblasten wie auch in plazentaren Explantkulturen eine Abnahme der Migrationsfähigkeit. In Trophoblasten mit reduzierte TCF-4 Expression wurde eine Abnahme der Integrin (ITGA1 und ITGA5) Expression detektierbar, was auf eine Verlangsamung des Differenzierungsprozesses hindeutet. Bekräftigt wurde diese Hypothese durch die Beobachtung das chemische Hemmung der Wnt-Signalkaskade zur morphologischer Veränderung isolierter Trophoblasten führt.

Zusammenfassend kann gesagt werden, TCF-4 ist ein Schlüsselfaktor im Differenzierung- und Migrationsprozess von extravillösen Trohoblasten.

7.2. ABSTRACT

Factors controlling trophoblast differentiation and migration are of great interest to better understand placental development and function. Since comparative gene chip analyses revealed T-cell factor 4 (TCF-4) as one of the most upregulated genes in invasive extravillous trophoblasts, the canonical Wnt signaling cascade in the human placenta is in focus of this study. In particular, the role of TCF-4 as part of the nuclear complex of the cascade in an activating as well as in a repressive context, is described.

Besides the analysis of expression patterns of members of the transcriptional complex including the LEF/TCF and, TLE families as well as Pygopus in the human placenta, inhibition of the cascade using siRNA-mediated TCF-4 silencing or a chemical inhibitor of TCF-4-β catenin binding, was performed to study the influence of TCF-4 on trophoblasts. Canonical Wnt reporter assays in SGHPL-5 cells, used as an invasive trophoblastic model system, showed reduced basal as well as induced activity in Wnt signaling-repressed cells compared to control cells. Migration of SGHPL-5 cells was found to be reduced under Wnt inhibition, whereas the proliferation rate of these cells was not affected. Furthermore, levels of MMPs and members of the PAI-uPA system were unchanged in TCF-4 repressed cells, but expression of the fibronectin receptor ITGA5, was shown to be reduced.

In extravillous trophoblasts (EVTs), isolated from first trimester placentae, we found that canonical Wnt signaling is active and inducible in vitro. Moreover, activity of canonical Wnt signaling in villous explant cultures showned an increase in more distally migrated EVT compared to proximal cell column trophoblasts.

In Wnt-inhibited EVTs, migration was reduced and villous explant cultures also showed a clear decrease of outgrowing and migrating EVTs upon inhibition of Wnt. Furthermore, the decrease of integrin alpha 1 and 5 in TCF-4 silenced EVTs indicates that the differentiation process of these cells was slowed down. Changes of EVT morphology under Wnt inhibition strenghten this assumption.

In summary we conclude that TCF-4 is an important factor for extravillous trophoblast migration and differentiation.

7.3. Figure legend

8. Curriculum vitae

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2005-2013	Studium an der Universität Wien, Österreich, Molekulare Biologie
1993	Diplom, Medizinisch technische Analytik
1991-1993	Schule für medizinisch technisch Laboratoriumsdienst am AKH Wien, Österreich
1991	Matura
1983-1991	Bundesrealgymnasium Villach, Kärnten, Österreich
1979-1983	Volkschule, St. Leonhard b. S., Kärnten, Österreich

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1996	Biomedizinische Analytikerin, Medizinische Universität Wien, Frauenheilkunde, Abteilung für Geburtshilfe und Feto-maternale Medizin, Reproduktionsbiologie Unit
1994-1996	Biomedizinische Analytikerin im Forschungslabor der Abteilung für Pulmologie, Innere Medizin am Allgemeinen Krankenhaus der Stadt Wien

Besondere Kenntnisse:

Sprachen:	Englisch
Software:	Adobe Photoshop u. Illustrator, Microsoft Office Produkte

Posterpräsentationen:

2011	*G. Meinhardt, S. Haider, J. Pollheimer, M. Knöfler:* TCF-4 is a critical regulator of trophoblast invasion *Geilo, Norwegen*

2003	*G. Meinhardt, P. Husslein and M.Knöfler* Expression patterns of basic helix-loop-helix proteins during extravillous trophoblast differentiation *Mainz, Deutschland*

Preise:

2011	Y.W. Loke Travel Award

8.1. Publications

Placenta. 2009 *Mar;30(3):284-91*

Expression, regulation and functional characterization of matrix metalloproteinase-3 of human trophoblast.

Husslein H, Haider S, **Meinhardt G**, Prast J, Sonderegger S, Knöfler M.

Placenta. 2005 *Aug;26(7):527-39*

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