

Research article

**QUANTITATIVE AND KINETIC PROFILE OF Wnt/ β -CATENIN
SIGNALING COMPONENTS DURING HUMAN NEURAL
PROGENITOR CELL DIFFERENTIATION**

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Abstract: ReNcell VM is an immortalized human neural progenitor cell line with the ability to differentiate *in vitro* into astrocytes and neurons, in which the Wnt/ β -catenin pathway is known to be involved. However, little is known about kinetic changes of this pathway in human neural progenitor cell differentiation. In the present study, we provide a quantitative profile of Wnt/ β -catenin pathway dynamics showing its spatio-temporal regulation during ReNcell VM cell differentiation. We show first that T-cell factor dependent transcription can be activated by stabilized β -catenin. Furthermore, endogenous Wnt ligands,

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Abbreviations used: APC – adenomatous polyposis coli; bFGF – basic fibroblast growth factor; Cdk – cyclin-dependent kinase; CK1 – casein kinase 1; DAPI – 4',6-diamidino-2-phenylindole; Dkk1 – Dickkopf 1; DMEM – Dulbecco's modified Eagle's medium; Dvl – dishevelled; EGF – epidermal growth factor; Fz – Frizzled; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; GFP – green fluorescent protein; GSK3beta – glycogen synthase kinase 3; HBSS – Hank's buffered salt solution; hNPC – human neural progenitor cell; LRP6 – low-density lipoprotein receptor-related protein 6; MAP2 – microtubule-associated protein 2; NPC – neural progenitor cell; Ror2 – receptor tyrosine kinase-like orphan receptor 2; Ryk – receptor-like tyrosine kinase; TCF – T-cell factor

pathway receptors and signaling molecules are temporally controlled, demonstrating changes related to differentiation stages. During the first three hours of differentiation the signaling molecules LRP6, Dvl2 and β -catenin are spatio-temporally regulated between distinct cellular compartments. From 24 h onward, components of the Wnt/ β -catenin pathway are strongly activated and regulated as shown by mRNA up-regulation of Wnt ligands (Wnt5a and Wnt7a), receptors including Frizzled-2, -3, -6, -7, and -9, and co-receptors, and target genes including Axin2. This detailed temporal profile of the Wnt/ β -catenin pathway is a first step to understand, control and to orientate, *in vitro*, human neural progenitor cell differentiation.

Key words: Wnt/ β -catenin pathway, Spatio-temporal dynamics, Quantitative kinetics

INTRODUCTION

Human neural progenitor cells (hNPCs) are considered as a therapeutic tool for treatment of neurological diseases such as Parkinson's and Huntington's diseases, spinal cord injury and stroke [1-3]. hNPCs can be first differentiated, *in vitro*, into the desired type of neurons, which are sequentially used for transplantation treatment [1]. ReNcell VM [4], a hNPC line derived from the ventral mesencephalon of a 10-week old fetus, has been extensively characterized and is known to differentiate, *in vitro*, into astrocytes, oligodendrocytes and neurons, e.g., dopaminergic neurons [4-6].

Wnt proteins are secreted morphogens involved in cell proliferation and differentiation, cellular polarity and apoptosis, cell migration, axon guidance, and synaptogenesis, and play an important role during embryogenesis and tissue formation [7]. Upon binding to Frizzled (Fz) receptors on the cell surface, Wnt proteins can trigger three different signaling pathways: the Wnt/ β -catenin pathway, the Wnt/calcium pathway, and the planar cell polarity (PCP) pathway. In the Wnt/ β -catenin pathway, a series of events occurs when Wnt proteins bind to the Fzs and their co-receptors LRP5/6, resulting in a stabilization of β -catenin in the cytosol by activation of Dishevelled (Dvl) family proteins. The Dvl proteins inhibit the degradation complex, which consists of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β) and casein kinase 1 (CK1), resulting in accumulation of β -catenin in the cytosol. Subsequently, β -catenin translocates into the nucleus, where it interacts with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors to drive specific expression of target genes [8]. Remarkably, Wnt signals regulate both proliferation and differentiation of NPCs in a stage-specific and cellular context-dependent manner [9, 10]. For example, Wnt1, Wnt3a and Wnt5a cooperate and regulate the proliferation and differentiation of dopaminergic progenitor cells during neurogenesis in the ventral midbrain [11].

However, little is known about the kinetic regulation of the Wnt signals during these processes although the specificity of signaling pathways rely on their

temporal and spatial dynamics, especially of the pathway's downstream signaling proteins [12]. Our previous studies showed that the Wnt/ β -catenin pathway and Wnt3a, a typical activator of the Wnt/ β -catenin pathway [13], regulate neurogenesis of ReNcell VM cells *in vitro* [14, 15]. Thus, in the present study, we investigate the kinetic and dynamic Wnt/ β -catenin pathway during physiological differentiation of ReNcell VM cells, in order to elucidate the role of this pathway during hNPC differentiation. We first elucidate whether Wnt/ β -catenin is inducible in ReNcell VM cells, characterize the differentiation of these cells upon arrest of the cell cycle by removal of growth factor, and investigate the presence of neurons and glial cells 72 h after differentiation. In the second part we analyze the kinetic and quantitative changes of the pathway signal molecules at mRNA and/or protein levels during cell physiological differentiation or upon Wnt3a stimulation. The results show that the key signaling proteins, the mRNAs of Wnt ligands and relative receptors, and other targeting molecules are dynamically regulated in time and space during cell differentiation, suggesting that the ReNcell VM cell is a good cell model to study hNPC differentiation and the role of the Wnt/ β -catenin pathway in this process. Our data help understand how the Wnt/ β -catenin pathway is regulated during ReNcell VM cell differentiation and provide reliable data that can be used for computational modeling of the pathway in the context of systems biology [16, 17].

MATERIALS AND METHODS

Cell culture

The ReNcell VM cell line used in this study is derived from the ventral mesencephalon of a 10-week-old human fetus and is immortalized by retroviral transduction with v-Myc oncogene (ReNeuron Ltd, Guildford, UK). ReNcell VM cells were cultivated according to the protocol described previously [5]. Briefly, cells were cultured in laminin (Trevigen, Gaithersburg, USA) coated cell culture flasks and maintained at 37°C with 5% CO₂ in DMEM/F12 supplemented with Glutamax, B27 media supplement, heparin sodium salt and gentamycin (Invitrogen, Karlsruhe, Germany). For proliferating culture, 20 ng/ml epidermal growth factor (EGF; Roche, Mannheim, Germany) and 10 ng/ml basic fibroblast growth factor (bFGF; Roche) were added to the medium. Proliferating cells were passaged when confluency reached approximately 80%. The medium was changed every 2-3 days. For initiation of differentiation, cells were washed with HBSS, and new medium without EGF and bFGF was added. Cells were harvested either in the proliferating state as a control (time point 0) or at time points of 0.5, 1, 2, 3, 4, 8, 12, 24, 48, 72, 96, and 120 h after initiation of differentiation. Cells were detached from flasks by adding Trypsin-EDTA (Gibco) in Benzonase (Merck, Darmstadt, Germany) and collected by centrifugation. Cells were directly used for the processes of sub-cellular fractionation.

RNA extraction

After trypsinization, cells were washed twice with HBSS and collected by centrifugation at 1500 rpm for 5 min at room temperature. Cell pellets were frozen in liquid nitrogen and stored at -80°C . RNA was extracted using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guidance. The quantity and quality of total RNA were determined by spectrometry (Nano-Drop; ThermoScientific, Wilmington, USA) and denaturing agarose gel electrophoresis, respectively.

Quantitative real-time RT-PCR

To quantify mRNA in cells, a LightCycler (Roche) in combination with the LightCycler 3.52 software was used. The first-strand cDNA was synthesized using FastLane cDNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR was performed using the FastStart DNA SYBRGreen Plus Kit (Roche) according to the manufacturer's instructions. PCR products were verified by size in agarose-gel electrophoresis, sequencing and melting point analysis. Relative changes of mRNA amount were calculated using the ddC_T method [18]. The tested gene mRNA amount was normalized to housekeeping gene Glucose-6-phosphate-dehydrogenase (G6PD). All samples were run in duplicate. Cycling parameters were as follows: initial denaturation at 95°C for 12 min; 45 cycles of 1 s at 95°C for denaturation, 5 s at 62°C for annealing and 10 s at 72°C for extension. Primer sequences were as follows: Axin2-FW: 5'-agctcagcagaggacaggaa-3', Axin2-RW: 5'-agctctgagccttcagcatc-3'; G6PD-FW: 5'-atcgaccactactctgggcaa-3', G6PD-RW: 5'-ttctgcatcacgtcccggga-3' [19]. All primer sequences exclude amplification of genomic DNA under the parameters described above.

TaqMan array

A custom 384-well format TaqMan Array (Applied Biosystems Inc, Foster, USA) was used for gene mRNA quantification. The first strand cDNA was synthesized with 400 ng total RNA as a template using the High Capacity cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. Each slot was filled with a cDNA equivalent derived from 200 ng whole RNA. Samples were run in duplicate on an ABI-7900HT real-time PCR system. Cycling parameters were as follows: 2 min at 50°C for activation of the Uracil DNA-Glycosidase; 20 s at 95°C for denaturation; followed by 45 cycles of 1 s at 95°C for denaturation and 20 s at 60°C for annealing and extension. The threshold line was set automatically by SDS 2.3 software and the threshold cycle (C_T) values were determined by the threshold line crossing the amplification curve. The PCR data were normalized to GAPDH. Target transcript abundance was quantified using the ddC_T method.

Cell cycle analyses

Propidium iodide (PI) staining was used for detection of the cell cycle status of proliferating and differentiating ReNcell VM cells. To prepare a single cell

suspension, cells were harvested by treating with Trypsin/Benzonase solution. The reaction was stopped using Trypsin-Inhibitor/Benzonase solution. Cells were fixed in ice-cold 70% ethanol for at least 1 h at -20°C . Before measurement fixed cells were incubated with 1 mg/ml RNaseA (Sigma-Aldrich, Munich, Germany) for 30 min at 37°C following incubation with 50 $\mu\text{g/ml}$ PI for 30 min at 37°C . Determination of DNA content was measured immediately after PI staining by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA) and analyzed using the Cell Quest Pro software (BD Bioscience). PI intercalates into double-stranded nucleic acids; therefore the fluorescence signal intensity of PI is directly proportional to the amount of DNA in each cell. Cells in G0/G1 phases of the cell cycle are defined by a DNA content of $2n$ and cells within the G2/M phases have a DNA content of $4n$, while S phase cells have DNA content greater than $2n$ and less than $4n$. Cell doublets and debris were excluded by gating out cells that are sub-diploid ($< 2n$).

Preparation of subcellular fractions

Cell sub-cellular fractions were prepared using the Qproteome Cell Compartment kit (Qiagen) according to the manufacturer's instructions. Purity of the fractions of the membrane, cytosol and nucleus were analyzed by immunoblotting using antibodies against the specific compartmental marker proteins: the mitochondrial inner membrane translocase subunit TIM23 (BD Biosciences, Franklin Lakes, NJ, USA) and glucose-regulated protein 78 (GRP78; Santa Cruz Biotechnology, Santa Cruz, USA, #1050, dilution 1:2000) for the membrane fraction; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology #25778, dilution 1:2000) for the cytosolic fraction and lamin A/C (BD Biosciences #612163, dilution 1:1000) for the nuclear fraction.

Protein determination and quantitative Western blot analysis

Protein concentrations in total cell lysates or sub-cellular fractions were estimated by the bicinchoninic acid assay (BCATM, Pierce, Rockford, IL, USA). Samples were diluted in 5x Laemmli-buffer and boiled at 95°C for 5 min. Lysates were loaded into gradient (4-15%) SDS-polyacrylamide gels (10-50 μg of proteins per lane) and separated electrophoretically by an electrophorator (Biorad, Hercules, USA). Prestained pEqGOLD marker IV (PEQLAB, Erlangen, Germany) was used as a molecular weight marker. Proteins were transferred (wet transfer) to nitrocellulose membrane by electroblotting (Amersham Biosciences, Freiburg, Germany). After blocking with 5% milk or 5% serum albumin (for detection of phosphorylated proteins) for 1 h, the membranes were incubated with primary antibodies, such as mouse anti- β -catenin (Santa Cruz #7963, dilution 1:1000), rabbit anti-Dishevelled-2 (Cell Signaling #3216, dilution 1:4000), rabbit anti-phospho-LRP6 (Ser¹⁴⁹⁰) (Cell signalling #2568, dilution 1:2000), rabbit anti-LRP6 (Cell Signaling #2560, dilution 1:4000), rabbit anti- β -actin (Delta Biolabs #070, dilution 1:1000) and rabbit anti-GAPDH

(Santa Cruz #25778, dilution 1:2000) overnight at 4°C. Blots were rinsed 3 times with TBST (Tris-HCl with 1% Tween 20; pH 7.6) and probed with appropriate fluorescent secondary antibodies such as goat anti-rabbit and goat anti-mouse Alexa Fluor 680 (Invitrogen #A-21076 and #A-21057), and goat anti-rabbit and goat anti-mouse IRDye 800 (Rockland, #611-131-122 and #610-131-003) (all diluted 1:10000). Visualization and quantification of proteins were performed using the Odyssey Infrared Imaging System (LI-COR Biosciences GmbH, Bad Homburg, Germany). For quantification of the target proteins, samples were loaded onto the gel in a randomized and non-chronological order to reduce systematic errors [20]. Expression of β -actin or GAPDH protein was used as a loading control to normalize the expression of the target proteins. Thereby relative expression levels of the target proteins were determined. For each quantified Western blot, the relative expression at a given time point is normalized to the mean of the time series. The resulting values are normalized to the one in proliferating cells (time point 0 h) which is set to 1.

Plasmid construction

The construction of expression plasmid pCAGGS-S33Y containing a mouse mutant S33Y β -catenin sequence with a C-terminal HA-tag has been described previously [14, 15].

TCF-reporter gene assay

TCF-dependent transcription in cells was analyzed by the TCF-reporter gene assay. pTCF-EGFP plasmids, in which EGFP protein expression is driven by a promoter containing six TCF binding sites [21], and pCAGGS-S33Y for expression of stabilized β -catenin [14, 15] were cotransfected into cells using Amaxa Nucleofector technology (Amaxa, Gaithersburg, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, GFP-positive cells were analyzed by FACS analysis.

FACS analysis

To quantify GFP-positive cells, cells were collected, washed and resuspended in PBS (with 0.5% BSA and 0.02% Na-azide). Measurement was done using FACSCalibur (Becton Dickinson, San Jose, USA) in combination with CellQuest Pro software. A green fluorescent signal defined all transfected cells. Mock transfected cells served as a negative control.

Immunocytochemistry

For immunocytochemistry, the following antibodies were used: rabbit polyclonal antibodies against GFAP (Dako, #Z0334), S100 (Dako, #Z0311), and against β -catenin (clone H102; Santa Cruz, #7199, dilution 1:300); mouse monoclonal antibodies against III-beta tubulin (Tuj-1; Santa Cruz, #51670) and Nextin (R&D, #MAB1259). All antibodies were used with a dilution of 1:500, except for clone H102. The secondary antibodies of Alexa 488-labeled goat anti-rabbit and anti-mouse IgG were used.

Cells cultured in chamber slides were washed with PBS and fixed with 4 % PFA in PBS for 20 min at room temperature. Then cells were washed with PBS and stored in PBS with 0.02 NaN₃ as a preservative at 4°C. To reduce the unspecific reaction, cells were first incubated in 5% normal goat serum together with 0.3% Triton-X100 in PBS for 30 min and then with primary antibody in PBS with 1% normal goat serum for 30 min. Then cells were washed 3 times with PBS and incubated for 30 min at room temperature with the appropriate secondary antibody. Finally, cells were mounted with the medium containing DAPI (VectaShield) for staining of nuclei. Fluorescence on slides was imaged under a fluorescent microscope (BZ-8000; Keyence Deutschland GmbH, Neu-Isenburg, Germany) or a Leica SP2 AOBS confocal laser scanning microscope (Leica, Germany). Digital images were adjusted in contrast and brightness with Photoshop software (Adobe Systems).

Statistical analysis

Results are reported as mean \pm SEM from at least three independent experiments. Statistical evaluation was carried out using the two-tailed Student's t-test. A difference was considered to be statistically significant when the p-value < 0.05 .

RESULTS

Wnt/ β -catenin pathway is inducible in ReNcell VM cells

β -catenin/TCF-dependent transcription is inducible in ReNcell VM cells

To verify whether TCF-dependent transcription is inducible by the Wnt/ β -catenin pathway in ReNcell VM, a pTCF-EGFP plasmid, in which an EGFP gene is driven by a promoter containing TCF binding sites, and a pCAGGS-S33Y plasmid for overexpression of stabilized β -catenin [14, 15] were cotransfected into ReNcell VM cells and the number of GFP-positive cells was measured 24 h after transfection by flow cytometry. Results show that compared to the control group with only pTCF-EGFP transfection, the number of GFP-positive cells in the cotransfected group increases by about 5-fold to 6% of the total cell population (Fig. 1A). These data suggest that TCF-dependent transcription can be activated by β -catenin in ReNcell VM cells.

Axin2 transcription is inducible in ReNcell VM cells upon LiCl treatment

LiCl inhibits the activation of GSK3 β , resulting in cytoplasmic β -catenin accumulation and is widely used to pharmacologically induce Wnt/ β -catenin signaling [22]. Therefore, we verified whether the hallmark of Wnt/ β -catenin signaling, i.e., Axin2 upregulation [23], is inducible in ReNcell VM cells upon treatment with LiCl (20 mM) in proliferation medium. The results show that Axin2 is upregulated 6-fold upon 8 h treatment with LiCl as detected by quantitative real-time PCR (qRT-PCR) (Fig. 1B). Thus, our data demonstrate that Wnt/ β -catenin-dependent signaling is inducible in ReNcell VM cells upon LiCl treatment.

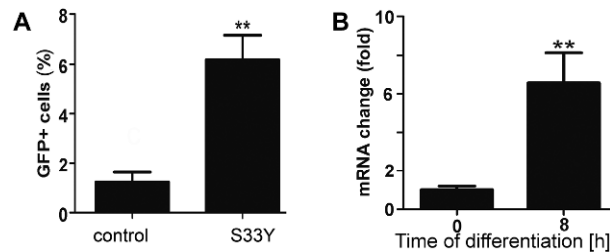


Fig. 1. Activation of TCF-reporter by FACS and induction of β -catenin-dependent signaling in ReNcell VM cells detected by real-time RT-PCR. A – Percentages of GFP-positive cells measured by flow cytometry at 24 h after cells were transfected with pTCF-EGFP together with either empty vector (control) or pCAGGS-S33Y (S33Y). B – After cells were treated with LiCl (20 mM) in proliferation medium for 8 h, the mRNA level of Axin2 was measured by real-time RT-PCR. Values are presented as mean \pm SEM from at least 3 independent experiments. ** $p < 0.01$ compared to control (time point 0 without LiCl treatment as a control).

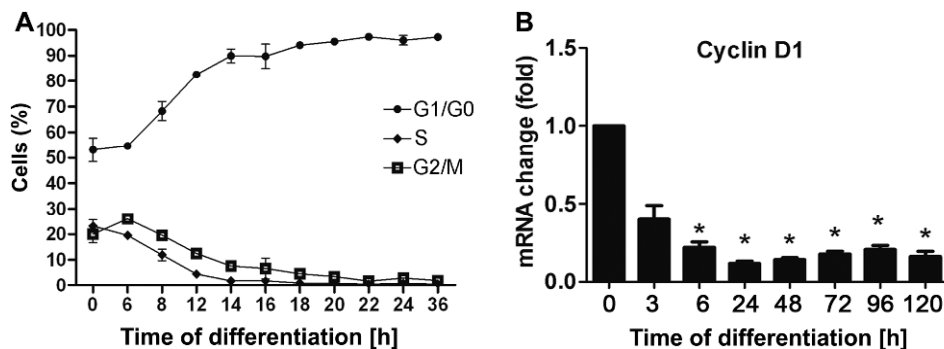


Fig. 2. Cell cycle analyses by FACS assay and expression of cyclin D1 during differentiation of ReNcell VM cells. A – The cells were harvested at distinct differentiation time points and the percentages of the cells in cell cycle phases were measured by flow cytometry after cells were stained with propidium iodide. B – Cyclin D1 mRNA levels at different time points (time point 0 with proliferating cells as a control) were detected by quantitative real-time RT-PCR. Values are normalized to GAPDH and presented as mean \pm SEM from three independent experiments. * $p < 0.05$ compared to control (0 h).

Cell cycle analysis of ReNcell VM cells during differentiation

The differentiation of ReNcell VM cells is triggered by the removal of growth factors from the culture medium. To follow the differentiation dynamics, the cell cycle and its controlling gene, i.e., cyclin D1, were first investigated. Results demonstrate that in the proliferating state (0 h, Fig. 2A), the percentage of cells in G0/G1 phases is $53.13 \pm 4.67\%$, in S phase $23.22 \pm 2.52\%$ and in G2 phase $20.05 \pm 3.37\%$. Twenty-four hours after induction of differentiation, the percentage of cells in G0/G1 phases reaches a very high level at $96.06 \pm 1.87\%$ (Fig. 2A), suggesting that almost all cells are arrested. Furthermore, the

expression of cyclin D1 – a member of the cyclin family functioning as a regulatory subunit of cyclin/cyclin dependent kinase (Cdk) holoenzymes, which is crucial for the progression of cells from G1 to S phase [24] – is strongly downregulated 3 h after induction of differentiation, proving the arrest of mitogen signaling in the differentiation process due to growth factor removal (Fig. 2B).

Cell morphological change and expression of different markers during differentiation

The cell morphology changes throughout the differentiation period. Generally, during differentiation, the cells extend axons and assume neuronal morphology (Fig. 3). Furthermore, 72 h after differentiation, the cells express the neuronal marker β III-tubulin (Fig. 3G), and astrocyte markers such as glial acidic fibrillary protein (GFAP) (Fig. 3H) and S100 (Fig. 3J), indicating that ReNcell VM cells differentiate into different cell types.

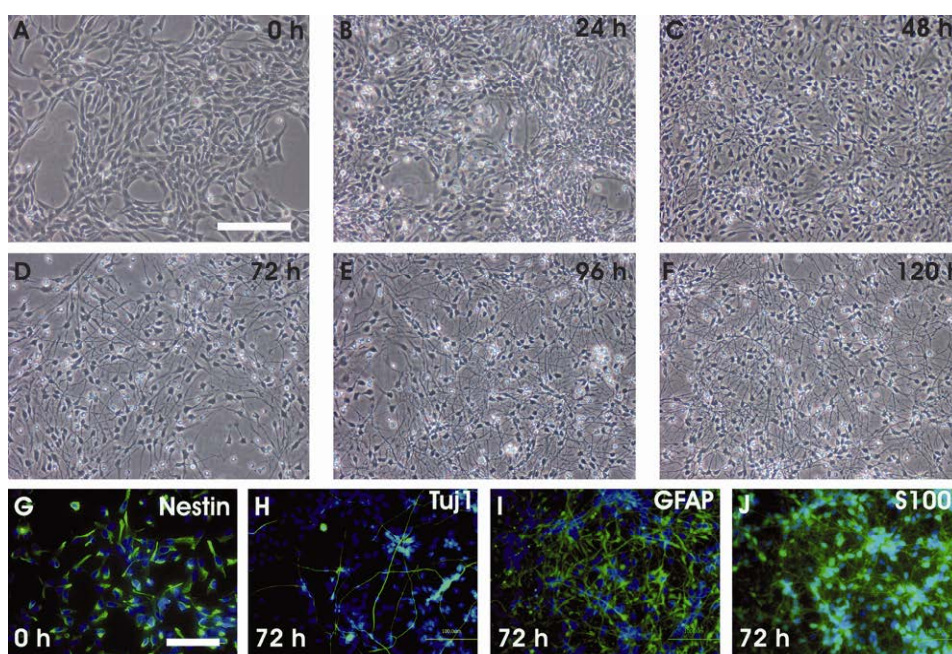


Fig. 3. Morphological changes and different marker protein expression during ReNcell VM cell differentiation. A-F – ReNcell VM cells were cultured in proliferation medium until 80% confluence (time point 0 h; A) and differentiated by removal of the growth factors. Representative phase contrast pictures are shown at 24 h (B), 48 h (C), 72 h d (D), 96 h (E) and 120 h (F) of differentiation. G-J – Imaging of immunostained ReNcell VM after 72 h of differentiation. Neurons stained against β III-tubulin (Tuj1; green) exhibit long neurites; glial cells stained with glial acidic fibrillary protein (GFAP, green) and S100 (green). Nuclei are stained with DAPI (blue). Scale bar = 20 μ M.

Temporal regulation of endogenous mRNAs of Wnt pathway components during differentiation of ReNcell VM cells

During physiological differentiation, endogenous Wnt ligands, Wnt receptors, and other pathway components show distinct temporal responses. Figs 4 and 5, and Tab. 1 display the mRNA expression profiles in term of fold change compared to proliferation as revealed by TaqMan array.

Tab. 1. Fold changes of the endogenous Wnt pathways' components in ReNcell VM cells at different time points during differentiation (C_T stands for threshold cycle).

| Time point | 1-5-fold decrease | 5-13-fold decrease | >13-fold decrease |
|------------|-------------------------------------------------------------------------------------------------|------------------------------------|------------------------------------------------------------------------------------------|
| 0-6 h | Fz5 Dkk1 | | |
| 6-48 h | WNT10b | | |
| | Maximum increase: 1-5-fold | Maximum increase: 5-13-fold | Maximum increase: >13-fold |
| 24-48 h | WNT5B Fz7 Fz9 c-Myc Kremen1 | Fz3 Fz6 LRP6 TUBB3 Ryk | WNT5A WNT7A Fz2 ROR2 Axin2 MAP2 |
| 72 h | Fz1 Fz4 LRP5 | | |
| | Low expression (C_T value >35) | | Not detected |
| | WNT3 WNT4 WNT7B WNT8A WNT8B WNT9B LEF1 Fz8 Fz10 KREMEN2 RSPO2 | | WNT1 WNT10A WNT11 WNT16 WNT2 WNT2B WNT3A WNT6 WNT9A RSPO3 |

The first observation concerns the downregulation of Wnt10b (Fig. 4D), Fz5 (Fig. 4K) and the pathway inhibitor Dickkopf 1 (Dkk1) (Fig. 5C) as early as 3 h after differentiation. A second phase of regulation takes place from 24 h onward after differentiation with the changes of different pathway components (Tab. 1), including the ligands Wnt5a (Fig. 4A) and Wnt7a (Fig. 4C); the receptors Fz2 (Fig. 4H), Fz3 (Fig. 4I), Fz6 (Fig. 4L), Fz7 (Fig. 4M), Fz9 (Fig. 4N), Ror2 (Fig. 5A) and Ryk (Fig. 5B); the co-receptor LRP6 (Fig. 4F); the Wnt target gene Axin2 (Fig. 5D) and c-Myc (Fig. 5E). The neuron-specific markers MAP2 (Fig. 5G)

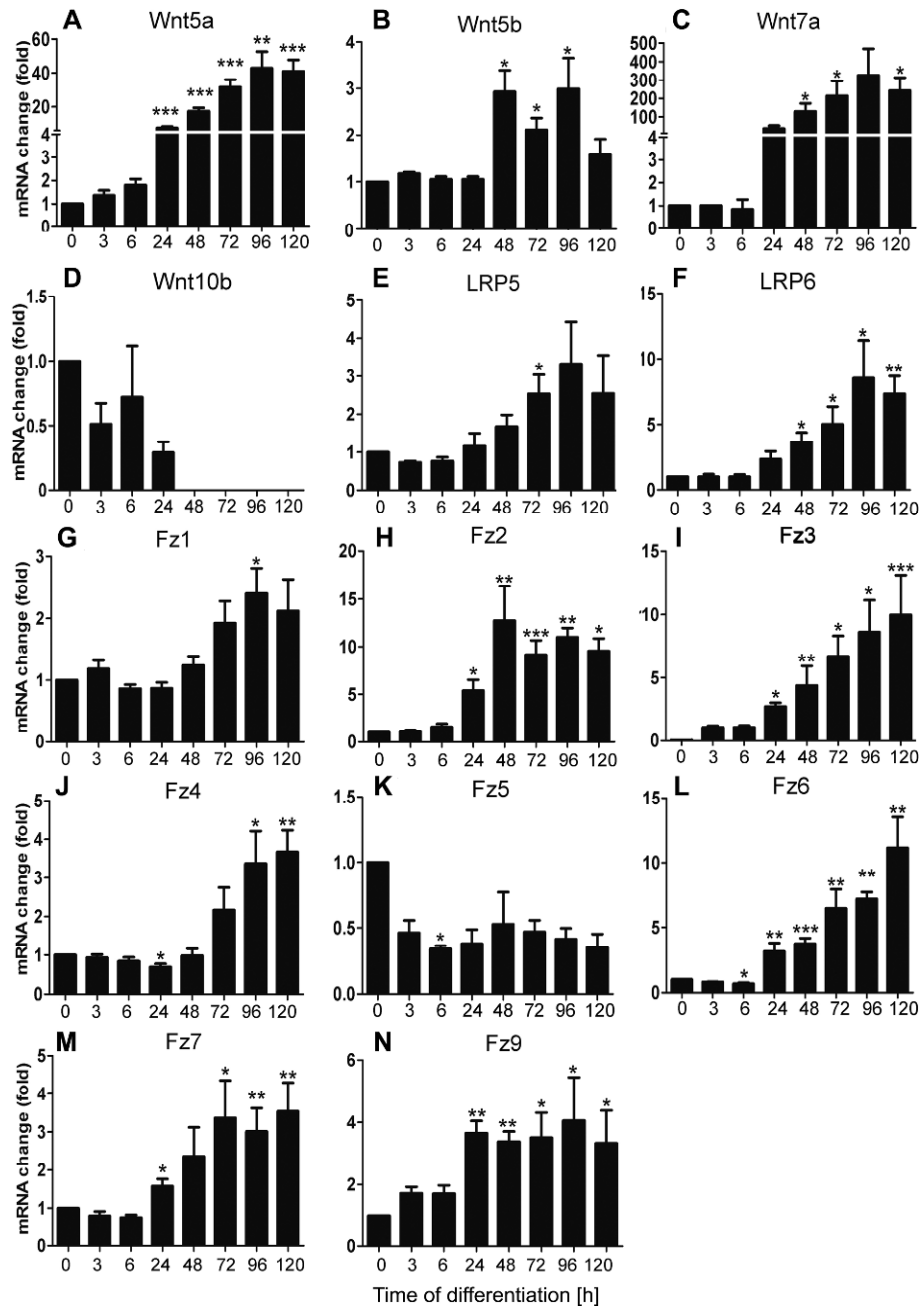


Fig. 4. Endogenous expression of Wnt ligands and relative receptors in ReNcell VM cells by TaqMan array. The mRNA levels of the Wnt ligands (A-D), the co-receptors (E, F) and the Fz receptors (G-N) at different time points (time point 0 with proliferating cells as a control) were detected by TaqMan qRT-PCR. Values are normalized to GAPDH and presented as mean \pm SEM from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (0 h).

and β III-tubulin (Fig. 5H) are also increased, consistent with the ability of ReNcell VM cells to differentiate into neurons [6] as shown in Fig. 3. A further upregulation phase is observed after 48 or 72 h of differentiation for Wnt5b (Fig. 4B), the receptors Fz1 (Fig. 4G) and Fz4 (Fig. 4J), the co-receptor LRP5 (Fig. 4E), and the modulator Kremen1 (Fig. 5F).

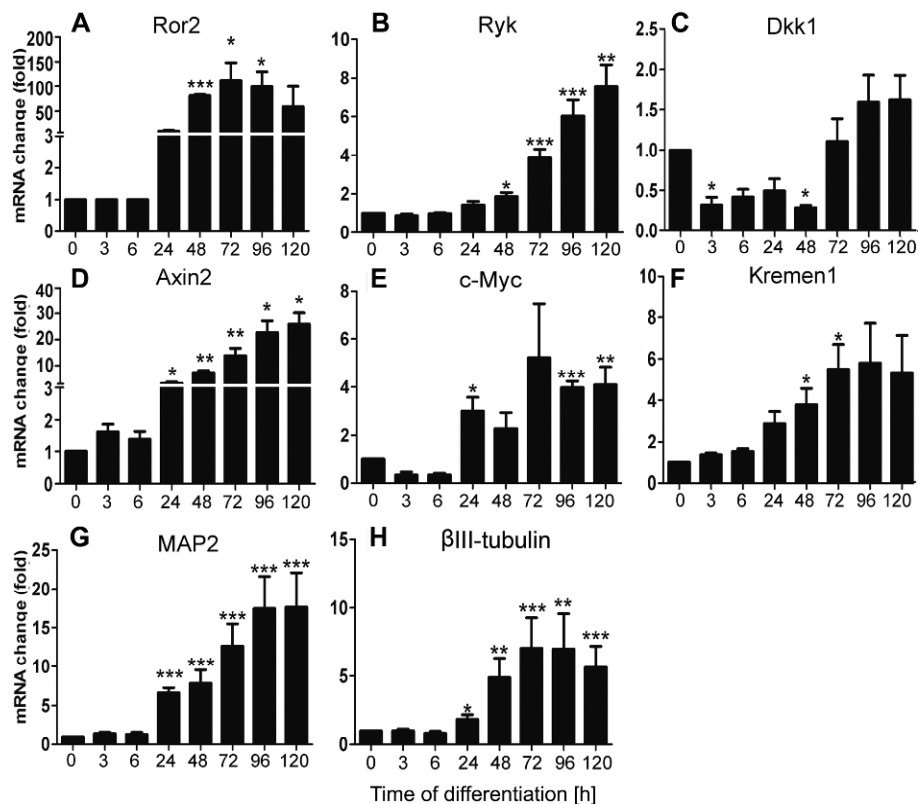


Fig. 5. Endogenous expression of other molecules including Wnt targeting genes in ReNcell VM cells by TaqMan array. The mRNA levels of Ror2 (A), Ryk (B), Dkk1 (C), Axin2 (D), c-Myc (E), Kremen1 (F), MAP2 (G), and β III-tubulin (H) at different time points (time point 0 with proliferating cells as a control) were detected by TaqMan qRT-PCR. Values are normalized to GAPDH and presented as mean \pm SEM from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (0 h).

Spatio-temporal regulation of signaling proteins during differentiation of ReNcell VM cells

First/early protein response during the first 3 h of differentiation

During ReNcell VM cell differentiation, total LRP6 (t-LRP6; two bands with the endoplasmic reticulum (ER) form of 180 kDa and the mature form of 190 kDa) [25] and phosphorylated LRP6-Ser¹⁴⁹⁰ (p-LRP6; 190 kDa) [26] were detected using different antibodies against LRP6. In the membrane subcellular fraction,

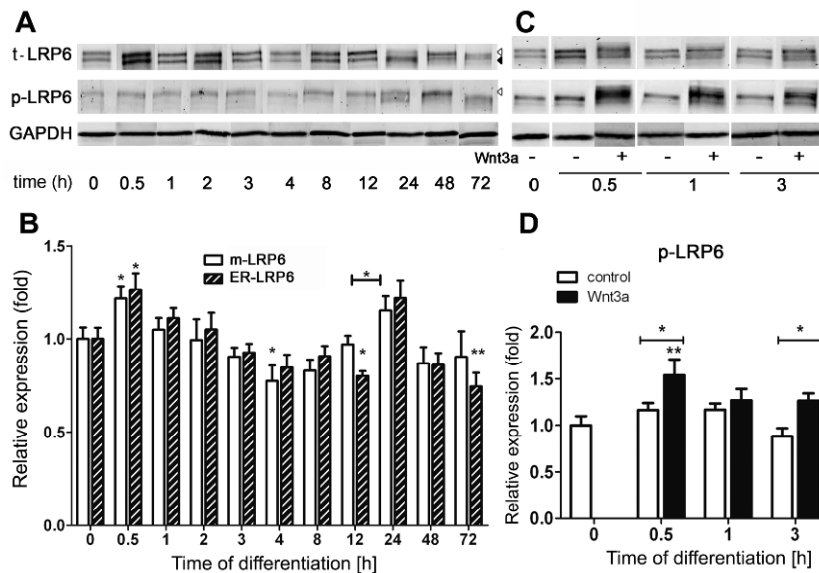


Fig. 6. Measurement of different forms of LRP6 during differentiation under native state or upon Wnt3a stimulation of ReNcell VM cells. A, C – Representative Western blots of total LRP6 (t-LRP6) and phosphorylated LRP6 on serine 1490 (p-LRP6) in membrane fraction under native state (A) or upon Wnt3a (100 ng/ml) stimulation (+) or without Wnt3a (-) (C). Time point 0 stands for control using proliferating cells. The white arrowhead for t-LRP6 shows the mature form of the receptor at 190 kDa (A, C). The black arrowhead shows the ER form of LRP6 at 180 kDa (A, C). The white arrowhead for p-LRP6 shows the mobility shift to 200 kDa (A, C). B, D – Quantified Western blot analysis of total LRP6 in membrane fraction at different time points (B) and of p-LRP6 with (+) or without (-) Wnt3a (100 ng/ml) at different time points (D). Time point 0 stands for control using proliferating cells, and GAPDH was used as a loading control. The signal intensities at 0 h are normalized to 1 and the values are presented as mean \pm SEM from at least 3 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to control.

the amount of t-LRP6 increases as early as 0.5 h after initiation of differentiation followed by a decrease (Fig. 6A, B), whereas the amount of p-LRP6 remains unchanged (Fig. 6A). Upon Wnt3a stimulation, both t-LRP6 and p-LRP6 increase immediately and p-LRP6 reaches a peak as early as 0.5 h after treatment, as detected by the shift of the original band at 190 kDa to 210 kDa (Fig. 6C,D) [27]. It is followed by a decrease of p-LRP6 throughout kinetic analysis although its amount remains at a significantly higher level than the control without Wnt3a (Fig. 6D).

Two forms of Dvl2 are found in the membrane (Fig. 7A) and cytosolic subcellular fractions (Fig. 7D): a mature one (m-Dvl2) and an active phosphorylated one (p-Dvl2). In the membrane fraction, both forms of Dvl2 increase as early as 0.5 h after initiation of differentiation (Fig. 7A) and are maintained until 2 h of differentiation. This is followed by a decrease before

a second increase at 12 h. In contrast, in the cytosol fraction, Dvl2 does not change during the first 8 h of differentiation and only slightly decreases at 12 h (Fig. 7D). Upon stimulation with Wnt3a, in the membrane fraction, Dvl2 responds quickly and increases immediately (Fig. 7B, C), while in the cytosolic fraction, only p-Dvl2 increases immediately, as detected by a shift from m-Dvl2 (black arrow) to p-Dvl2 (white arrow) (Fig. 7E).

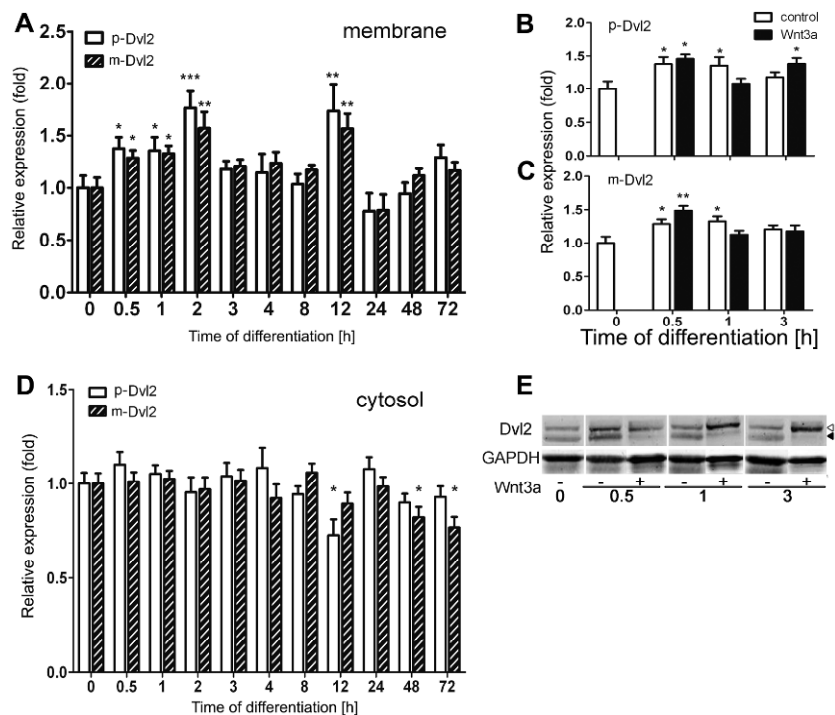


Fig. 7. Measurement of Dishevelled 2 protein levels in the membrane and cytosol fractions during ReNcell VM cell differentiation. A-C – Kinetics of Dishevelled 2 (Dvl2) under native differentiation (A, D) or with (+) or without (-) Wnt3a (100 ng/ml) stimulation (B, C, E) in the membrane (A-C) or the cytosol (D, E) fractions of ReNcell VM cells. Protein amount was quantified by Western blot analysis along the differentiation time course for the phosphorylated Dvl2 (p-Dvl2) and the mature, mobile fraction of Dvl2 (m-Dvl2). (D, E) Representative Western blots show Dvl2 abundance in the cytosol fraction (E) upon Wnt3a stimulation. The white arrowhead indicates p-Dvl2 at 95 kDa and the black arrowhead m-Dvl2 at 90 kDa. Time point 0 stands for control using proliferating cells, and GAPDH was used as a loading control. The signal intensities at 0 h are normalized to 1 and the values are presented as mean \pm SEM from at least 3 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to control.

In the cytosol, β -catenin protein decreases significantly at 1 h after differentiation (Fig. 8A, B), whereas in the nucleus it increases between 0.5 and 1 h after differentiation (Fig. 8C, D). Upon Wnt3a stimulation, in the cytosol fraction, β -catenin decreases significantly at 0.5 h after differentiation and then increases

to 1.5-fold after 3 h of differentiation (Fig. 8E, F). In the nucleus fraction, a fast and large increase of β -catenin to 2-fold after 0.5 h of differentiation is observed (Fig. 8G, H), suggesting that Wnt3a can accelerate this process compared to the native state.

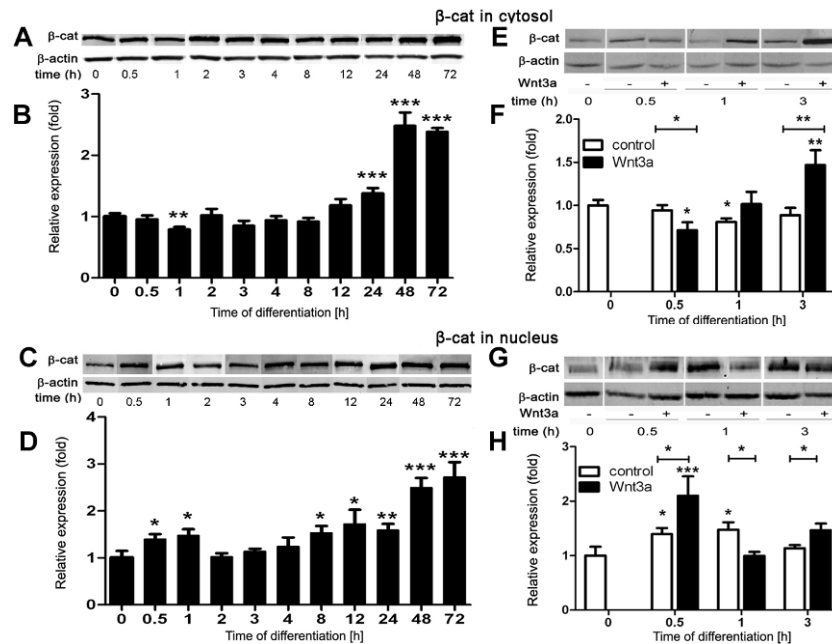


Fig. 8. β -catenin accumulation in cytosol and nuclear fractions during ReNcell VM cell differentiation. A-D – Representative Western blots of total β -catenin (β -cat) in the cytosol fraction (A) and in the nucleus fraction (C) from which protein amount was quantified (B, D, respectively) along the differentiation of ReNcell VM cell differentiation in the physiological state. The quantification indicates a significant decrease of β -catenin in the cytosol at 1 h (B), which is correlated with a significant increase in the nucleus between 0.5 and 1 h of differentiation (D). Late accumulation of β -catenin occurs at 24 h in the cytosol fraction and already at 8 h in the nucleus fraction. E-H – Kinetics of β -catenin for the first 3 h of differentiation with (+) or without (-) Wnt3a (100 ng/ml). Representative Western blots of total β -cat in the cytosol fraction (E) and in the nucleus fraction (G), from which protein abundance was quantified (F, H, respectively). Wnt3a accelerates cytosolic β -catenin decrease (F; at 0.5 h instead of 1 h in native condition) and enhances nuclear β -catenin increase at 0.5 h (H). Time point 0 stands for control using proliferating cells, and β -actin was used as a loading control. The signal intensities at 0 h are normalized to 1 and the values are presented as mean \pm SEM from at least 3 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to control.

Second/late protein response 24 h after differentiation

In the membrane fraction t-LRP6 significantly increases at 24h of differentiation (Fig. 6B), but p-LRP6 increases at 48 h (Fig. 6A) and both decrease at 72 h (Fig. 6A, B). In the membrane fraction, Dvl2 is maintained at a low level at 24 h and increases

gradually at 72 h (Fig. 7A), whereas in the cytosol, Dvl2 decreases significantly at 48 and 72 h (Fig. 7D).

In the cytosol fraction, β -catenin accumulation increases constantly from 24 h onwards to 2.5-fold at 72 h (Fig. 8A, B); in the nucleus fraction, it gradually increases from 8 h onwards to 2.7-fold at 72 h (Fig. 8C, D). β -catenin expression analyzed by immunocytochemistry correlates with these data (Fig. 9). For example, in proliferating cells, β -catenin is mainly distributed at the cell membrane and in the cell periphery (Fig. 9A, B), whereas in differentiated cells (72 h after differentiation), β -catenin is found mainly in the cytosol and in the nucleus (Fig. 9C, D).

Interestingly, we found that the changes of LRP6, Dvl2 and β -catenin in the subcellular fractions show a biphasic-dependent regulation manner with an initial increase peak followed by a decrease and then a further increase (Figs. 6-8). Taken together, the results described above show that LRP6, Dvl2 and β -catenin are temporally and spatially regulated during ReNcell VM cell differentiation.

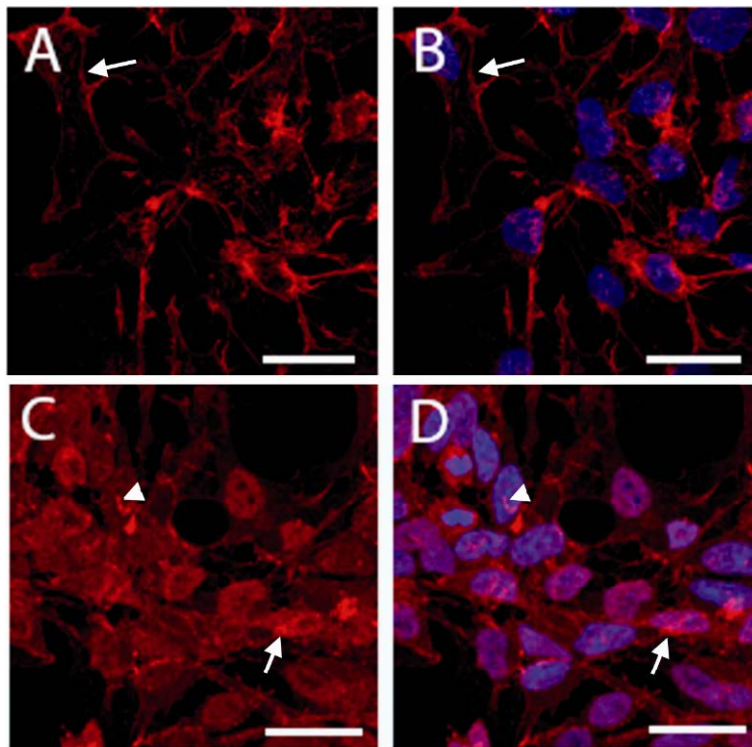


Fig. 9. Confocal imaging of immunostained β -catenin during ReNcell VM cell differentiation. (A-B) In proliferating cells (time 0 h), β -catenin is mainly located at the cell membrane and periphery (white arrow). During differentiation, cells exhibit increasing nuclear β -catenin levels, (C-D) at 72 h, β -catenin levels are elevated in cytosol (white arrowhead) and especially in nuclei (white arrow) as indicated by colocalization of chromatin (blue, Hoechst) and β -catenin (red) resulting in purple color. Scale bar = 20 μ M.

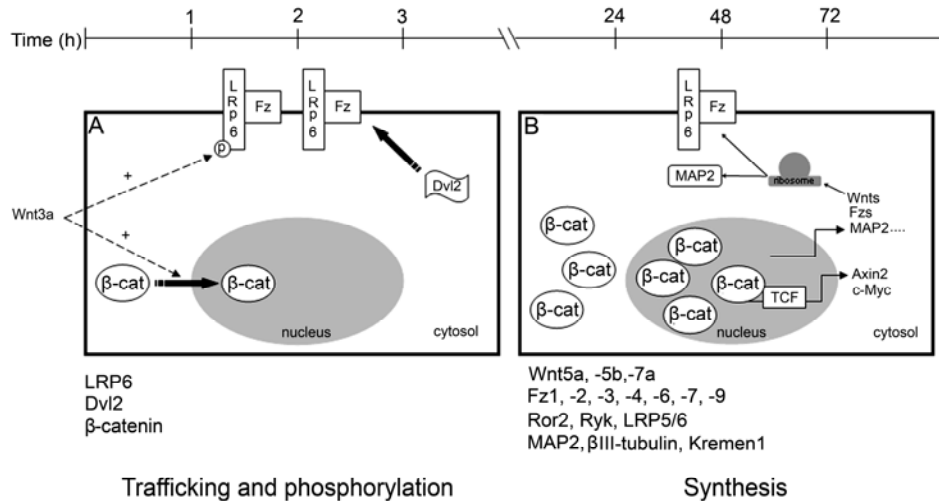


Fig. 10. A spatio-temporal model for the regulation of Wnt/ β -catenin pathway components, during ReNcell VM cell differentiation. Trafficking is observed during the first wave of regulation (first 3 h of differentiation). The proteins are relocated (thick black arrow), i.e. β -catenin from the cytosol to the nucleus, Dvl2 from the cytosol to the membrane. Presence of Wnt3a in the cell environment increases β -catenin trafficking and LRP6 phosphorylation (dashed arrows). B – Synthesis is observed during the second and third waves of regulation (after 24 h of differentiation). Accumulation of β -catenin in the cytosol and in the nucleus is accompanied by an increase of mRNA expression, such as Wnt ligands, Wnt receptor mRNAs, as well as Wnt target genes such as Axin2. Translation of these mRNAs via the ribosome might lead to increased protein expression after 72 h of differentiation.

DISCUSSION

In the present study, we investigate the dynamics of Wnt pathway components and signaling proteins during differentiation of ReNcell VM cells and provide a detailed biochemical profile of Wnt/ β -catenin signaling referring to both mRNA and protein levels. In ReNcell VM cells, we show first that TCF-dependent transcription can be activated by β -catenin and that Axin2, a target gene of Wnt/ β -catenin signaling, is inducible upon stimulation with LiCl, an inhibitor of GSK3 β (Fig. 1). Additionally, we prove that proliferating ReNcell VM cells are sensitive to the withdrawal of growth factors from the culture medium, which is sufficient to induce cell proliferation arrest, as supported by the fact that 96% of the cells remain in G0/G1 phases 24 h after differentiation and the decrease of cyclin D1 from 3 h onward after differentiation (Fig. 2). Furthermore, the removal of the growth factors triggers the Wnt/ β -catenin pathway, as indicated by the upregulation of Axin2 (Fig. 5), and by the fact that the endogenous Wnt ligands, Wnt receptors and signaling molecules are temporally regulated at mRNA levels during ReNcell VM cell differentiation (Figs. 4, 5). Further analyses of the core proteins of the Wnt/ β -catenin pathway reveal that the signaling proteins of LRP6, Dvl2 and β -catenin have a response

to the removal of the growth factors, e.g., phosphorylation of Dvl2 and β -catenin accumulation with spatio-temporal modifications during differentiation (Figs. 6-9). The biphasic regulation of LRP6, Dvl2, and β -catenin, with an initial peak followed by a decrease and a later increase, which is known as a typical dynamic for a signaling pathway, is also found (Figs. 6-8). Upon stimulation with Wnt3a, the amounts of the core components increase (Fig. 6-9). Finally, the differentiated ReNcell VM cells can develop potentially into various neural cell types, e.g., neurons and astrocytes, as detected by immunostaining (Fig. 3). Taken together, all these data demonstrate that the components of the Wnt/ β -catenin pathway are involved in the differentiation of ReNcell VM cells and, remarkably, its activation is temporally and spatially regulated.

For the analysis of gene expression in progenitor cells, real-time PCR is a good choice due to its reliability, affordability, and ease of performance. The most applied approach for normalization is the use of reference genes, also known as housekeeping genes [28]. These genes should have similar mRNA levels in all samples analyzed and they should not be regulated significantly by the experimental treatment or condition used. For example, Willems et al. [29] selected GAPDH, Pgc1, and Ubc as the most stable reference genes in human embryonic stem cells. Although membrane GAPDH in some cell types changes considerably and is not recommended to be used as an internal loading control, the membrane GAPDH in ReNcell VM cells both in the physiological state and upon Wnt3a stimulation is quite stable during cell differentiation (Fig. 6A, C), suggesting that GAPDH is a candidate for loading control in ReNcell VM cells.

Two different regulation waves of endogenous Wnt signaling molecules during ReNcell VM cell differentiation

ReNcell VM cells express a wide range of Fz receptors, co-receptors and Wnt ligands in a time-dependent manner with a balance of component expression during cell differentiation. The most relevant, for example, is Axin2, the direct target gene of β -catenin/TCF signaling, which is strongly upregulated 24 h after differentiation.

The first wave is the down-regulation of Dkk1, Wnt10b, and Fz5 from 3 h onward. Dkk1 can antagonize Wnt signaling by an LRP6-dependent mechanism preventing Wnt-induced Fz-LRP6 complex formation [30] or inducing internalization and degradation of LRP6 [31], or by an LRP6-independent mechanism [32]. Wnt10b is involved in cell growth in the presence of growth factors [33] and maintains self-renewal of stem cells [34]. Fz5 mediates cell growth of endothelial cells, possibly via its binding to Wnt10b [35], and its down-regulation in human progenitor cells (NT2/D1 cells) inhibits cell growth [36]. Therefore, we assume that the early down-regulation of Dkk1, Wnt10b and Fz5 might help to stop cell proliferation and triggers the differentiation of ReNcell VM cells.

Furthermore, Wnt5a and Wnt7a, which are known to trigger the Wnt/ β -catenin pathway [37-39], are up-regulated from 24 h of differentiation in the second wave

(Fig. 4). Wnt7a promotes neuronal differentiation in mouse NPCs [9] and triggers the Wnt/ β -catenin pathway when binding to Fz5 in the presence of LRP6 in rat CNS cells [38]. Interestingly, Fz7 is a specific receptor for Wnt7a [40] and both are upregulated during ReNcell VM cell differentiation (Fig. 4). Therefore, we can speculate that the binding of Wnt7a to Fz7 triggers ReNcell VM cell differentiation. Wnt5a and Wnt5b regulate the proliferation and differentiation of chondrocytes [41] and Wnt5a can promote dopaminergic neurogenesis [42]. Previous studies have shown that ReNcell VM cells are inducible to differentiate into dopaminergic neurons [4, 6]. Therefore, whether the upregulation of Wnt5a and Wnt5b is responsible for activation of the Wnt/ β -catenin pathway during ReNcell VM cell differentiation should be further analyzed.

Two kinetic phases of signaling protein regulation during ReNcell VM cell differentiation

Our data show that the intracellular signaling proteins LRP6, Dvl2, and β -catenin are temporally and spatially regulated, through a biphasic kinetic with the first phase during the first 3 h of differentiation involving protein phosphorylation and protein shuttling between different subcellular compartments, and a second phase after 24 h of differentiation involving protein phosphorylation and accumulation changes that accompany mRNA increases. Wnt3a stimulation increases the phosphorylation of membrane-associated LRP and cytosolic Dvl2, and the β -catenin shuttling from the cytosol into the nucleus.

Interestingly, in the first phase, we found that LRP6 phosphorylation of the PPP(S/T)P motifs, a trigger of pathway activation [26], occurs only upon Wnt3a stimulation, but not in the physiological state (Fig. 6). This suggests either that LRP6 phosphorylation is not necessary, as proposed by a previous study [43], or that the sensitivity of the antibody used here is not sufficient to detect low physiological levels of phosphorylation. Moreover, we observed an increase of p-LRP6 protein at 24 h after physiological differentiation and this change is correlated with an increase of cytosolic β -catenin (Fig. 8A) at the same time point, consistent with the notion that LRP6 phosphorylated on PPP(S/T)P motifs increases active β -catenin [44]. By TaqMan array we found that LRP6 mRNA increases only after 24 h during differentiation, suggesting that the increase of the ER form of LRP6 (180 kDa) observed by Western blot at the same time point reflects the synthesis and turnover of the receptor. These data suggest that the ReNcell VM cell line is a good cell model to study the dynamics of the co-receptor LRP6 in regulation of the Wnt/ β -catenin pathway.

Dvl2 is highly regulated during ReNcell VM cell differentiation. Interestingly, the increase of Dvl2 in the membrane fraction, upon Wnt3a stimulation, is similar to the one in the physiological state (Fig. 7). In contrast, the cytosolic p-Dvl2 is immediately increased by a shift from m-Dvl2. This has been described as the notion of the phosphorylation-dependent Dvl2 mobility shift in a dopaminergic neuronal cell line [45]. However, due to our subcellular analysis,

we show that this shift only occurs in the cytosol upon Wnt3a stimulation. Why Wnt3a regulates Dvl2 in a spatially dependent manner should be further investigated.

β -catenin is highly regulated in a spatio-temporal manner during ReNcell VM cell differentiation: an early shuttle of the protein from the cytosol to the nucleus is suggested by a decrease of cytosolic β -catenin and an increase of nuclear one, which can be accelerated upon Wnt3a stimulation. Acceleration of protein trafficking upon Wnt3a stimulation has also been reported in mouse F9 teratocarcinoma cells [46]. From 24 h of differentiation onward, the accumulation of β -catenin in both nucleus and cytosol (Figs. 8, 9) suggests a very pronounced and effective response to the pathway stimulation. Indeed, a close correlation between nuclear β -catenin accumulation and the upregulation of specific gene transcription such as Axin2 is found in our analysis (Figs. 1B, 5).

It is noteworthy that the differentiation of ReNcell VM cells is not homogeneous due to the diversity of the cell types obtained after 72 h (Fig. 3), as well as the delay in the cell cycle arrest after withdrawal of growth factors (Fig. 2). Synchronization of the ReNcell VM cells should allow a homogeneous analysis of the pathway during differentiation. However, such a method remains delicate to apply to hNPCs and brings more bias in the analysis. We used a computational systems biology approach to clarify whether the temporal dynamics observed are intrinsic to the pathway activation [17] (Mazemondet et al., manuscript under revision). However, as over 50% of the cells are synchronized and start to differentiate after growth factor removal, and that 90% of the cells are arrested in the G0/G1 phase at 14 h (Fig. 3), we assume that the time course observed is representative of the ReNcell VM cell population during differentiation.

Taken together, our data show that growth factor removal and Wnt3a stimulation change the key components of the Wnt/ β -catenin pathway by protein phosphorylation and compartment redistribution during early ReNcell VM differentiation (Fig. 10A). As cell differentiation continues from 24 h onward, Wnt/ β -catenin pathway activation results in expression of target genes associated with synthesis of signaling components, as reported previously in other cells [47-50] (Fig. 10B), which further control the cell differentiation in a context- and stage-dependent manner.

CONCLUSION

The endogenous expression of the Wnt ligands, their receptors and the signaling molecules is temporally regulated during hNPC cell differentiation. Furthermore, the kinetics of trafficking and phosphorylation of the key signaling proteins LRP6, Dvl2 and β -catenin are spatio-temporally controlled at early differentiation stages, followed by mRNA synthesis after activation of the Wnt/ β -catenin pathway from 24 h onward during differentiation. These data definitely prove for the first time that Wnt/ β -catenin signaling is involved in the differentiation of ReNcell VM cells.

Competing interests. The authors declare that they have no competing interests.

Authors' contributions. OM carried out the Western blot and participated in data analysis. RH performed the real-time PCR experiments and TaqMan qRT-PCR and participated in data analysis. Both also contributed to writing the manuscript. JF performed the TCF-reporter gene assay and cell cycle analysis, and analyzed the data. DK participated in TaqMan qRT-PCR array. BMB performed the confocal immunofluorescence microscopy. AMU, DGW and MF helped draft the manuscript and contributed to the strategy making. AR conceived the study and contributed to drafting the manuscript. JL participated in the design of the study and wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Lindvall, O., Kokaia, Z. and Martinez-Serrano, A. Stem cell therapy for human neurodegenerative disorders-how to make it work. **Nat. Med.** 10 Suppl (2004) S42-50.
2. Clelland, C.D., Barker, R.A. and Watts, C. Cell therapy in Huntington disease. **Neurosurg. Focus** 24 (2008) E9.
3. Locatelli, F., Bersamo, A., Ballabio, E., Lanfranconi, S., Papdimitriou, D., Strazzer, S., Bresolin, N., Comi, G.P. and Corti, S. Stem cell therapy in stroke. **Cell. Mol. Life Sci.** 66 (2009) 757-772.
4. Donato, R., Miljan, E.A., Hines, S.J., Aouabdi, S., Pollock, K., Patel, S., Edwards, F.A. and Sinden, J.D. Differential development of neuronal physiological responsiveness in two human neural stem cell lines. **BMC Neurosci.** 8 (2007) 36.
5. Hoffrogge, R., Mikkat, S., Scharf, C., Beyer, S., Christoph, H., Pahnke, J., Mix, E., Berth, M., Uhrmacher, A., Zubrzycki, I.Z., Miljan, E., Völker, U. and Rolfs, A. 2-DE proteome analysis of a proliferating and differentiating human neuronal stem cell line (ReNcell VM). **Proteomics** 6 (2006) 1833-1847.
6. Morgan, P.J., Ortinau, S., Frahm, J., Kruger, N., Rolfs, A. and Frech, M.J. Protection of neurons derived from human neural progenitor cells by veratridine. **Neuroreport** 20 (2009) 1225-1229.
7. Logan, C.Y. and Nusse, R. The Wnt signaling pathway in development and disease. **Annu. Rev. Cell Dev. Biol.** 20 (2004) 781-810.
8. Komiyama, Y. and Habas, R. Wnt signal transduction pathways. **Organogenesis** 4 (2008) 68-75.
9. Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. **Development** 131 (2004) 2791-2801.

10. Mutoyama, Y., Kondoh, H. and Takada, S. Wnt proteins promote neuronal differentiation in neural stem cell culture. **Biochem. Biophys. Res. Commun.** 313 (2004) 915-921.
11. Castelo-Branco, G., Wagner, J., Rodriguez, F.J., Kele, J., Sousa, K., Rawal, N., Pasolli, H.A., Fuchs, E., Kitajewski, J. and Arenas, E. Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. **Proc. Natl. Acad. Sci. USA** 100 (2003) 12747-12752.
12. Kholodenko B.N. Cell-signaling dynamics in time and space. **Nat. Rev. Mol. Cell Biol.** 7 (2006) 165-176.
13. Nakamura, T., Sano, M., Songyang, Z. and Schneider, M.D. A Wnt- and beta -catenin-dependent pathway for mammalian cardiac myogenesis. **Proc. Natl. Acad. Sci. USA** 100 (2003) 5834-5839.
14. Hübner, R., Schmöle, A.C., Liedmann, A., Frech, M.J., Rolfs, A. and Luo, J. Differentiation of human neural progenitor cells regulated by Wnt-3a. **Biochem. Biophys. Res. Commun.** 400 (2010) 358-362.
15. Schmöle, A.C., Brenführer, A., Karapetyan, G., Jaster, R., Pews_Davtyan, A., Hübner, R., Ortinau, S., Beller, M., Rolfs, A. and Frech, M.J. Novel indolylmaleimide acts as GSK-3 β inhibitor in human neural progenitor cells. **Bioorg. Med. Chem.** 18 (2010) 6785-6795.
16. Klipp, E. and Liebermeister, W. Mathematical modeling of intracellular signalling pathways. **BMC Neuroscience** 7 (2006) S10.
17. Mazemondet, O., John, M., Maus, C., Uhrmacher A. and Rolfs, A. Integrating diverse reaction types into stochastic models – a signaling pathway case study in the imperative pi-Calculus. In: **Proceedings of Winter Simulation Conference**, 2009, 931-943.
18. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Res.** 29 (2001) e45.
19. Ohl, F., Jung, M., Radonic, A., Sachs, M., Loening, S.A. and Jung, K. Identification and validation of suitable endogenous reference genes for gene expression studies of human bladder cancer. **J. Urol.** 175 (2006) 1915-1920.
20. Schilling, M., Maiwald, T., Bohl, S., Kollmann, M., Kreuts, C., Timmer, J. and Klinmüller, U. Computational processing and error reduction strategies for standardized quantitative data in biological networks. **FEBS J.** 272 (2005) 6400-6411.
21. Angers, S. and Moon, R.T. Proximal events in Wnt signal transduction. **Nat. Rev. Mol. Cell Biol.** 10 (2009) 468-477.
22. Stambolic, V., Ruel, L. and Woodgett, J.R. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. **Curr Biol.** 6 (1996) 1664-1668.
23. Jho, E.H., Zhang, T., Domon, C., Joo, C.K., Freund, J.N. and Costantini, F. Wnt/beta-catenin/Tcf signalling induces the transcription of Axin2, a negative regulator of the signalling pathway. **Mol. Cell Biol.** 22 (2002) 1172-1183.
24. Blagosklonny, M.V. and Pardee, A.B. The restriction point of the cell cycle. **Cell Cycle** 1 (2002) 103-110.

25. Li, Y., Wenyan L., Xi, H. and Guojun, B. Modulation of LRP6-mediated Wnt signaling by molecular chaperone Mesd. **FEBS Lett.** 580 (2006) 5423-5428.
26. Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z. and He, X. A mechanism for Wnt coreceptor activation. **Mol. Cell** 13 (2004) 149-156.
27. Khan, Z., Vijayakumar, S., De La Torre, T.V., Rotolo, S. and Bafico, A. Analysis of endogenous LRP6 function reveals a novel feedback mechanism by which Wnt negatively regulates its receptor. **Mol. Cell Biol.** 27 (2007) 7291-7301.
28. Winer, J., Jung, C.K., Shackel, I. and Williams, P.M. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. **Anal. Biochem.** 270 (1999) 41-49.
29. Willems, E., Mateizel, I., Kemp, C., Gauffman, G., Sermon, K. and Leyns, L. Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells. **Int. J. Dev. Biol.** 50 (2006) 627-635.
30. Semenov, M.V., Tamai, K., Brott, B.K., Kuehl, M., Sokol, S. and He, X. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. **Curr. Biol.** 11 (2001) 951-961.
31. Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B.M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A. and Niehrs, C. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. **Nature** 417(6889) (2004) 664-667.
32. Semenov, M.V., Zhang, X. and He, X. DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation. **J. Biol. Chem.** 283 (2008) 21427-21432.
33. Yoshikawa, H., Matsubara, K., Zhou, X., Okumara, S., Kubo, T., Murase, Y., Shikauchi, Y., Esteller, M., Herman, J.G., Wei, X. and Harris, C.C. WNT10B functional dualism: beta-catenin/Tcf-dependent growth promotion or independent suppression with deregulated expression in cancer. **Mol. Biol. Cell** 18 (2007) 4292-4303.
34. Kirikoshi, H. and Katoh, M. Expression and regulation of WNT10B in human cancer: up-regulation of WNT10B in MCF-7 cells by beta-estradiol and down-regulation of WNT10B in NT2 cells by retinoic acid. **Int. J. Mol. Med.** 10 (2002) 507-511.
35. Ishikawa, T., Tamai, Y., Zorn, A.M., Yoshida, H., Seldin, M.F., Nishikawa, S. and Taketo, M.M. Mouse Wnt receptor gene *Fzd5* is essential for yolk sac and placenta angiogenesis. **Development** 128 (2001) 25-33.
36. Snow, G.E., Kasper, A.C., Busch, A.M., Schwarz, E., Ewings, K.E., Bee, T., Spinella, M.J., Dmitrovsky, E. and Freemantle, S.J. Wnt pathway reprogramming during human embryonal carcinoma differentiation and potential for therapeutic targeting. **BMC Cancer** 9 (2009) 83.
37. van Amerongen, R., Mikels, A. and Nusse, R. Alternative Wnt signaling is initiated by distinct receptors. **Sci. Signal.** 1 (2008) re9.
38. Caricasole, A., Ferraro, T., Iacovelli, L., Barletta, E., Caruso, A., Melchiorri, D., Terstappen G.C. and Nicoletti, F. Functional characterization of WNT7A

- signaling in PC12 cells: interaction with a FZD5-LRP6 receptor complex and modulation by Dickkopf proteins. **J. Biol. Chem.** 278 (2003) 37024-37031.
39. Carmon, K.S. and Loose, D.S. Secreted frizzled-related protein 4 regulates two Wnt7a signaling pathways and inhibits proliferation in endometrial cancer cells. **Mol. Cancer Res.** 6 (2008) 1017-1028.
 40. Le Grand, F., Jones, A.E., Seale, V., Scime, A. and Rudnicki, M.A. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. **Cell Stem Cell** 4 (2009) 535-547.
 41. Yang, Y., Topol, L., Lee, H. and Wu, J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. **Development** 130 (2003) 1003-1015.
 42. Castelo-Brance, G., Sousa, K.M., Bryja, V., Pinto, L., Wagner, J. and Arenas, E. Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. **Mol. Cell Neurosci.** 31 (2006) 251-262.
 43. Beagle, B., Mi, K. and Johnson, G.V.W. Phosphorylation of PPP(S/Y)P motif of the free LRP6 intracellular domains is not required to activate the Wnt/beta-catenin pathway and attenuate GSK3beta activity. **J. Cell Biochem.** 108 (2009) 886-895.
 44. Wu, G., Huang, H., Abreu, J.G. and He, X. Inhibition of GSK3 phosphorylation of beta-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. **PloS One** 4 (2009) e4926.
 45. Bryja, V., Schulte, G. and Arenas, E. Wnt-3a utilizes a novel low dose and rapid pathway that does not require casein kinase 1-mediated phosphorylation of Dvl to activate beta-catenin. **Cell Signal.** 19 (2007b) 610-616.
 46. Yokoyama, N., Yin, D. and Malbon, C.C. Abundance, complexation, and trafficking of Wnt/beta-catenin signaling elements in response to Wnt3. **J. Mol. Signal.** 2 (2007) 11.
 47. Müller, H.A., Samanta, R. and Wieschaus, E. Wingless signaling in the Drosophila embryo: zygotic requirements and the role of the frizzled genes. **Development** 126 (1999) 577-586.
 48. Cadigan, K.M. and Nusse, R. Wnt signaling: a common theme in animal development. **Genes Dev.** 11 (1997) 3286-3305.
 49. Sato, A., Kojima, T., Ui-Tei, K., Miyata, Y. and Saigo, K. Frizzled-3, a new Drosophila Wnt receptor, acting as an attenuator of Wingless signaling in wingless hypomorphic mutants. **Development** 126 (1999) 4421-4430.
 50. Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W. and Behrens, J. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. **Mol. Cell Biol.** 22 (2004) 1184-1193.