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Complex Expression Pattern of Wnt Ligands and Frizzled Receptors in Human Placenta and its Trophoblast Subtypes

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Abstract

Canonical Wingless (Wnt) signalling provoked by exogenous and endogenous Wnt ligands was recently shown to play a crucial role in the invasive differentiation of human trophoblasts. To gain insights into the expression pattern of the developmental regulators, we analysed all human Wnt ligands and their frizzled (FZD) receptors in the human placenta and different trophoblast model systems using semi-quantitative PCR. Fourteen out of 19 Wnt ligands and 8 out of 10 FZD receptors were detectable in placental tissues, however, expression patterns varied with gestational age and between different trophoblast subtypes suggesting cell-specific functions. Besides Wnt ligands acting through the canonical pathway, non-canonical ligands such as Wnt-5a, which may also activate alternative Wnt signalling pathways or inhibit canonical Wnt signalling, could be identified. Western blot analyses revealed secretion of Wnt-5a from primary trophoblast cultures and trophoblastic cell lines. To evaluate the potential role of Wnt-5a, SGHPL-5 trophoblast cells were transfected with luciferase reporter plasmids harbouring eight T-cell factor (TCF) DNA-recognition sequences which are exclusively activated through the canonical Wnt signalling pathway. Luciferase assays revealed that Wnt-3a-induced reporter activity was repressed by recombinant Wnt-5a indicating an antagonistic role in trophoblasts. The data suggest that a complex network of Wnt ligands and FZD receptors may regulate developmental processes of the human placenta.

Keywords

Wnt; Frizzled; Placenta; Trophoblast; Differentiation

1. Introduction

Wnt signalling plays an important role in proliferation, differentiation and cell motility of normal development and cancer cell progression [1,2]. Wnt molecules comprise a large family of secreted glycoproteins, which interact with specific surface receptors, the different members of the FZD family [3]. While Wnt can act through several pathways, the canonical, β -catenin-dependent signalling has been extensively characterised. Upon formation of a receptor complex containing Wnt, FZD and low-density lipoprotein receptor-related protein-5/6 (LRP-5/6) cytoplasmic β -catenin gets stabilised and is transferred to the nucleus where it interacts with transcription factors of the T cell-specific factor (TCF) family. Upon binding β -catenin converts TCFs into transcriptional activators inducing target genes for proliferation and invasion [4, 5]. However, Wnts can also bind FZDs independently of LRP,

activate or inhibit canonical Wnt signalling depending on the receptor context and induce non-canonical signal transduction pathways involving Ca^{2+} as a second messenger [6-8].

Recent evidence suggested that Wnt signalling may also play a role in placental development and human trophoblast differentiation. Several mRNAs encoding Wnt ligands and FZD receptors were detected in human term placenta [9-13]. In addition, endometrial expression of Wnt3 and Dickkopf-1 (Dkk1), which inhibits canonical Wnt signalling by disrupting binding of LRP-5/6 to the Wnt/FZD complex, was observed [14], suggesting paracrine mechanisms regulating implantation and/or trophoblast invasion. The TCF family members TCF-3 and TCF-4 are predominantly expressed in invasive trophoblasts and activation of the canonical Wnt signalling pathway promotes trophoblast migration and invasion [15]. The fact that Dkk-1 also blocked basal proliferation and invasion of trophoblast cultures suggested that canonical Wnt signalling could be regulated in an autocrine manner [15]. Therefore, we here investigated the full range of Wnt and FZD mRNAs of the human placenta and its different cell types and performed initial experiments on the potential role of Wnt-5a in trophoblasts.

2. Materials and methods

2.1. Cultivation of primary trophoblasts and trophoblastic cell lines

Placental tissues of early ($n = 6$, between 8th and 12th week of gestation) pregnancy were obtained from legal abortions. Placental tissue of late ($n = 6$, between 38th and 40th week) pregnancy was obtained from caesarean sections. Utilisation of tissues was approved by the local ethical committee. Villous cytotrophoblasts of early ($n = 5$) and late gestation ($n = 6$) with a purity of at least 98% were isolated by Kliman method and immuno-depletion of HLA-I-positive cells as previously mentioned [15,16]. Primary trophoblasts were seeded on plastics at a density of 5×10^5 cells/cm² and cultivated in DMEM containing 10% fetal calf serum (FCS, Pall, East Hills, NY, USA) for 24 h. Villous fibroblasts of first trimester placentae ($n = 4$) were also isolated by trypsinisation and gradient centrifugation (between = 25% and 35% Percoll) and passaged two times in DMEM supplemented with 10% FCS. Fibroblasts were characterised by vimentin immunocytochemistry (100% of cells), a contamination with trophoblasts was excluded by cytokeratin 7 staining. Villous explants of first trimester placentae ($n = 3$) were seeded on Matrigel and after 72 h invaded trophoblasts were separated from attached villi as previously mentioned [17]. JEG-3 choriocarcinoma cells were cultivated in DMEM + 10% FCS. SGHPL-5 cells, exhibiting properties of extravillous cytotrophoblasts, were cultivated in a 1:1 mixture of DMEM/Ham's F-12 supplemented with 10% FCS (GibcoBRL Life Technologies, Paisley, UK) as described [18]. Trophoblastic HTR-8/SVneo cells were cultivated in RPMI 1640 (GibcoBRL) plus 5% FCS as mentioned [19].

2.2. RNA extraction and semi-quantitative RT-PCR

Total RNA isolation of cultured cells was performed using TRI-Reagent as suggested by the manufacturer (Molecular Research Center Inc., OH, USA). Placental tissue samples were first minced using a Braun microdismembrator (Mikro-Dismembrator S, B. Braun Biotech International, Melsungen, Germany) before supplementation of TRI-Reagent. Quality and quantity of RNA was checked using the Agilent Bioanalyzer 2100 (Agilent Palo Alto, CA, USA). Two micrograms of total RNA of each sample were transcribed into first-strand cDNA using SuperScript™ (10 U/μl, Invitrogen, Carlsbad, CA, USA). Semi-quantitative PCR amplification (45 s 96 °C, 1 min 55–63 °C, 1 min 72 °C) was performed with PCR Reagent System (Invitrogen) in a RoboCycler Gradient 96 (Stratagene, Amsterdam, Netherlands) using 0.5 U Taq polymerase (Invitrogen). Primer sequences, gene bank accession numbers, target regions as well as annealing temperatures were taken from the

published literature and are depicted in Table 1. Cycle numbers were additionally optimised within the linear range of individual PCR reactions. In all experiments, possible DNA contaminations were checked by negative control RT-PCR in which reverse transcriptase was omitted in the RT step. Primers for Wnt-3a, Wnt-8a, Wnt-8b, Wnt-9a, Wnt-16, FZD8 and FZD9 failed to detect mRNA expression in human placenta, however, gave rise to signals in different non-placental cell lines and tissues (data not shown). GAPDH (5'-CCATGGAGAAGGCTGGGG-3', sense primer, 5'-CAAAGTTGTCATGGATGACC-3', anti-sense primer) was used as loading control (185 bp). All PCR products were analysed on 1.5% agarose gels containing ethidium bromide and photographed under UV radiation.

2.3. Western blot analyses

For Wnt-5a Western blot analyses primary trophoblasts, cell lines and floating villi of first trimester placentae were incubated overnight in serum-free medium containing 50 µg/ml Heparin (H-3149, Sigma). Cell lines were seeded at a density of 8×10^5 cells/cm². Twenty microlitres of each supernatant (approximately 2 µg of protein) were separated on 12.5% PAA gels and blotted onto nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany) in a Tris/Glycine transfer buffer (pH 11.5). Protein concentration was determined by BioRad Assay Reagent according to the manufacturer's instructions (BioRad, Hercules, CA, USA). After blocking with 3% non-fat milk the membrane was incubated with goat anti-human Wnt-5a antibody (AF645, final concentration: 0.4 µg/ml, R&D Systems, Minneapolis, MN, USA) overnight at 4 °C. Subsequently, filters were incubated with peroxidase-linked, rabbit anti-goat IgG (final dilution: 1:50000, Southern Biotechnology Associates, Birmingham, AL, USA). Detection was performed with Enhanced Chemiluminescence System (Amersham Pharmacia Biotech., Buckinghamshire, UK) and signals were visualised on autoradiography films. Antibodies against Wnt-5a detected specific signals at approx. 48 kDa. As a positive control recombinant, mouse Wnt-5a (50 ng, 645-WN, R&D Systems) was utilised.

2.4. Luciferase reporter assays

SGHPL-5 cells were grown to a confluence of 80% and co-transfected with luciferase plasmids containing either multimeric LEF/TCF cognate sequences (pSuper(8x)TopFlash) or mutated binding sites (pSuper(8x)FopFlash) and CMV-β-Gal vectors as described recently [15]. Briefly, cells cultivated in 24 wells were incubated with 1.5 µg luciferase reporter plasmid and 0.5 µg pCMV-β-Gal in 1.5 µl lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfection efficiency determined by in situ β-Galactosidase staining kit (Stratagene, La Jolla, CA, USA) was between 15% and 20%. After 7 h medium was changed and cells were stimulated with 50 ng/ml Wnt-3a and/or 50–200 ng/ml Wnt-5a for an additional 14 h. Luciferase activity was determined on a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany) using a luciferase assay system (Promega, Madison, WI, USA). 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 [20]. For each sample luciferase and β-Gal assays were performed in duplicate and mean values were calculated.

2.5. Statistics

Statistical analyses were performed by non-parametric Wilcoxon test using SPSS 14 (SPSS Inc., Chicago, IL, USA). A *p* value of less than 0.05 was considered statistically significant.

3. Results

To assess the full range of human Wnt ligands and FZD receptors different placental tissues, trophoblast primary cultures and trophoblastic cell lines were analysed by semi-quantitative RT-PCR using conditions mentioned in Table 1. Wnt and FZD mRNA expression patterns are summarised in Table 2. Of the 19 known Wnt ligands 14 were expressed in first trimester placenta, whereas 5 Wnt ligands (Wnt-3a, Wnt-8a, Wnt-8b, Wnt-9a, Wnt-16) could neither be detected in placental tissues nor in the different trophoblast cultures. 8 of 10 FZD receptors are present in early placenta, FZD8 and FZD9 are absent from all placental cell types. Whereas several Wnt ligands (Wnt1, Wnt2, Wnt-2b, Wnt-3, Wnt-6, Wnt-7a, Wnt-7b, Wnt-9b, Wnt-10a, Wnt-10b) which may activate the canonical signalling pathway were detected in first trimester villous and invasive trophoblasts, expression of non-canonical Wnts (Wnt-4, Wnt-5a, Wnt-5b, Wnt-11) was also noticed. Wnt-5a is of particular interest since it can activate or inhibit canonical Wnt signalling pathways depending on the receptor context [8]. To gain insights into the potential role of Wnt-5a in the placenta, secretion of the protein from placental cell cultures was investigated using Western blotting. Soluble Wnt-5a could be detected in supernatants of villous trophoblasts, of villous explant cultures and of HTR-8/SVneo and SGHPL-5 cells, whereas the ligand was absent from JEG-3 cells (Fig. 1). Recombinant Wnt-5a did not induce the canonical Wnt reporter pSuper(8x)TopFlash in SGHPL-5 cells which however could be activated 10.3 fold by canonical Wnt-3a (Fig. 2) as also previously noticed [15]. In contrast, Wnt-5a suppressed Wnt-3a-mediated transcription to 63% and 67% at a concentration of 200 ng/ml and 150 ng/ml, respectively, suggesting that Wnt-5a may antagonise canonical Wnt signalling in trophoblasts. Compared to Wnt-3a stimulated cells, Wnt-5a did not significantly inhibit luciferase expression at a dose of 100 or 50 ng/ml.

4. Discussion

Semi-quantitative RT-PCR analyses revealed that numerous Wnt ligands and FZD receptors are expressed in the human placenta and its different trophoblast subtypes. Previous data have shown that LRP-6 and Dkk-1 are produced in placental tissue, primary trophoblast and trophoblast cell lines [15]. Therefore, the results suggest that multiple FZD/LRP-receptor complexes transducing canonical Wnt signalling could be formed and modulated by inhibitory molecules such as Dkk family members. In first trimester villous cytotrophoblasts canonical Wnt signalling, which is thought to be regulated by specific Wnt-FZD combinations [21], may involve putative ligand-receptor pairs of Wnt-1, Wnt-2, Wnt-2b, Wnt-7a, Wnt-7b, Wnt-9b, Wnt-10a, Wnt-10b with FZD1, FZD5, FZD7 or FZD10 [21-25]. It is likely that complexes of these proteins with LRP-6 control cytotrophoblast proliferation of early pregnancy in an autocrine manner since inhibition of the canonical Wnt pathway by Dkk-1 decreased basal cell growth in vitro [15]. Moreover, the Wnt ligands Wnt-1, Wnt-7b, Wnt-10a, Wnt-10b are abundantly expressed in first trimester cytotrophoblasts but largely absent from term trophoblasts. This could be explained by downregulation of mRNA expression during gestation and/or the small number of cytotrophoblasts in term placenta as compared to early tissue. Interestingly, Wnt-10a and Wnt-10b were also absent from invasive trophoblasts and villous fibroblasts which may indicate that these Wnt ligands are specifically associated with trophoblast proliferation. Similarly, FZD5 may primarily control trophoblast growth since its expression strongly decreased during invasive differentiation. However, other canonical Wnt ligands such as Wnt-1 or Wnt-2b are abundantly expressed in extravillous trophoblasts, suggesting that they may control Dkk-1-dependent trophoblast migration and invasion [15].

In addition the data show that trophoblasts express ligands such as Wnt-4, Wnt-5a, Wnt-5b and Wnt-11, which are known to activate non-canonical signalling pathways independently

of LRP-5/6. Non-canonical Wnt signalling involves Ca^{2+} -dependent activation of protein kinase C and calcium/calmodulin-dependent kinase II (CamKII) or induction of Rho-A and Jun-N-terminal kinase (JNK) [7]. Since Wnt-5a is present in all trophoblast cell types except JEG-3 and secreted from primary trophoblasts and cell lines, it is likely that the ligand can activate non-canonical Wnt signalling in the placenta. It has not yet been tested whether Wnt-5a could modulate trophoblast proliferation or invasion. However, Wnt-5a impaired Wnt-3a-induced luciferase activity of pSuper(8x)TopFlash, suggesting that the ligand may antagonise processes mediated through canonical Wnt signalling. Additionally, recombinant Wnt-5a could not induce luciferase expression of the TCF reporter plasmid, suggesting that the accessory proteins required for Wnt-5a-induced canonical Wnt signalling [8] are lacking in trophoblasts. Interestingly, Wnt-5a expression has also been detected in early and late phase endometrium, suggesting that decidual expression of the ligand may exert paracrine effects on invading trophoblasts [14].

In conclusion, the data suggest that several different Wnt signalling pathways could be active in human placenta depending on the cell-specific expression and distribution of individual Wnts and FZD receptors. The fact that canonical Wnts such as Wnt-3a can also interact with the majority of FZD receptors in a LRP-independent manner and activate MAPK and PI3K signalling pathways adds further complexity to the Wnt network [21,26]. Further experiments are necessary to identify specific Wnt-FZD receptor complexes present in placenta and to more precisely delineate the role of alternative Wnt signalling pathway in trophoblast differentiation.

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Fig. 1.

Western blot analyses of soluble Wnt-5a secreted from placental cells. Serum-free supernatants of trophoblast cell lines, third trimester villous trophoblasts and first trimester floating explant cultures were separated on PAA gels, transferred to membranes and incubated with Wnt-5a antibodies as described above. One out of 6 experiments with similar results is shown. As a positive control recombinant, mouse Wnt-5a (50 ng) was utilised. Marker bands and the specific Wnt-5a signal are indicated.

Fig. 2.

Luciferase assays of SGHPL-5 cells after co-transfection of either pSuper(8x)TopFlash or pSuper(8x)FopFlash (negative control) and CMV- β Gal plasmids. After stimulation with Wnt-3a and/or Wnt-5a, luciferase activity was determined in protein extracts and normalised to β -Gal activity as described above. For comparison, values of unstimulated controls transfected with pSuper(8x)TopFlash were arbitrarily set to 1 in each experiment. Bars represent mean values of 8 independent transfections of 4 different experiments; error bars indicate S.D. * $p < 0.05$.

Primer sequences, target regions, accession numbers, fragment lengths, cycle numbers, annealing temperatures and citations of all human Wnt ligands and FZD receptors used in semi-quantitative PCR reactions

Name	Accession number	Target region	Fragment length (bp)	TM (°C)	Cycle number	Sense primer	Anti-sense primer	Published in
Wnt-1	NM_005430	1056–1309	253	60	30	5'-CACGACCTCGTC TACTTCGAG-3'	5'-ACAGACACTCGTGCA GTACGC-3'	[27]
Wnt-2	NM_003391	575–955	380	57	30	5'-CCAGCCTTTT GGCAGGGTC-3'	5'-GCATGTCTCTGAGAG TCCATG-3'	[14]
Wnt-2b	NM_024494	854–1175	321	60	25	5'-AAGATGGTGCCA ACTTCACCG-3'	5'-CTGCCCTTCTTGGGG CTTTGC-3'	[27]
Wnt-3	AB067628	570–1009	439	56	30	5'-TGAACAAGCAC AACACGAG-3'	5'-CAGTGGCATTTTT CCTTCC-3'	[14]
Wnt-3a	NM_033131	245–571	326	55	35	5'-CAGGAACCTACGTG GAGATCATG-3'	5'-CCATCCCACCAACTC GATGTC-3'	[28]
Wnt-4	NM_030761	685–1114	429	57	30	5'-CCTTCTCAC AGTCGTTTG-3'	5'-CACAGCCGTCGAT GGCCTT-3'	[14]
Wnt-5a	NM_003392	2283–2424	141	56	35	5'-GGGAGGTTGGC TTGAACATA-3'	5'-GAATGGCACGCAAT TACCTT-3'	[14]
Wnt-5b	BC001749	1234–1739	505	56	30	5'-AGATCGTGGACCA GTACATCTG-3'	5'-TTACGGAAACCACTCTAC ATTCTG-3'	[29]
Wnt-6	NM_006522	201–613	412	60	35	5'-TGGTGTGCGTA GTACAGTGC-3'	5'-CCATCCTGTGGCCAG CAGTTC-3'	[30]
Wnt-7a	D83175	49–460	411	63	35	5'-GCTGCTGGGCCACC TCTTCTCA-3'	5'-CCCGGTGGTACAGGCCTT GCTTCT-3'	[14]
Wnt-7b	NM_058238	766–1099	333	61	35	5'-GGGCCACCTGCT GAAAGGAGAA-3'	5'-TTGACGAAAGCAGCACCA GTGGA-3'	[31]
Wnt-8a	NM_031933	95–386	291	59	35	5'-GGCGATGGGGAAC CTGTTTATG-3'	5'-CTCCAGCAGAGCTGAT AGCATG-3'	[32]
Wnt-8b	Y11094	1745–2017	272	57	35	5'-ATGTCTTTGGGGTT GGTCTCTAG-3'	5'-TTGCTAGGAGGAAGAA GGTACAG-3'	[14]
Wnt-9a	NM_003395	1056–1631	575	57	35	5'-TGGAGTGCAGG CAGTGCACG-3'	5'-AAAAAGCCTCTCAGTCC TTGCAG-3'	[33]
Wnt-9b	NM_003396	1075–1464	389	58	35	5'-GTGAGGAGGAG CTTGTGTAC-3'	5'-CCAGGAAGCAAGATGG AATAGC-3'	[33]
Wnt-10a	NM_025216	1685–2098	413	60	35	5'-GCATCACCGAG TGGGTCAGC-3'	5'-TCTGCCCGTCAGCTT CCATTC-3'	[30]
Wnt-10b	NM_003394	1022–1218	196	58	35	5'-GAATGCGAAATCCA CAACAACAG-3'	5'-TTGCGGTTGTGGGTATC AATGAA-3'	[27]

Name	Accession number	Target region	Fragment length (bp)	T _M (°C)	Cycle number	Sense primer	Anti-sense primer	Published in
Wnt-11	NM_004626	748–1342	594	60	25	5'-GTAAAGTGCCATGGGGTGTCT-3'	5'-GCTTCCAAAGTGAAAGGCAAAG-3'	[34]
Wnt-16	NM_057168	312–538	226	59	35	5'-TGTCGTGCTGTGTCCCTTAC-3'	5'-ATCATGTCAGTTCCA TCTCTC-3'	[35]
FZD1	NM_003505	2194–2357	163	61	30	5'-GCCCATGAGCCC GGACTTCAC-3'	5'-TCAGACTGTAGTCTC CCCTTG-3'	[36]
FZD2	NM_001466	954–1439	485	62	30	5'-TACCCAGAGCGGCCT ATCATTTT-3'	5'-ACGAAGCCGGCCAGGAGG AAGGAC-3'	[37]
FZD3	AB039723	1294–1624	330	55	30	5'-AAGGCTTCCACAGT GACACAAAG-3'	5'-AGAGGAGAGAAACCCCAAC TACCAC-3'	[38]
FZD4	NM_012193	1872–2087	215	56	30	5'-GGAAATGGTTGGG TGAAGCTG-3'	5'-TTTTTGATGCTGGG GTCGGG-3'	[38]
FZD5	AB043702	939–1422	483	60	30	5'-GGGCCCGTTTCGTGTG CAAGTGTCG-3'	5'-ATGCCGGCGGCCAGG AACGAG-3'	[39]
FZD6	AF072873	1529–2090	561	61	30	5'-AGTCTTCAGCGGCTT GTAICTTGT-3'	5'-GCTCCGTCCGGCTTTC ACCTCT-3'	[14]
FZD7	AB017365	1745–1959	214	59	30	5'-ACAGACTTAGCCACA GCAGCAAAG-3'	5'-TTTCCAAATCACCC CTCGCC-3'	[38]
FZD8	NM_031866	6–316	310	58	30	5'-TGGAGTGGGGT TACCTGTG-3'	5'-AGCGGCTTCTTGTA GTCCTC-3'	[38]
FZD9	NM_003508	955–1351	396	60	30	5'-TTCTTCTCCAC CGCCTTCAC-3'	5'-CAGGATGACGATGGT CTTGAG-3'	[38]
FZD10	NM_007197	1784–2186	402	59	30	5'-ATCGGGCTCT TCTCTGTC-3'	5'-GACTGGCAGGGAT CTCATA-3'	[40]

Table 2
Semi-quantitative RT-PCR analyses of all human Wnt ligands and FZD receptors in the placenta

Name	1st trim. fibroblast	1st trim. CTB	3rd trim. CTB	3rd trim. placenta	3rd trim. placenta	1st trim. Explant/Villi	1st trim. Explant/EVT	HTR-8/Svneo	SGHPL-5	JEG-3
Wnt-1	+++	+++	-	++	+	+++	+++	-	-	-
Wnt-2	++	+	+++	+++	+++	++	++	++	+++	+
Wnt-2b	++	+++	+++	+++	+++	+++	+++	+++	+++	++
Wnt-3	+	+	++	++	+	+	+	++	+++	+++
Wnt-4	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
Wnt-5a	++	++	+++	++	+++	++	++	+++	+++	-
Wnt-5b	++	+	++	++	++	++	++	+++	+++	+
Wnt-6	+	+	+	+	+	+	+	-	+	+++
Wnt-7a	-	+	+	+	+	+	+	+	-	-
Wnt-7b	++	+++	-	+	+	+	++	+++	+	+++
Wnt-9b	+	++	+	+	+	+	++	+++	+	+
Wnt-10a	-	+++	-	+	+	+	-	-	++	-
Wnt-10b	-	+++	-	+	+	+	-	+	+++	++
Wnt-11	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
FZD1	+	+	+	+++	+	+	-	-	+	-
FZD2	++	-	-	+++	+	++	-	+++	+++	+
FZD3	+	+	++	+++	+	+	+	+++	+++	++
FZD4	+	-	-	+++	+	++	-	++	+++	-
FZD5	+	+++	++	+++	-	+	+	+	+	++
FZD6	+++	++	+++	+++	++	++	++	+++	+++	+++
FZD7	+++	+	+	++	++	++	+	+	+++	++
FZD10	-	+++	+++	++	+++	++	+	+	-	+++

Equal amounts of RNA (each $n = 3$) were isolated from first trimester fibroblasts, first trimester cytotrophoblasts, third trimester cytotrophoblasts, total placental tissue of early and late pregnancy, from villi and extravillous trophoblasts (EVT) of first trimester explant cultures and from trophoblastic HTR-8/SVneo, SGHPL-5 and JEG-3 cells. PCR reactions were performed as described above and PCR fragments were separated on agarose gels. Relative expression levels/brightness of DNA fragments were estimated by two independent investigators. (+++), (++), (+), (-) indicate strong, medium, weak and absent mRNA expression, respectively.