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PLACENTA
Placenta 28, Supplement A, Trophoblast Research, Vol. 21 (2007) S97–S102

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

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Accepted 14 November 2006

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

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0143-4004/$ - see front matter © 2007 Published by IFPA and Elsevier Ltd.
doi:10.1016/j.placenta.2006.11.003

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/cm^2 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′-CAAAGTTGTCATG GATGACC-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/cm^2. 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