Activation of the Canonical Wingless/T-Cell Factor Signaling Pathway Promotes Invasive Differentiation of Human Trophoblast

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The molecular mechanisms governing invasive differentiation of human trophoblasts remain largely elusive. Here, we investigated the role of Wnt- β -catenin-T-cell factor (TCF) signaling in this process. Reverse transcriptase-polymerase chain reaction and Western blot analyses demonstrated expression of Wnt ligands, frizzled receptors, LRP-6, and TCF-3/4 transcription factors in total placenta and different trophoblast cell models. Immunohistochemistry of placental tissues and differentiating villous explant cultures showed that expression of TCF-3/4 strongly increased in invading trophoblasts. Some of these cells also accumulated dephosphorylated β -catenin in the nucleus. Wnt3A treatment of primary cytotrophoblasts and SGHPL-5 cells induced activity of TCF-luciferase reporters. Accordingly, the ligand provoked interaction of TCF-3/4 with β -catenin as assessed in electrophoretic mobility shift assays (EMSAs) and up-regulation of Wnt/TCF target genes as observed by Western blot analyses. Wnt3A stimulated trophoblast migration and invasion through Matrigel, which could be blocked by addition of Dickkopf-1, mediating inhibition of canonical Wnt signaling. Dickkopf-1 also reduced basal migration, invasion, and proliferation of cytotrophoblasts, suggesting expression of endogenous Wnt ligand(s). Immunohistochemistry revealed that the percentage of extravillous trophoblasts containing nuclear β -catenin was significantly higher in placentas of complete hydatidiform mole pregnancies as compared to normal placentas. Thus, canonical Wnt signaling may promote invasive trophoblast differentiation, and exaggerated activation of the pathway could contribute to trophoblastic hyperplasia and

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The invasive differentiation program of human trophoblasts represents an essential process of placentation and therefore plays a critical role in fetal growth and survival. Extravillous trophoblasts invading uterine tissue originate from proliferative trophoblast cell columns that attach to the decidualized endometrium. Between the 10th and 18th weeks of pregnancy, these cells establish the vascular connection between mother and fetus, ensuring continuous supply of nutrients and oxygen. They transform maternal spiral arteries into vessels of low resistance by replacing endothelial and vascular smooth muscle cells, thereby increasing blood flow to the placenta.¹ Analyses of anchoring villi *in situ* and of differentiating villous explant cultures *in vitro* suggest that the molecular processes controlling invasive trophoblast differentiation could have similarities with the mechanisms governing tumor invasion and metastasis. During invasion trophoblast cells lose markers of the polarized epithelium, such as $\alpha 6 \beta 4$ integrin, transiently down-regulate the adherens junction protein E-cadherin, and induce matrix metalloproteinases and receptors for fibronectin and collagens, ie, $\alpha 5 \beta 1$ and $\alpha 1 \beta 1$ integrins.^{2–5} In contrast to tumor cells, however, growth, cell cycle exit, and invasion of normal trophoblasts are tightly controlled by placental and decidual proteins.^{6,7}

Failures in this process are associated with different gestational diseases. Shallow invasion of decidual tissue and incomplete transformation of spiral arteries are hallmarks of preeclampsia and severe intrauterine growth restriction.^{8,9} In contrast, trophoblastic hyperplasia with a potential of malignant transformation occurs in complete hydatidiform moles (CHMs) lacking fetal development whereas extensive invasion has been noticed in malig-

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nant choriocarcinomas.^{10,11} Despite these facts few signaling cascades regulating normal trophoblast invasion have been elucidated and downstream transcription factors governing the differentiation program are primarily uncharacterized.¹² As a consequence, molecular mechanisms and sequential events leading to the pathogenesis of these gestational diseases remain unknown.

To gain insight into the invasive differentiation process, we here investigated Wingless (Wnt)/T cell-specific factor (TCF) signaling in the human placenta and different trophoblast cell models. In the canonical pathway Wnt ligands, which comprise a large family of secreted developmental regulators,^{13,14} bind to the heterodimeric Frizzled(fzd)/low-density lipoprotein receptor-related protein-5/6 (LRP-5/6) receptors, thereby activating Dishevelled (Dvl).^{15,16} Once activated, Dvl inhibits glycogen synthase kinase 3 β (GSK-3 β), which marks β -catenin for degradation by N-terminal phosphorylation.¹⁷ Wnt-dependent inactivation of GSK-3 β results in cytoplasmic accumulation and nuclear translocation of dephosphorylated β -catenin.^{18–20} Nuclear β -catenin provides the activation domain of the lymphoid enhancer binding factor 1 (LEF-1)/TCF transcription factor family, which induces growth- and invasion-associated genes such as cyclin D1, *c-myc*, MMP-7, and MT1-MMP.^{21–25}

Wnt signaling is thought to play a critical role in tumorigenesis because nuclear β -catenin, dominant active forms of LEF-1/TCF and mutations in upstream regulatory genes such as adenomatous polyposis coli are common in human epithelial cancer.^{26,27} Recent data suggested that Wnt signaling could also be involved in placental development and trophoblast differentiation. Homozygous mutation of Wnt2, Wnt7b, and LEF-1/TCF in mice results in different placental pathologies.^{28–30} Implantation in mice has been shown to be associated with increased expression of Wnt4.³¹ In the human endometrium, expression of Wnt3 and Dickkopf-1 (Dkk1),³² which inhibits canonical Wnt signaling by disrupting binding of LRP-5/6 to the Wnt/Frizzled ligand-receptor complex,³³ changes throughout the menstrual cycle.³⁴ In addition, several mRNAs encoding Wnt ligands are expressed in the human placenta.^{35–39} Therefore, we studied expression and localization of LEF-1/TCFs in the differentiating anchoring villus and investigated the influence of Wnt-dependent activation of these factors on trophoblast proliferation, migration, and invasion. Moreover, we examined nuclear expression of β -catenin in normal placentas and tissues from CHM. The data suggest that canonical Wnt signaling regulates invasive trophoblast differentiation. In gestational diseases such as CHMs, elevated nuclear localization of dephosphorylated β -catenin may indicate abnormal activation of the signal transduction pathway.

Materials and Methods

Tissue Collection

Placental tissues of early (between the 6th and 12th weeks of gestation, $n = 38$) and mid pregnancy (between the 18th and 22nd weeks of gestation, $n = 12$) were obtained from legal abortions with the permission of the

ethical committee of the Medical University of Vienna. Informed consent was obtained from women donating their placentas. Tissue samples were used for immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blotting. Paraffin-embedded placentas of CHM pregnancies ($n = 13$) between the 7th and 14th weeks of gestation (7th week, $n = 1$; 8th week, $n = 2$; 9th week, $n = 4$; 10th week, $n = 3$; 11th week, $n = 2$; 14th week, $n = 1$) were retrieved from the archive of the Department of Clinical Pathology, Medical University of Vienna. Based on the original report of the Department of Obstetrics and Gynecology, which included data from high-resolution ultrasound and determination of hCG levels, histological evaluation was performed. All cases were further investigated by immunohistochemical p57KIP2 staining as previously mentioned.^{40,41}

Cultivation of Cell Lines

JEG-3 choriocarcinoma cells and trophoblastic SGHPL-5 were cultivated in Dulbecco's modified Eagle's medium (DMEM) and in a 1:1 mixture of DMEM and Ham's F-12, respectively, supplemented with 10% fetal calf serum, 2 mmol/L glutamine, and 0.05 mg/ml gentamicin (all purchased from Invitrogen, Carlsbad, CA) as previously mentioned.^{42,43} SGHPL-5 cells exhibit features of invasive, extravillous trophoblasts because they were shown to express HLA class I, cytokeratin 7, and hCG.⁴⁴ All experiments using SGHPL-5 cells were performed between passages two and five.

First Trimester Villous Explant Culture

Preparation and cultivation of villous explants of different first trimester placentas ($n = 15$) between the 8th and 10th weeks of gestation were performed as described elsewhere.⁴⁵ Briefly, mesenchymal villi were dissected under the microscope and grown on Matrigel-coated 24-well plates (Becton Dickinson, Bedford, MA) for 24 and 72 hours. On extracellular matrix contact explant cultures formed anchoring villi and expressed invasive cell markers of the extravillous trophoblast, ie, α 1 β 1 and α 51 integrins and HLA-G1.^{5,46} In addition, extravillous trophoblast outgrowths were obtained 5 days after seeding of dissected villi on plastics. At the end of the culture period, invaded/migrated extravillous trophoblasts were mechanically separated from attaching villi, and protein/mRNA were isolated using the TRI-Reagent method as described elsewhere.⁴³ Both mRNA and protein were tested for the presence of the extravillous trophoblast-specific markers cytokeratin 7 and HLA-G1 using specific PCR primer sets⁴⁶ or the following antibodies: anti-cytokeratin 7 (clone OV-TL 12/30, mouse, 10 μ g/ml; DAKO, Glostrup, Denmark) or anti-HLA-G1 (clone MEM-G/1, mouse, 2 μ g/ml; Exbio, Praha, Czech Republic), respectively (data not shown).

Purification and Cultivation of First Trimester Cytotrophoblasts and Fibroblasts

Villous cytotrophoblasts were isolated at early gestation (between the 10th and 12th weeks, $n = 10$) by enzymatic dispersion and Percoll (5 to 70%) density gradient centrifugation as described.^{47,48} Furthermore, cells were immunopurified by depleting CD45-positive cells and fibroblast by using monoclonal anti-CD45 antibody (CD45RB, clone PD7/26, 0.2 $\mu\text{g}/10^6$ cells; DAKO) and anti-fibroblast-specific antigen antibodies (1:100, clone ASO2; Dianova, Hamburg, Germany), respectively, as described elsewhere.^{49,50} Cell preparations were routinely checked by immunocytochemistry using anti-cytokeratin 7 and anti-vimentin antibodies (clone Vim 3B4, 1.2 $\mu\text{g}/\text{ml}$; DAKO) to detect trophoblasts (99.8%) and contaminating stromal cells (0.2%), respectively. Pure trophoblasts were resuspended in DMEM containing 10% fetal calf serum and cultivated in uncoated or Matrigel-coated 24-well plates (Costar, Corning, NY). Villous fibroblasts of first trimester placentas ($n = 5$) were isolated after gradient centrifugation of trypsinized placental material (between 25% and 35% Percoll) and passaged two times in DMEM supplemented with 10% fetal calf serum. Fibroblasts were characterized by vimentin immunocytochemistry (100% of cells), and the absence of contaminating trophoblasts was confirmed by cytokeratin 7 staining.

Immunohistochemistry

For cryosectioning placental tissues or explant cultures were fixed in 2% paraformaldehyde, soaked in 0.5 mol/L sucrose/phosphate-buffered saline (PBS), covered with OCT compound (Sakura, Zoetermonde, The Netherlands), frozen in liquid nitrogen, and stored at -80°C as described recently.⁴⁶ For immunohistochemistry, tissues (4- μm serial sections) were postfixated with 1% paraformaldehyde (10 minutes) and treated with 0.1% Triton X-100/PBS (5 minutes). After incubation in blocking solution (NEN, Boston, MA), slides were incubated overnight with primary antibodies followed by 1-hour treatment with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 $\mu\text{g}/\text{ml}$; Molecular Probes, Eugene, OR) or fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (5 $\mu\text{g}/\text{ml}$; Molecular Probes). The following primary antibodies were used: anti-active β -catenin (anti-ABC, clone 8E7, mouse, 1.5 $\mu\text{g}/\text{ml}$; R&D Systems, Minneapolis, MN), anti-Ki-67 (clone Ki-S5, mouse, 5 $\mu\text{g}/\text{ml}$; Chemicon, Temecula, CA), anti-p57KIP2, (C-20, rabbit, 2 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology, Santa Cruz, CA), anti-TCF-3/4, (clone 6F12-3, mouse, 10 $\mu\text{g}/\text{ml}$; Upstate, Lake Placid, NY), anti-TCF-4 (clone 6H5-3, mouse, 5 $\mu\text{g}/\text{ml}$; Upstate) and anti-LEF-1 (anti-LEF, clone 2D12, mouse, 5 $\mu\text{g}/\text{ml}$; Upstate). Finally, all sections were counterstained with 1 $\mu\text{g}/\text{ml}$ of DAPI (4'6 diamidine-2'-phenylindole dihydrochloride) from Roche (Mannheim, Germany) and covered with fluoromount G (Soubio, Birmingham, AL). Sections were analyzed by fluorescence microscopy (Olympus BX50, Hamburg, Germany) and digitally photographed. For paraffin embedding, placental tissue was prepared as previously described.⁵¹ Briefly, after fixation (4%

formaldehyde, 24 hours at 4°C), samples were dehydrated and embedded in paraffin (Merck). Serial sections (4 to 5 μm) were prepared, deparaffinized, and finally heated in a microwave oven (2×5 minutes, 850 W). Blocking procedures and antibody staining were performed as described above.

RNA Extraction and Semiquantitative RT-PCR

Total RNA isolation using TRI-Reagent was performed as suggested by the manufacturer (Molecular Research Center Inc., OH). Before supplementation of TRI-Reagent, tissue samples were first minced using a Braun microdismembrator (Mikro-Dismembrator S; B. Braun Biotech International, Melsungen, Germany). For RT-PCR analysis, first-strand cDNA synthesis using 2 μg of total RNA and SuperScript (10 U/ μL ; Invitrogen) was performed. Semiquantitative PCR amplification (45 seconds at 96°C , 1 minute at 56°C , 1 minute at 72°C) was performed with PCR reagent system (Invitrogen) in a Robo-Cycler Gradient 96 (Stratagene, Amsterdam, The Netherlands) using 0.5 U *Taq* polymerase (Invitrogen). Cycle numbers were optimized within the linear range of individual PCR reactions. In all experiments, possible DNA contamination was assessed by negative control RT-PCR in which reverse transcriptase was omitted in the RT step. Sequences of the forward and reverse primers to identify mRNA expression were 5'-TAAAGTGCCCGTGGTGC-AG-3' and 5'-TCTGTTCATGCTGAGGCTTCAC-3' for LEF-1 (485 bp DNA fragment, 30 cycles), 5'-CATCTGCAGCTCTGCCATTGTGAC-3' and 5'-AGGGATGATCG-CCACTGGCAAG-3' for TCF-3 (328 bp, 30 cycles), 5'-CGAGACGCCAAGTCACAGAC-3' and 5'-TTGACCAATGAACCTCGATAAAC-3' for TCF-4 (451 bp, 30 cycles), 5'-AAGATGGTGCCAACTTCACCG-3' and 5'-CTGCCTTCTTGGGGGCTTTGC-3' for Wnt2b (321 bp, 30 cycles), 5'-GGGCCACCTGCTGAAGGAGAA-3' and 5'-TTGACGAAGCAGCACCAGTGGAA-3' for Wnt7b (333 bp, 30 cycles), 5'-AAGGCTCCACAGTGACACAAGG-3' and 5'-AGAGGAGAGAAACCCCAACTACCAC-3' for fzd3 (330 bp, 30 cycles), 5'-AGTCTTCAGCGGCTTGATCTTGT-3 and 5'-GCTCCGTCGCTTTACCTCT-3' for fzd6 (561 bp, 30 cycles), and 5'-CATCCTCGTCTTCACTCATC-3' and 5'-GGCTCGAGGTCTGTCTGCT-3' for LRP-6 (550 bp, 30 cycles). GAPDH (sense primer, 5'-CCATGGAAGAGCTGGGG-3', and anti-sense primer, 5'-CAAAGTTGTCATGGATGACC-3') was used as loading control (185 bp, 20 cycles). The PCR products were analyzed on 1.5% agarose gels containing ethidium bromide and photographed under UV radiation. All PCR fragments were sequence verified on a 16-capillary sequencer by using the nonradioactive ABI PRISM Terminator cycle sequencing ready reaction kit as specified by the supplier (Applied Biosystems, Foster City, CA).

Preparation of Protein Lysates and Western Blot Analysis

Cells were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.8), 1 mmol/L KCl, 0.3% Triton X-100, and protease

inhibitor cocktail (1:100; Sigma, St. Louis, MO). Protein quantity was evaluated by Bradford assay (Pierce, Rockford, IL), and quality was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. A portion (20 μ g) of total protein lysates was separated on denaturing polyacrylamide gels (10%), transferred to filters, and incubated overnight (4°C) with primary antibodies in Tris-buffered saline-0.3% Tween-20 (TBST) containing 0.5% nonfat dry milk as described recently.⁴³ Monoclonal anti-TCF-3/4 and anti-TCF-4 antibodies (Upstate) were used at a concentration of 2 or 1 μ g/ml, respectively. Anti-LRP-6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a concentration of 0.4 μ g/ml. Anti- β -catenin (clone 14, mouse; Transduction Laboratories, Lexington, KY) and anti-cyclin D1 (M20, rabbit; Santa Cruz Biotechnology) antibodies were used at 0.5 μ g/ml and 0.2 μ g/ml, respectively. After three washing steps in TBST, blots were incubated with peroxidase-linked anti-mouse IgG (1:80,000, NA 931; Amersham Pharmacia Biotech, Buckinghamshire, UK) or with peroxidase-linked anti-rabbit IgG (1:50,000; Amersham). Signals were developed using the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To monitor equal protein loading, blots were stripped in a buffer containing 100 mmol/L β -mercaptoethanol, 2% sodium dodecyl sulfate, and 6.25 mmol/L Tris-HCl, pH 6.7, at 50°C and subsequently incubated with an anti-GAPDH antibody (clone 6C5, 4 μ g/ml; Ambion, Austin, TX). PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) was used as a molecular size marker. Western blot density data were analyzed using alphaEaserFC software (Alpha Innotech, CA).

Proliferation Assays

For detection of DNA synthesis as a marker of proliferation, BrdU labeling and Detection Kit I was used according to the manufacturer's instructions (Roche Diagnostics, Vienna, Austria). Briefly, purified first trimester cytotrophoblasts were seeded on uncoated or Matrigel-coated 48-well culture dishes (10,000 cells/well; Costar) and incubated with 10 μ mol/L BrdU for 24 hours in the presence or absence of recombinant Wnt3A (100 ng/ml, R&D Systems) and/or recombinant Dickkopf-1 (1 μ g/ml; R&D Systems). Additionally, cells were immunocytochemically stained with Ki-67 antibodies (5 μ g/ml; Chemicon) after 24 hours of cultivation. Subsequently, cells were fixed for 20 minutes in Ethanol-Fixans (15 mmol/L glycine/70% ethanol, -20°C) and Ki-67 protein or incorporated BrdU were detected with monoclonal anti-Ki-67 or BrdU antibodies, respectively. After counterstaining with DAPI, the number/percentage of BrdU- and Ki-67-positive nuclei was counted in ~250 to 300 cytotrophoblasts. Proliferation of SGHPL-5 cells was evaluated by counting of cumulative cell numbers. Cells (5000 per 300 μ l) were seeded in 24-well dishes in the absence or presence of 100 ng/ml of recombinant Wnt3A and counted after 24, 48, 72, 96, and 120 hours using a multichannel electronic cell counter (CASY-I; Schärfe Systems, Reutlingen, Germany). Medium containing Wnt3A was changed every second day.

Figure 1. Analyses of components of the Wnt signaling pathway in different placental tissues and primary cell cultures. Extraction of mRNA and protein from cells and tissues was performed as described in Materials and Methods. Representative examples of experiments performed with at least three different placental tissues or cell preparations are shown. **A:** Semiquantitative RT-PCR analyses. Detection of GAPDH cDNA fragment was used as a control of equal mRNA quantities. Primer sequences, cycle numbers, and length of PCR products are mentioned in Materials and Methods. **B:** Western blot analyses. Separation of proteins and immunodetection using antibodies specifically recognizing LRP-6 (180 kD), TCF-4 (66 kD), or both TCF-3 and TCF-4 (66 kD, 80 kD) were performed as described in Materials and Methods. After stripping of filters, antibodies against GAPDH (36 kD) were used to monitor protein loading. LRP-6, TCF-3, TCF-4, GAPDH signals, and marker bands (left) are indicated; unspecific signals are depicted by open circles.

Invasion and Migration Assays

Matrigel invasion assays were performed using BD BioCoat growth factor-reduced Matrigel invasion chambers (BD Bioscience) according to the manufacturer's instructions. Briefly, SGHPL-5 cells were trypsinized, washed twice with

PBS, and seeded on rehydrated Matrigel inserts at a concentration of 15,000 cells/200 μ l of culture medium. Primary cytotrophoblast were plated on Matrigel-coated invasion chambers at a concentration of 100,000 cells/200 μ l in DMEM. Before seeding, cells were incubated for 30 minutes at 37°C in the presence or absence of recombinant Wnt3A and/or different doses of Dkk1. After 48 hours cells on the upper side of the inserts were removed by a cotton swap. For evaluation cells on the lower surface were fixed in 3.7% formaldehyde/PBS (10 minutes) and treated with 0.1% trypsin/PBS (5 minutes). After blocking with 1% bovine serum albumin (30 minutes), cells were stained with the cytokeratin 7 antibody and counterstained with DAPI. Slides were digitally photographed and analyzed by alphaEaserFC software (Alpha Innotech). For migration assays, SGHPL-5 cells (15,000 cells/200 μ l) and primary trophoblasts (50,000 cells/200 μ l) were seeded on 8- μ m pore Transwell inserts (Costar). After 24 hours migration was evaluated as described above.

Luciferase Reporter Assays

Cells were co-transfected with plasmids⁵² containing either multimeric LEF/TCF cognate sequences (pTOPFLASH) or mutated binding sites (pFOPFLASH) and CMV- β Gal vectors as described recently.⁴⁷ After 12 hours cells were stimulated with 100 ng/ml of Wnt3A for an additional 14 hours. Luciferase activity was determined on a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) using a commercial luciferase assay system (Promega, Madison, WI). Activity of β -galactosidase was quantitated on a photometer by determining the conversion of the chromogenic substrate chlorophenol red- β -D-galactopyranoside (Roche Diagnostics, Vienna, Austria) at 570 nm as described.⁵³ For each sample luciferase and β -Gal assays were performed in duplicates, and mean values were calculated.

EMSA

Nuclear extracts were prepared using NE-PERT nuclear and cytoplasmic extraction reagent according to the manufacturer's instructions (Pierce, Rockford, IL). Annealing/labeling of complementary oligonucleotide sequences and EMSA were performed as described elsewhere.⁴⁷ Oligonucleotides were derived from the optimized TCF binding site (wtTCF sense, 5'-CCCTTTGATCTTACC-3', and wtTCF anti-sense, 5'-GGTAAGATCAAAGGG-3') as described.^{52,54} For competition experiments unlabeled wtTCF cognate sequences and mutated binding sites (mutTCF sense, 5'-CCCTTTGGCCTTACC-3', mut TCF anti-sense 5'-GGTAAGGCCA-

AAGGG-3') were used at 50-fold molar excess. Binding reactions were performed in a buffer containing 3 μ g of nuclear extract, 4 fmol of ³²P-labeled double-stranded oligonucleotide, 16 mmol/L HEPES, 1 mmol/L dithiothreitol, 60 mmol/L KCl, 1 mmol/L ethylenediamine-tetraacetic acid, 100 ng poly(dI:dC), and 10% glycerin. In supershift experiments, anti-TCF-3/4 (1 μ g), anti-TCF-4 (1 μ g), or anti- β -catenin (0.5 μ g) antibodies were added after 30 minutes, and binding reactions were incubated for an additional 15 minutes at room temperature. Electrophoresis of protein-DNA complexes was performed on 4% polyacrylamide gels (3% glycerin) at 4°C (25 mA). Gels were dried and exposed to films (Hyperfilm MP, Amersham Pharmacia Biotech).

Statistical Analysis

Statistical analyses were performed with SPSS 10 (SPSS Inc., Chicago, IL) using Student's paired *t*-test or one-way analysis of variance. A *P* value <0.05 was considered statistically significant.

Results

Expression of Factors Involved in Canonical Wnt Signaling in Placental Tissue and Cell Cultures

To assess the potential role of Wnt/TCF signaling in invasive trophoblast differentiation, LEF-1/TCF expression was investigated in different placental tissues, cell lines, and primary cultures. Because TCF-1 is restricted to the T-cell lineage, we focused on LEF-1, TCF-3, and TCF-4 which are produced in different epithelial cell types.^{54,55} Semiquantitative RT-PCR analyses revealed that LEF-1 was present in first trimester villous fibroblasts, in villi dissected from villous explant cultures cultivated on Matrigel, and in total placenta of early and late gestation (Figure 1A). However, LEF-1 was absent from all trophoblast cell types (Figure 2C), suggesting that the factor could play a role in villous stromal cell proliferation and/or differentiation. In contrast, TCF-3 and TCF-4 mRNA expression could be detected in trophoblast cell lines and in the different primary cultures. In addition, selected Wnt ligands and frizzled receptors were investigated. Wnt2B, Wnt7B, fzd3, fzd6, and LRP-6 mRNAs were expressed in all tissues and cell types analyzed, suggesting that functional Wnt receptors (fzd/LRP-6) could be formed in human trophoblasts. Wnt3A could not be detected by RT-PCR analyses (data not shown). Western blot analyses indicated that TCF-3 and TCF-4 protein were weakly expressed in villous fibroblasts and term placenta but were more strongly produced in early gestation placenta, primary

Figure 2. Immunohistochemical analyses of LEF-1/TCF and β -catenin localization in placental tissues and villous explant cultures. Serial sections were immunostained with different antibodies, counterstained with DAPI, and analyzed by fluorescence microscopy as described above. Representative placental sections prepared from the 6th week (*n* = 5), 8th to 12th weeks (*n* = 14), and 18th to 22nd weeks (*n* = 12) of pregnancy and from first trimester villous explant cultures (8th week, *n* = 5; 10th week, *n* = 3) are shown. Ci, cell island; vCTB, villous cytotrophoblast; CC, cell column; EVT, extravillous trophoblast; VC, villous core; ABC, active β -catenin. **Arrowheads** indicate representative cytotrophoblasts expressing nuclear ABC. **A:** Villus of a 6-week placenta. **B:** Eight- and ten-week placentas. The **inset** depicted in ABC-1 is shown at higher magnification below (ABC-2). **C:** Midgestation anchoring villi attached to the decidua. Specific antibodies are indicated on the **left**, the corresponding DAPI counterstains are shown at the **right**. The **inset** depicted in ABC-1 is shown at a higher magnification in ABC-2 (digitally zoomed). **D:** Differentiating villous explant (10th week) seeded on Matrigel for 24 hours. Pictures were taken at a 400-fold and 1000-fold (ABC) magnification. Original magnifications: $\times$ 400 [**A**, **B** (week 8)]; $\times$ 200 [**B** (week 10), **C**]; $\times$ 1000 (ABC).

extravillous trophoblasts, and trophoblast cell lines (Figure 1B). LRP-6 protein was more uniformly expressed.

Invasive Differentiation of Cytotrophoblasts Is Associated with Induction of TCF-4 and Nuclear Accumulation of Dephosphorylated β -Catenin

Subsequently, we investigated localization of the Wnt-dependent transcription factors in placental tissues as well as differentiating villous explant cultures using immunohistochemistry (Figure 2). Analyses of placental villi from early gestation (6th week) revealed expression of TCF-3/4 and, to lesser extents, TCF-4 in nuclei of villous cytotrophoblasts (Figure 2A). Interestingly, formation of proliferative, Ki-67-positive cell islands was accompanied by increasing nuclear expression of TCF-3/4 and TCF-4, the latter being predominantly expressed in distal areas of the island. The active, dephosphorylated β -catenin (ABC) was mainly detected at the cell membrane of cytotrophoblasts suggesting localization at adherens junctions. A closer examination of cell columns at the 8th week of pregnancy indicated that TCF-4 is mainly expressed in distal, noncycling, p57KIP2-positive cells, which eventually develop into invasive, extravillous trophoblasts on contact with the maternal, uterine tissue (Figure 2B). In contrast, anti-TCF-3/4 antibodies also recognized signals in proliferating, Ki-67-positive nuclei, suggesting that TCF-3 expression could be associated with growth of the cell column. ABC expression decreased in distal areas of cell columns of 10-week placentas, and several cytotrophoblasts harboring both membrane and nuclear localization of the protein could be detected. Nuclear expression of ABC was also observed in several of the villous cytotrophoblasts (not shown). At the 20th week of gestation, TCF-3 and TCF-4 were abundantly expressed in cytotrophoblasts invading the decidua, whereas placental expression of LEF-1 was restricted to the villous core (Figure 2C). Numerous invasive trophoblasts exhibited nuclear ABC staining, suggesting that invasive differentiation could be associated with the formation of active β -catenin/TCF transcription factor complexes. To confirm differentiation-dependent induction of TCF-4, expression was evaluated in short-term (24 hours) villous explant cultures seeded on Matrigel (Figure 2D). Similar to the *in vivo* situation, TCF-4 was predominantly detectable in noncycling, p57KIP2-positive trophoblasts but not in proximal, Ki-67-positive regions of the cell column. TCF-3/4 antibodies detected signals in both proliferative and differentiated cytotrophoblasts. In some invasive cells, detaching from each other in distal zones of the explant, diffuse cytoplasmic and nuclear staining of ABC was observed.

Wnt3A Induces Active β -Catenin/TCF Transcription Factor Complexes in a Trophoblastic Cell Line and Primary Cytotrophoblasts

To study whether trophoblast cells might form active β -catenin/TCF complexes, SGHPL-5 cells were treated with recombinant Wnt3A (Figure 3). The DNA-binding

activities of TCFs and their association with β -catenin were analyzed by EMSA (Figure 3A). Specific TCF complexes interacting with the radiolabeled TCF recognition sequence were detected. Binding was competed in the presence of unlabeled wild-type but not mutated TCF cognate sequences and was partially or completely supershifted with anti-TCF-4 or anti-TCF-3/4 antibodies, respectively. On Wnt3A treatment the DNA-binding complex reacted with the ABC antibody, suggesting Wnt-dependent nuclear translocation and association of β -catenin with TCF. Luciferase assays using a reporter harboring multimeric TCF recognition sequences (TOP-FLASH) confirmed functionality of β -catenin/TCF complexes (Figure 3B). Compared to untreated cultures, supplementation of Wnt3A increased luciferase activity of the plasmid 4.2-fold whereas the mutated FOP-FLASH reporter was unresponsive in SGHPL-5 cells. Similarly lithium chloride, an inhibitor of GSK-3 β ,⁵⁶ provoked interaction of TCFs with β -catenin and elevated TOP-FLASH reporter activity (not shown). Wnt3A-dependent translocation of β -catenin was also investigated in purified first trimester cytotrophoblasts containing 27% ABC-positive nuclei in unstimulated cultures (Figure 3C). Wnt3A treatment significantly increased the number of ABC-positive nuclei (58%). In the presence of Wnt3A and recombinant Dkk1, 25% of cytotrophoblast nuclei were β -catenin-positive, whereas addition of Dkk1 alone significantly decreased the number of ABC-positive nuclei to 11%, suggesting inhibition of endogenous Wnt signaling. Luciferase assays revealed 1.5-fold induction of the TOP-FLASH reporter on Wnt3A addition, suggesting ligand-dependent formation of activating β -catenin/TCF complexes in primary cytotrophoblasts (Figure 3D). Compared to FOP-FLASH luciferase activity, TOP-FLASH reporter expression was 1.7-fold higher in nonstimulated cells, indicating the presence of endogenous β -catenin/TCF complexes.

Wnt3A Induces β -Catenin/TCF Target Genes in Trophoblastic Cells and Primary Cytotrophoblasts

As a consequence of Wnt3A treatment induction of the β -catenin/TCF target genes cyclinD1,²¹ TCF-4,⁵⁷ and β -catenin⁵⁸ were observed in SGHPL-5 cells (Figure 4A). Compared to controls, TCF-4, cyclin D1, and β -catenin were 2.6-, 3.5-, and 3.1-fold elevated, respectively, after 24 hours of Wnt3A-stimulation. Compared to controls (100%), treatment with Dkk1 alone reduced endogenous cyclin D1 and β -catenin levels to 39% and 41%, respectively. The ligand also increased protein expression of Wnt/TCF target genes in first trimester cytotrophoblasts (Figure 4B). Cyclin D1 and β -catenin rose 2.6- and 2.8-fold, respectively, after 24 hours of Wnt3A stimulation. Similarly to SGHPL-5 cells, reduction of cyclin D1 and β -catenin to 53% and 62%, respectively, was observed in the presence of Dkk1.

Figure 3. Analyses of formation and activity of β -catenin/TCF complexes. SGHPL-5 cells or first trimester cytotrophoblasts were stimulated with Wnt3A (100 ng/ml) for 14 hours. **A:** EMSA of Wnt3A-treated SGHPL-5 cells. Nuclear extraction and EMSA of controls and Wnt3A-stimulated cells were performed as described in Materials and Methods. Specific DNA-binding complexes containing TCF-3/4 are indicated; bands supershifted by different antibodies (AB) are indicated by asterisks. Open circles mark unspecific signals. **B:** Luciferase assays of SGHPL-5 cells after co-transfection of either TOP-FLASH or FOP-FLASH and CMV- β Gal plasmids. After Wnt3A stimulation luciferase activity was determined in protein extracts and normalized to β -Gal activity as described above. For comparison, values of FOP-FLASH (unstimulated) were arbitrarily set to 100% in each experiment. Bars represent mean values of six independent transfections of three different cultures, error bars indicate SD. * $P < 0.05$. **C:** Wnt-dependent accumulation of ABC in nuclei of primary cytotrophoblasts. Cells were seeded on chamber slides and treated with Wnt3A, Wnt3A and Dkk1 (1 μ g/ml), or Dkk1 alone, fixed, and immunocytochemically stained with ABC antibodies and DAPI. The ratio of ABC-positive nuclei versus DAPI of each condition was determined on digital pictures using α EaserFC software. Eight chamber slides in parallel (each 100 to 150 nuclei) of two different Kliman preparations were counted. **D:** Luciferase assay of primary cytotrophoblasts. Luciferase assay and normalization of values was performed as described above. Mean values were calculated from four independent transfections of two different preparations. * $P < 0.05$.

Figure 4. Western blot analyses of canonical Wnt/β-catenin target genes. Preparation of protein lysates, separation, and immunodetection using antibodies specifically recognizing TCF-4 (66 kD) β-catenin (92 kD), or cyclin D1 (35 kD) were performed as described in Materials and Methods. After stripping of filters, antibodies against GAPDH (36 kD) were used to evaluate protein loading. Signals at 24 hours of Wnt3A stimulation were quantitated using αEaserFC software and normalized to GAPDH signals. Unspecific signal is marked by open circle. **A:** Protein expression in SGHPL-5 cells after 6, 14, and 24 hours of Wnt3A stimulation (100 ng/ml). For evaluation of canonical Wnt signaling, cells were stimulated for 24 hours in the absence or presence of Wnt3A and/or Dkk1 (1 μg/ml). **B:** Protein expression in primary cytotrophoblasts after 24 hours of Wnt3A treatment in the absence or presence of Dkk1.

Activation/Inhibition of the Canonical Wnt-Signaling Pathway Affects Proliferation of First Trimester Cytotrophoblasts

To assess whether Wnt signaling could alter trophoblast proliferation, cumulative cell numbers of SGHPL-5 cells were determined in the presence of Wnt3A (Figure 5A). Despite Wnt-dependent induction of proliferation-associated genes, such as cyclin D1, Wnt3A did not affect SGHPL-5 cell numbers within a period of 120 hours. In contrast, DNA synthesis of cytotrophoblasts cultivated on plastic increased on Wnt3A treatment (Figure 5B). Compared to controls ($8.1 \pm 1.5\%$ SD of nuclei), numbers of BrdU-labeled and Ki-67-stained cells significantly rose to $12.31 \pm 2\%$ SD and $12.1 \pm 3\%$ SD, respectively, on Wnt3A treatment. On plating on Matrigel enhanced BrdU labeling ($18.7 \pm 1.5\%$ SD) could be observed; however,

Figure 5. Proliferation of SGHPL-5 cells and primary cytotrophoblasts in the presence of Wnt3A. **A:** Determinations of cumulative cell numbers of Wnt3A-treated and untreated cultures were performed as described in Materials and Methods. Mean values $\pm$ SD are derived from two different experiments performed in duplicates. **B:** Proliferation of first trimester cytotrophoblasts. Cells were treated for 24 hours in the absence or presence of Wnt3A (100 ng/ml) and/or Dkk1 (1 μg/ml). BrdU labeling (24 hours) and Ki-67 staining were performed as described in Materials and Methods. The percentage of BrdU-labeled and Ki-67-labeled nuclei is depicted. Mean values $\pm$ SD are derived from two different experiments performed in triplicates. * $P < 0.05$.

Wnt3A did not significantly alter proliferation ($21.5 \pm 3.2\%$ SD). Supplementation of Dkk1 significantly decreased BrdU-labeling of both plastic- and Matrigel-seeded cytotrophoblasts to $4.3 \pm 2.1\%$ SD and $11.7 \pm 2.5\%$ SD, respectively, suggesting that autocrine Wnt-signaling could be blocked by the inhibitor.

Wnt3A Treatment Increases Trophoblast Migration and Invasion

Furthermore, the influence of Wnt3A and Dkk1 treatment on trophoblast migration and invasion was investigated. Wnt3A dose dependently increased migration of SGHPL-5 cells through uncoated filters (Figure 6A). Compared to controls, treatment with 100 ng/ml increased migration to $240 \pm 35\%$ SD, whereas addition of Dkk1 in the absence of Wnt3A decreased migration to $55 \pm 40\%$ SD. Invasion of SGHPL-5 cells through Matrigel-coated chambers was elevated to $270 \pm 31\%$ SD in the presence of Wnt3A. Inducible invasion was reduced to basal

Figure 6. Migration and invasion assays of trophoblastic SGHPL-5 cells and purified first trimester cytotrophoblasts. Transwell migration and invasion assays through Matrigel were performed as described above. Cells at the underside of the filters were digitally counted and analyzed using imaging software. Wnt3A and Dkk-1 were added at 100 ng/ml and 1 µg/ml, respectively, if not indicated otherwise. Values of untreated cultures (n.c., negative control) were arbitrarily set at 100%. * $P < 0.005$. **A:** Migration and invasion of SGHPL-5 cells. Bars represent mean values of six (migration) and four (invasion) different experiments, respectively, performed in duplicates, error bars indicate SD. **B:** Migration and invasion of primary cytotrophoblasts. Bars represent mean values of two (migration) and three (invasion) different experiments performed in duplicates, error bars indicate SD.

levels in the presence of Dkk-1. Addition of 0.5, 1, and 2 µg of the inhibitor alone decreased invasion to $63 \pm 25\%$ SD, $56 \pm 22\%$ SD, and $58 \pm 31\%$ SD, respectively. Similarly, motility of purified cytotrophoblasts was stimulated by Wnt3A, although to a lesser extent (Figure 6B). Migration and invasion increased to $185 \pm 32\%$ SD and $200 \pm 56\%$ SD, respectively. Compared to controls, Dkk1 decreased migration and invasion of primary cells to $55 \pm 18\%$ SD and $62 \pm 10\%$ SD, again suggesting inhibition of endogenous Wnt-ligands/autocrine Wnt signaling.

Elevated Expression of Nuclear β -Catenin in Extravillous Trophoblasts of CHMs

CHM is characterized by trophoblastic hyperplasia and extensive formation of extravillous trophoblasts due to their androgenetic origin, with all 46 chromosomes being paternally derived.^{10,11} To study whether Wnt/ β -catenin could play a role in the pathogenesis of the disease, 13 CHMs were investigated by immunohistochemistry (Figure 7). For evaluation of histopathological diagnosis, p57KIP2 expression was investigated in villous tissue of CHM placentas. This cyclin-dependent kinase inhibitor, which is paternally imprinted, was recently shown to be a useful tool for the diagnosis of CHM because its expression is primarily absent in villous cytotrophoblasts.⁴⁰ According to these criteria, p57KIP2 was found to be expressed in 0 to 1.5% of villous cytotrophoblasts of 11 placentas, confirming CHM, whereas two placentas were excluded from further investigations because p57KIP2 expression was observed in more than 7% of villous cytotrophoblasts. Representative examples of villi from CHM placentas lacking p57KIP2 expression are shown (Figure 7A). Subsequently, numbers of ABC-positive nuclei were counted in trophoblast cell column/islands of CHM and normal age-matched placentas. On the average, $8 \pm 3\%$ of extravillous trophoblasts of normal placentas were ABC-positive, whereas the active protein could be detected in $45 \pm 13\%$ of extravillous trophoblasts of CHMs ($P < 0.01$). TCF-4 and ABC localization in extravillous trophoblasts of CHMs are depicted (Figure 7B). Interestingly, the vast majority of ABC-positive extravillous trophoblasts expressed the protein exclusively in the nucleus, suggesting that nuclear accumulation of active β -catenin may contribute to the premalignant status of CHMs.

Discussion

Developmental processes of the human placenta such as the invasive differentiation pathway are tightly controlled by trophoblast and decidual products expressed at the fetal/maternal interface.^{6,7} Several growth factors including IGF-II, EGF, or HGF promote trophoblast migration and invasion acting through activation of extracellular signal-regulated kinases (ERKs) or the phosphoinositide 3-kinase (PI3K)-signaling pathway.⁵⁹ However, definitive target genes of these signaling cascades have not been

Figure 7. Immunohistochemical analyses of normal and CHM placentas. Placental tissues of 13 paraffin-embedded CHMs (between the 7th and 14th weeks of gestation) and 9 age-matched controls (between the 8th and 12th weeks of gestation) were stained with different antibodies, counterstained with DAPI, and analyzed by fluorescence microscopy as described in Materials and Methods. **A:** CHMs were stained with antibodies against p57KIP2, Ki-67, and cytokeratin 7. Two representative examples of villous CHM tissue (8th and 10th weeks of pregnancy), lacking p57KIP2 expression in villous cytotrophoblasts, and a normal 8-week placenta (control) are shown. **B:** Serial sections of two representative CHMs (9th and 10th weeks of gestation). VC, villous core; EVT, extravillous trophoblast; CC, cell column. Note the abundance of extravillous trophoblasts lacking membrane-bound ABC. CK7, cytokeratin 7; ABC, active β -catenin. For statistical evaluation, the ratio of nuclear ABC versus DAPI was counted in areas containing extravillous trophoblasts/cell columns. In total 1706 and 1415 nuclei of extravillous trophoblasts of CHMs (11 placentas) and normal tissues (9 placentas), respectively, were evaluated. Original magnifications: $\times 200$ (A); $\times 400$ (B).

evaluated in human trophoblasts. In addition, the role of transcription factors that control commitment and differentiation of invasive trophoblasts has not been defined. Key regulatory factors that control proliferation and differentiation of murine trophoblasts, such as Hand1 or Mash-2,^{60,61} are either absent in the human placenta⁴² or are not restricted to a distinct trophoblast subtype,⁶² questioning their role in extravillous trophoblast formation.

Invasive differentiation of trophoblasts shares some features with the process of epithelial-mesenchymal transition. Conversion of epithelial to fibroblast-like cell types occurs during development, but it has also been implicated in the transformation of early stage tumors into invasive malignancies.^{63,64} Differing from cancer cells, extravillous trophoblasts do not up-regulate fibroblast-specific genes and maintain epithelial markers such as cytokeratin 7 during invasion *in vivo* and *in vitro*.^{46,65} However, some of the critical steps of epithelial-mesenchymal transition occur in invading trophoblasts. For example, E-cadherin, which maintains epithelial integrity, is transiently down-regulated in the proximal invasion zone of the anchoring villus.⁹ In this area we could also observe reduction of membrane-bound β -catenin (Figure 2), suggesting that impaired cell-cell contact might be one of the initial events of the trophoblast invasion process. Moreover, recent data demonstrated that deeply invaded, single-cell cytotrophoblasts showed E-cadherin-negative breaches in the cell membrane, suggesting partial epithelial-mesenchymal transition.⁶⁶

Because expression of Wnt ligands and activation of β -catenin/TCF have been implicated in epithelial-mesenchymal transition, development, and cell differentiation,^{67–70} we here investigated the particular signaling pathway and its downstream target genes in different trophoblast cell models. The data suggest that TCFs could be critically involved in invasive trophoblast differentiation. Immunohistochemical analyses of early placental tissues revealed that formation of precursors of the invasive trophoblast, residing in cell islands, associates with strong induction of TCF-4. During formation of larger cell island/cell columns, TCF-4 became restricted to non-cycling p57KIP2-positive trophoblasts that eventually develop into migratory cells on matrix contact. Therefore, we concluded that expression of TCF-4 in precursors of the invasive trophoblasts might render the cells susceptible to ligand-dependent activation of the transcription factor. The fact that active nuclear β -catenin could be detected in a considerable number of TCF-positive extravillous trophoblasts of the placental bed suggested that β -catenin/TCF DNA-binding complexes could be involved in the invasive differentiation process. In addition, immunohistochemistry using the TCF-3/4 antibody revealed nuclear staining in Ki-67-positive trophoblasts of the cell column and in villous cytotrophoblasts. Thus, in the presence of ABC, TCFs might also control trophoblast proliferation. However, contradictory data with respect to expression of nuclear β -catenin in placental villi have been published. Two studies demonstrated that β -catenin is predominantly found at the membrane of villous cytotrophoblasts^{71,72} whereas one publication showed that the protein localizes mainly to cytotrophoblast nu-

clei.⁷³ However, none of these studies have used an antibody exclusively detecting dephosphorylated β -catenin. Using this ABC-specific antibody, we observed nuclear expression in ~25% of isolated cytotrophoblasts, suggesting that a considerable amount of these cells may contain active TCF/ β -catenin complexes. Experiments using recombinant Dkk1 indicated that the endogenous TCF/ β -catenin heterodimers are likely functional in trophoblast cultures. Treatment with the Wnt antagonist in the absence of exogenous Wnt3A reduced numbers of ABC-positive nuclei and expression of critical TCF target genes, such as cyclin D1 and β -catenin, in Western blot analyses.

Several lines of evidence support the idea that formation of TCF/ β -catenin heterodimers can be amplified in isolated cytotrophoblasts and in trophoblastic SGHPL-5 cells. Treatment of the two cellular models with recombinant Wnt3A provoked nuclear translocation of dephosphorylated β -catenin, DNA-binding of the TCF/ β -catenin transcription factor, and elevated expression of a luciferase reporter harboring TCF recognition elements. Compared to FOP reporters, higher basal levels of TOP reporter expression were noticed in unstimulated primary cytotrophoblasts. Moreover, Wnt-dependent induction of the TCF luciferase plasmid was less pronounced in the primary cultures than in SGHPL-5 cells. Interestingly, in Western blot analyses higher basal levels of ABC could be detected in cytotrophoblasts than in the cell line (J. Pollheimer and M. Knöfler, unpublished observation). This could be because of elevated production of Wnt ligands or other secreted factors in the primary cells, possibly inactivating GSK-3 β ⁷⁴ and rise ABC in an autocrine manner.

Expression of Wnt-2, -3, -4, -5a, -7a, and -8b has been detected in human endometrial cells suggesting that these factors could potentially control invading trophoblasts in a paracrine manner.³⁴ Accordingly, addition of recombinant Wnt3A increased invasion and migration of both SGHPL-5 cells and primary cytotrophoblasts. In agreement with the suggestion that the canonical Wnt signaling pathway could be involved, Wnt3A-dependent effects were primarily abolished in the presence of Dkk1. Moreover, basal invasion and migration of both SGHPL-5 cells and primary cytotrophoblasts were reduced by this inhibitor, suggesting that autocrine Wnt signaling may contribute to the functional properties of differentiated trophoblasts. Indeed, mRNAs of different Wnt ligands are expressed in placental villi,^{35–39} although expression and localization of the encoding proteins have not been investigated so far. As part of an ongoing study, we here demonstrate mRNA expression of Wnt2b, Wnt7b, fzd3, fzd6, and LRP-6 in all trophoblast cell types strengthening the idea that these and other Wnt/fzd factors could play a role in autocrine Wnt signaling. Notably, reduction of invasion/migration was more pronounced in the presence of Dkk-1 alone as compared to the treatment with both Wnt3A and Dkk-1. This suggests that the inhibitor may not completely block Wnt3A-dependent effects. Indeed, it was recently shown that the ligand can also activate ERK phosphorylation through a noncanonical, β -catenin-independent Wnt pathway.⁵⁸

Whereas β -catenin/TCF-dependent invasion was evident, effects of recombinant Wnt3A on trophoblast proliferation were less pronounced. Although the Wnt ligand induced cyclin D1 in SGHPL-5 cells, cumulative cell numbers did not change during 5 days of treatment. Recombinant Dkk1 reduced endogenous cyclin D1 levels, but the inhibitor could not repress proliferation of the cell line (J. Pollheimer and M. Knöfler, unpublished observation). Therefore, we conclude that despite the presence of endogenous Wnt ligand(s), proliferation of SGHPL-5 cells is independent of Wnt signaling. This might be explained by the fact that for generation of a stable cell line cytotrophoblast cultures had been transformed with SV40 large T antigen.⁴⁴ In contrast, proliferation of villous cytotrophoblasts was associated with canonical Wnt signaling. Whereas stimulation of BrdU labeling by Wnt3A was weak, Dkk1 significantly reduced proliferation of cultures either cultivated on plastics or Matrigel. Therefore, we conclude that endogenous Wnt ligand(s) regulate proliferation of cytotrophoblasts in an autocrine manner.

Wnt/TCF signaling has been shown to promote cell proliferation and migration by activating critical target genes associated with either growth, such as cyclin D1 and *c-myc*, or invasion, such as MMP-7 and MMT1-MMP.^{21–25} Similarly, Wnt3A-dependent induction of cyclin D1 could provoke elevated proliferation of cytotrophoblasts, whereas Wnt-inducible genes promoting trophoblast invasion have yet to be elucidated. However, MMP-7 and MT1-MMP are expressed in invasive, extravillous trophoblasts,^{75,76} supporting the idea that these enzymes could also be Wnt-targets in the human placenta.

Analyses of placentas of CHMs support the hypothesis that canonical Wnt signaling could be critically involved in trophoblast proliferation. In these tissues numerous trophoblasts were detected in cell islands/columns lacking membrane-bound β -catenin. Instead these cells expressed considerable amounts of nuclear ABC, which could provoke elevated levels of active TCF/ β -catenin complexes. Nuclear β -catenin protein has been observed in different types of tumor cells and its increasing nuclear abundance has been shown to correlate with the malignancy of some cancers.^{77–79} Therefore, we conclude that nuclear ABC might reflect the hyperproliferative and/or the preinvasive and premalignant status of CHMs. Similar to tumor cells,⁸⁰ recruitment of β -catenin to the nucleus might be caused by changes in proteins regulating β -catenin stability, such as adenomatous polyposis coli, or by elevated expression of Wnt ligands. However, activity of GSK-3 β can be lowered by various growth factors^{81,82} and nuclear translocation of β -catenin can also be provoked through activation of integrin-linked kinase.⁸³ Therefore, several mechanisms could contribute to high levels of nuclear β -catenin in CHM placentas. On the other hand, a recent study using microarray analyses demonstrated sixfold elevated expression of Dishevelled two mRNA in villi of CHM placentas, suggesting that the canonical Wnt pathway could indeed be affected.⁸⁴

Recently, β -catenin/TCF was suggested to play a role in the process of trophoblast genome multiplication. Although activation of the Wnt/TCF pathway has not been demonstrated, nuclear accumulation of β -catenin was

noticed in differentiating bovine trophoblasts forming binucleate cells by DNA-endoreduplication.⁸⁵ Therefore, Wnt/TCF signaling could also be involved in the development of polyploid nuclei of the human extravillous trophoblast.^{86,87}

In conclusion, the data suggest that Wnt/ β -catenin/TCF plays a critical role in invasive trophoblast differentiation. Exaggerated activation of the pathway in CHM could be part of the mechanism elevating nuclear β -catenin, which may promote abnormal trophoblastic hyperplasia and/or local invasion.

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