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Wnt3a induces neuronal differentiation and crosstalk between Wnt/Ca^{2+}-pathway and Wnt/β-catenin pathway

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To

My

Family,

Friends & Teachers

‘‘Imagination is more important than Knowledge’’

-Albert Einstein

Contents

1 Introduction

Loss of neurons and oligodendrocytes in the brain and/or spinal cord induces progressive dysfunction of the central nervous system (CNS), leading to neurological diseases, such as Parkinson's disease, Alzheimer's disease (AD), Huntington disease, amyotrophic lateral sclerosis, and multiple sclerosis (MS). Finding effective therapies for treatment of these diseases are big challenge in the field of human medicine. Recently, stem cell-based cellular therapy for neurodegenerative disease has been focused (Fig. 1) and translation medicine develops quickly by transferring new techniques from bench to clinic. Therefore, the understanding of signaling pathways in differentiating neural stem cell is of great interest (Lunn et al., 2011).

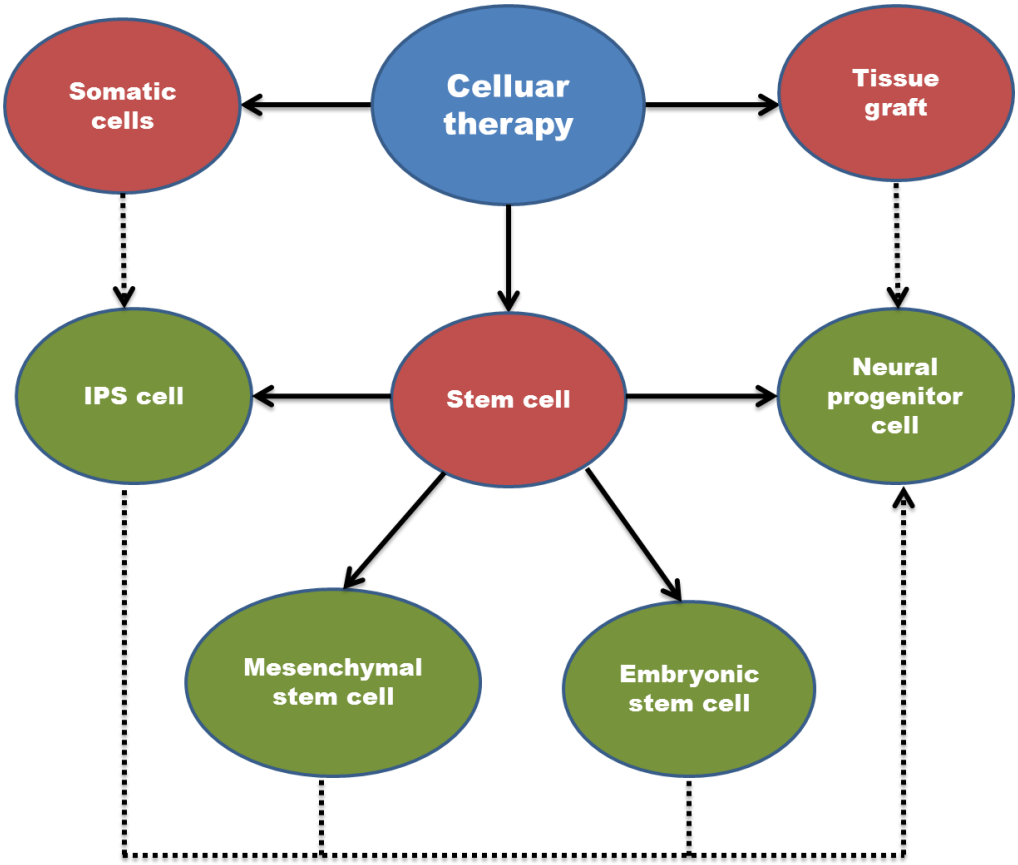

Figure 1. Overview of stem cell technology and stem cell-based cellular therapies.
The cellular therapy is involved in the treatment of diseases using cells or tissue grafts. Different types of stem cells may be utilized and each possesses exclusive characteristic and advantages depending on desired outcomes. Selection of proper stem cells for the treatment of neurodegenerative diseases is essential for cellular therapies from bench to bedside. Here solid arrows represent divisions within the each category and the dashed the source of NPCs and iPS cells.

1.1 Stem Cells and Neurogenesis

Stem cells have two important characteristic features: self-renewal and ability to differentiate into different types of cells. Stem cells are essential for maintenance of body function by supplying new cells to replace the lost cells induced by normal homeostasis or aging, e.g., thousand million new blood cells are produced by the bone marrow every day in humans. In different tissues and developmental stages, stem cells vary in numbers and types. Based on the ability to develop into other types of cells, stem cells are classified into different types: totipotent, pluripotent, and multipotent types (Fig. 2). Totipotent stem cells are the one developing from a zygote into a 16-cell stage, which have the greatest differentiating potential to develop into any one of the three germ layers plus placental cells, giving rise to a functional organism. Pluripotent stem cells, also called as embryonic stem cells (ESCs), are separated from the inner cell mass of the blastocyst, which can continue to develop into any types of cells excluding placenta. The multipotent stem cells can develop into more than one cell type, which are limited than pluripotent cells, e.g., neural progenitor cells (NPCs) or adult stem cells. All types of these cells can be cultivated and grown by in vitro system.

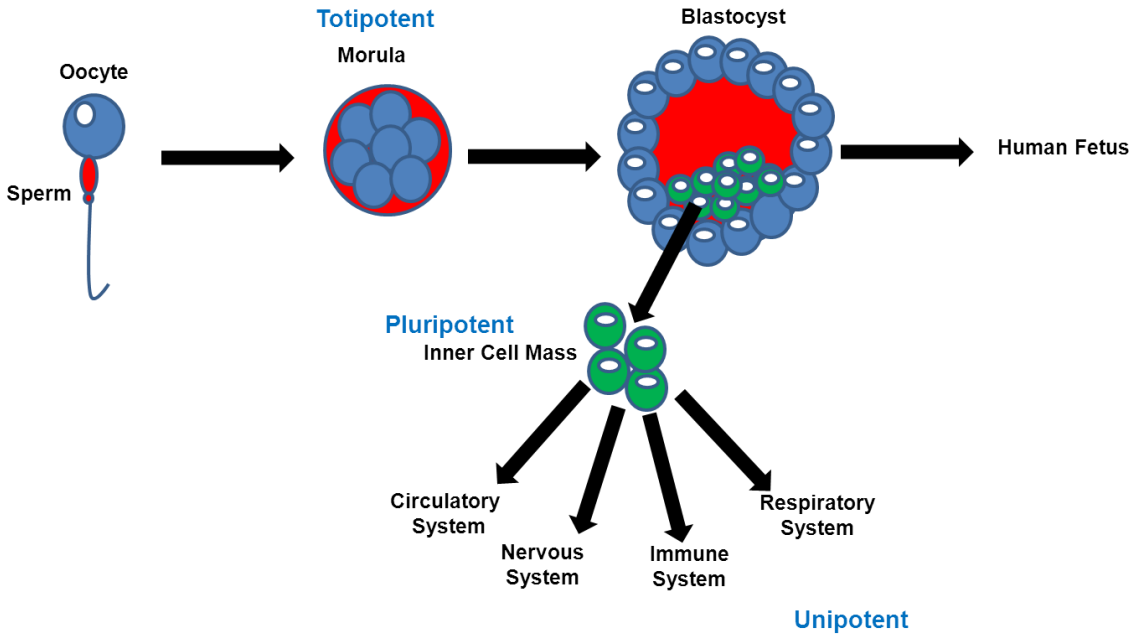

Figure 2. Stem cells and cell potency.
The cells from the morula stage are totipotent and have the ability to become all tissues and placenta. The embryonic stem cells originated from the inner cell mass of the blastocyst are pluripotent and can form any tissue, excluding placenta in the body.

After the three germ layers are established, cells in some part of the ectoderm develop into the neural ectoderm, which forms the neural tube. The rostral part of the neural tube develops into the brain, including the telencephalon, diencephalon, midbrain, cerebellum and hindbrain; while the caudal part into the spinal cord (Fig. 3). NPCs in the neural tube are multipotent and undergo asymmetric divisions, producing neurons, astrocytes, and oligodendrocytes. Recently, adult stem cells have also been recognized and identified in the specific regions of the subventricular zone in the cortex and the dentate gyrus in the hippocampus (Fig. 3; Temple and Alvarez-Byulla, 1999; Temple, 2001).

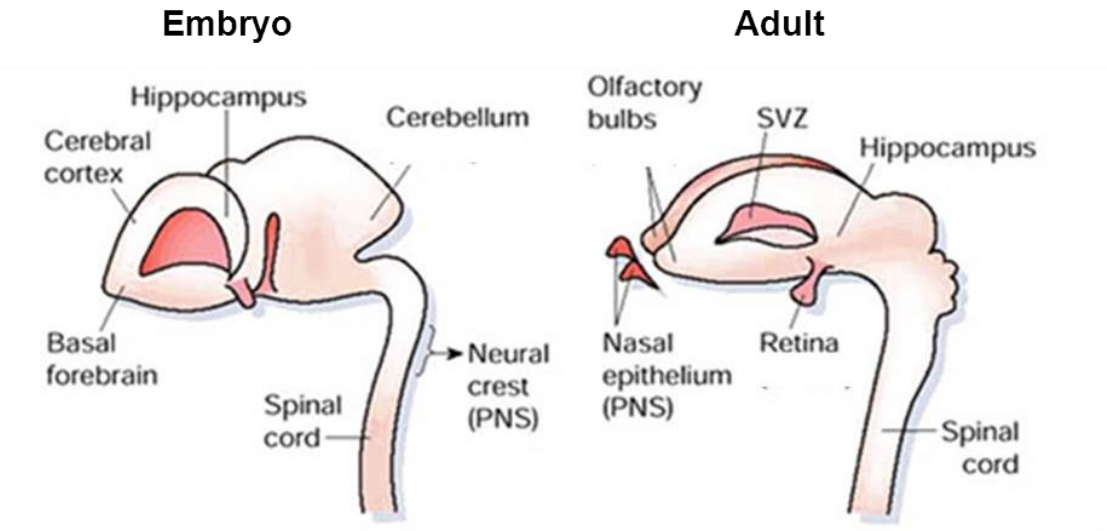

Figure 3. The developmental and adult CNS.
Schematic pictures show embryonic developmental (left) and adult (right) CNS. The different parts of the CNS are indicated. The location of adult stem cells can be isolated from the subventricular zone (SVZ) (Figure adapted from Temple, 2001).

1.2 Stem Cell-Based Therapy

Replacing lost cells via transplantation with a specific type of stem cells is considered as an efficient treatment for various neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis. For example, millions of people are affected by Parkinson's and Alzheimer's diseases, but no efficient treatment can cure patients, although the symptoms can be improved by drug and other methods. However, in the animal model, Parkinson's disease has been almost successfully treated using transplantation of human and mouse embryonic stem cells. Dopamine producing neurons from hESCs efficiently engraft into mice and rat, and trails are

going on with monkeys for the further confirmation (Kriks et al., 2011). Furthermore, transplantation with adipose derived stem cells (ADSCs) in the mouse model also improves the memory and learning ability, providing a basic knowledge and potential platform for treatment of the patients with Alzheimer's disease (Kim et al., 2012).

In recent years, research in the field of the stem cell therapy develops quickly and the stem cell-based therapies are substantial (Nikolic et al., 2009). Clinical trials are on the way for the use of stem cells, especially with autologous transplants, although the therapeutic benefit for patients is still in discussion (Trounson et al., 2011).

1.3 The Wnt Signaling

The ''Wnt'' is derived from the abbreviation of two genes - Wg (wingless gene from Drosophila) and Int-1 (integration site-1 gene; Nusse and Varmus, 1982). Members of the Wnt family are secreted lipid-modified glycoproteins, which have been found in all animal species and play key roles in various intracellular processes, such as proliferation, differentiation, and cell apoptosis in physiological processes during embryonic development. The dysregulation of the Wnt signaling during developmental processes induces various diseases, e.g., cancer, type II diabetes (Grant, 2006), and osteogenesis imprefecta (Fahiminiya et al., 2013).

The activation of the Wnt signaling pathway is triggered through the binding of the Wnt ligand to its receptor, such as the Frizzled (Fz) receptor - a seven pass trans-membrane domains protein - and the co-receptor low-density receptor-related protein 5/6 (Lrp5/6) (Fig. 4; Bhanot et al., 1996). According to the receptors and their activated effects, Wnt signals are mainly divided into two different pathways:

1. Canonical or the Wnt/β-catenin dependent pathway
2. Non-canonical or the Wnt/β-catenin independent pathway, including the Wnt/ Ca^{2+} dependent pathway and the planar cell polarity (PCP) pathway.

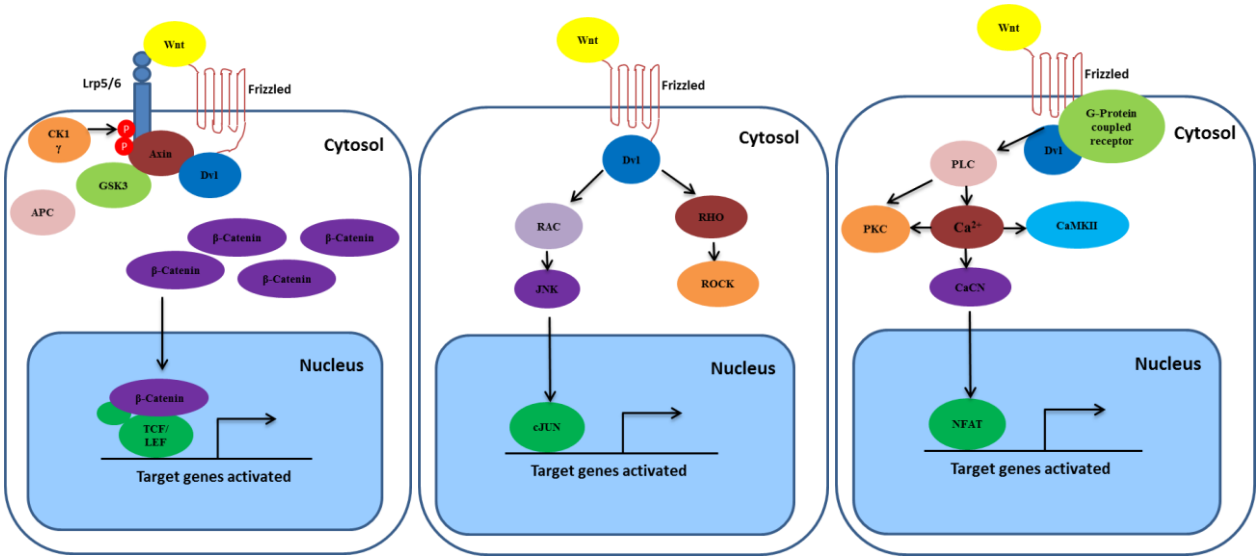

Figure 4. Schematic representation of Wnt signalings.
(A): *In the canonical Wnt pathway, the binding of Wnt ligand to its Frizzled (Fz) and Lrp5/6 receptor complex induces the stabilization of β-catenin through activated dishevelled and β-catenin degradation complex (GSK3β, Axin and casein kinase 1). The stabilized β-catenin is translocated into the nucleus, where β-catenin forms a complex with TCF/LEF, inducing the target gene expression. β-catenin exported from nucleus is degraded through the proteasome pathway.* ***(B):*** *In the Wnt/Ca^{2+} pathway, the binding of Wnt to Fz activates hetromeric G-proteins and dishevelled, triggering protein kinase C (PKC) and calcium/calmodulin-dependent kinase 2 (CaMK2) activation, mediating the cell adhesion and motility.* ***(C):*** *In non-canonical or PCP pathway, the binding of Wnt ligand to Fz activates dishevelled, which subsequently activates the small GTPases, Rho and Rac, mediating cell movements and cytoskeleton changes.*

1.3.1 Wnt/β-Catenin Dependent Pathway

The Wnt/β-catenin dependent pathway is an ancient, conserved and one of the well described signaling cascade, which is triggered by the binding of the canonical Wnt to its receptor Fz and Lrp5/6, leading to degradation of the axin, adenomatous poliposis coli (APC), and glycogen synthase kinase3β (GSK3β) complex, resulting in accumulation of β-catenin in the cytosol. In the Wnt/β-catenin dependent pathway, β-catenin acts as a main transcriptional co-activator to trigger expression of target genes. In order to understand easily, this pathway can be described in an OFF (without Wnt) and an ON (with Wnt) model (Fig. 5).

OFF Model

In the absence of the Wnt ligand, β-catenin is phosphorylated in the positions of serine-33/37 and threonine-41 (Kimelman and Xu, 2006) by GSK3β-Axin-APC degradation complex through ubiquitin degradation mediated by β-transducin repeat

containing E3 ubiquitin protein ligase (β-TrCP), resulting in a low concentration of β-catenin in the cytosol (Mac Donald et al., 2009). However, it is not totally understood yet how β-catenin is phosphorylated under the degradation complex.

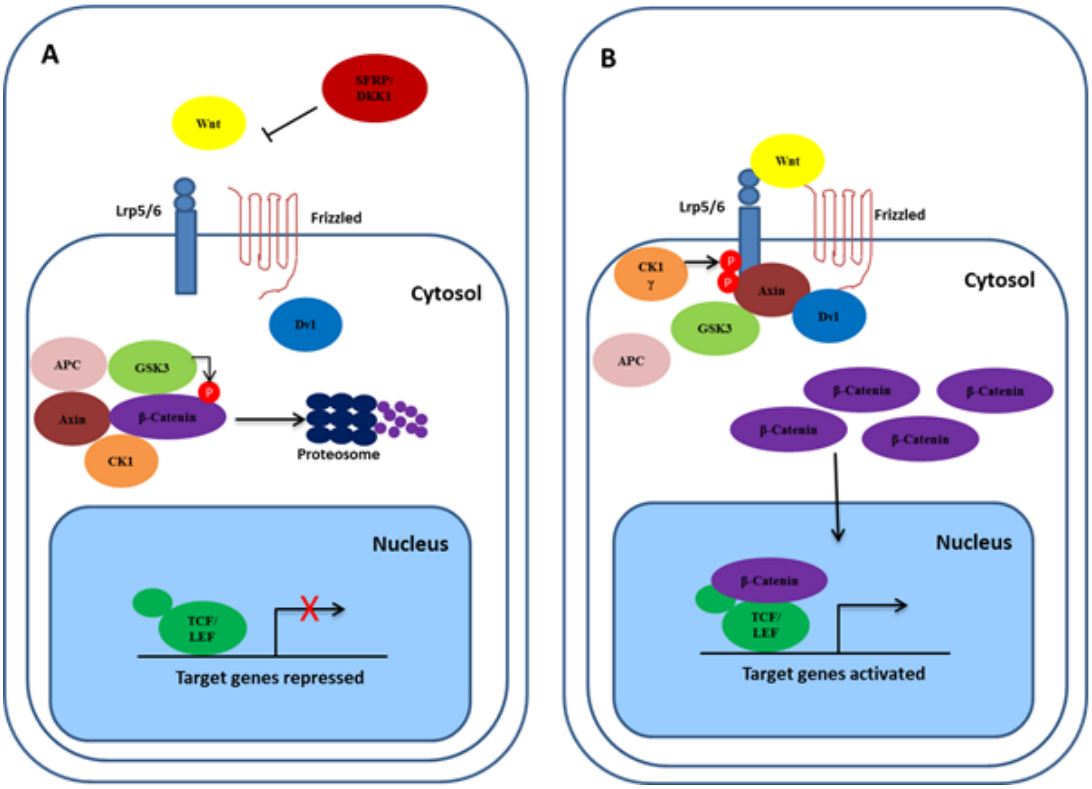

Figure 5. Wnt/β-catenin dependent signaling pathway.
(A): Off model. *In the absence of Wnt, cytosolic β-catenin is continuously phosphorylated by the β-catenin destruction complex, which consists of Axin, GSK3β, APC, and CK1. The β-TrCP recognizes the phosphorylated form of β-catenin and degrade the target in an ubiquitin-dependent manner.* ***(B): On model.*** *The binding of Wnt ligand to Fz and Lrp5/6 initiates degradation of GSK3β-Axin-APC complex and phosphorylation of Lrp5/6, resulting in accumulation of cytosolic β-catenin, which enters the nucleus and interacts with TCF/LEF to activate the Wnt target genes.*

ON Model

The binding of the Wnt ligand to Fz and its co-receptor Lrp5/6 leads to internalization of Lrp6 phosphorylation through GSK3β or casein kinase-1 (CK1). The phosphorylated Lrp6 acts as a docking site for Axin, resulting in a stabilization of cytosolic β-catenin against the GSK3β distraction complex in a dishevelled (Dvl) dependent manner. Accumulated cytosolic β-catenin enters into the nucleus and binds to T-cell factor (TCF)/lymphoid enhancer factor (LEF), triggering transcription of the Wnt target genes, e.g., *Axin2*.

1.3.2 Wnt/β-Catenin Independent Pathway

Wnt/β-catenin-independent pathway plays a key role for regulation of convergent extension movement during embryonic development (Sheldhal et al, 2003). This pathway doesn't require an involvement of β-catenin and represents another diverse array of signaling pathways. Based on their specific receptor used, it is further divided into: the Wnt/ Ca^{2+} dependent pathway and the planar cell polarity (PCP) pathway.

Wnt/ Ca^{2+} Dependent Pathway

The binding of the Wnt ligand to its Fz receptor leads to activation of heterotrimeric G-protein, followed by subsequent activation of phospholipase C (PLC), which cleaves phosphotidaylinositol-4,5-biphophate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphophate (IP3). Released IP3 from the membrane diffuses through cytosol and triggers Ca^{2+} release from the endoplasmic reticulum (ER), resulting in activation of DAG and protein kinase C (PKC). Released Ca^{2+} activates calcium-calmodulin-dependent kinase II (CaMKII) and calcineurin (CaCN) (Kühl et al., 2000). Phosphorylation of CaCN induces transport of the nuclear factor activated T-cell (NFAT), resulting in the expression of target genes in different types of cells, including neuronal cells (Feske et al., 2003; Hogan et al., 2003).

Planar Cell Polarity Pathway

Wnt/PCP pathway is described first in the orientation of the facet for eyes and of hairs on wings in *Drosophila.* Studies in vertebrates show that PCP pathway controls cell movement and tissue polarity, inner ear development and neural tube closure (Seifert and Mlodzik, 2007). The binding of the Wnt ligand to its Fz and a co-receptor (ROR or RYK) activates Rho associated kinase (ROCK) and c-Jun terminal kinase (JNK) signalings (Sethi and Vidal-Puig, 2010). The binding of the Wnt ligand to Fz can also lead to activation of heterotrimeric G-proteins Ras homolog A (RHOA) and Ras-related C3 botulinum toxin substrate-1 (RAC1) via Dvl. All these signals are involved in actin remodeling (Schlessinger et al., 2009). Interestingly, ROR2 mediated PCP pathway inhibits β-catenin dependent signaling (Winkel et al., 2008), but RYK activates it (Berndt et al., 2011). More factors involved in the cooperation between Wnt/β-catenin-dependent and independent pathways have been also found out (Dworkin et al., 2011).

1.4 Proline-Rich Tyrosine Kinase 2

Enzymes that transfer phosphate group from adenosine triphosphate (ATP) molecule to side chains of specific amino acids in target proteins are referred to as protein kinases, which are divided into phosphatases and tyrosine or serine/threonine kinases (Hunter, 1995). Based on the structure and location, protein tyrosine kinases (PTKs) are subdivided into the receptor PTK and non-receptor PTK (Neet and Hunter, 1996). Focal adhesion kinase (FAK) and proline-rich tyrosine kinse 2 (Pyk2) are two types of non-receptor PTKs. Pyk2 is also named as related adhesion focal tyrosine kinase (RAFTK), Ca^{2+} dependent tyrosine kinase (CADTK), protein tyrosine kinase 2β (PTK2B) and cell adhesion kinase β (CAKβ). Pyk2 is expressed in hematopoietic cells, platelets, fibroblasts, and cells in the CNS (Stanzione et al., 2001). Pyk2 can be activated by an increased intracellular Ca^{2+} concentration, a change in osmolality, upon ultra violet irradiation and inflammatory cytokine stimulation, and various extracellular signals. Structurally, FAK and Pyk2 are very similar and Pyk2 contains a central catalytic domain flanked by N-terminal FERM domain and carboxy-terminal focal adhesion targeting regions (Fig. 7; Lipinski and Loftus, 2010). The N-terminal FERM domain mediates protein-protein and protein-membrane interactions. The tyrosine residue at 402 (Y^{402}) position is a major auto-phosphorylation site of Pyk2 and lies in FERM domain, which is regulated by Ca^{2+}/calmodulin dependent catalytic activity. The phosphorylation of Pyk2 at Y^{402} and Y^{881} positions facilitates the binding of the SH2 domain to Src and GRB2 (growth factor receptor bound 2), respectively (Dikic et al., 1996; Avraham et al., 2000).

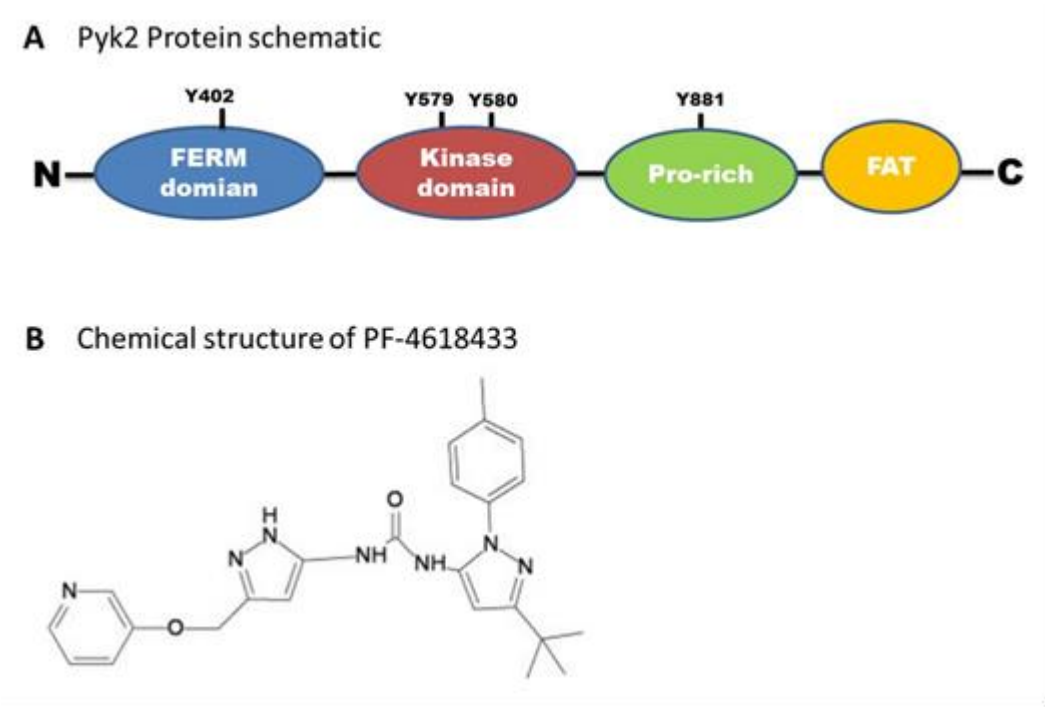

Figure 6. The schematic structure of Pyk2 and the Pyk2 inhibitor PF-4618433.
(A): *The N-terminal FERM domain of Pyk2 mediates the protein-protein interactions. Phosphorylation of Pyk2 at tyrosine-402 (Y402) is a major auto-phosphorylation site regulated by* Ca^{2+} *and the phosphorylation at tyrosine-881 position facilitates the binding of Pyk2 to GRB2. At the carboxy terminus, Pyk2 contains a focal adhesion targeting (FAT) domain that binds to paxillin.* ***(B):*** *The chemical structure of Pyk2 inhibitor PF-4618433.*

Studies demonstrate that Pyk2 is involved in MAPK (mitogen activated protein kinase) signaling (Lipinski and Loftus, 2010). Upon stress stimulus, Pyk2 activates JNK (c-Jun N-terminal kinase) signaling (Tokiwa et al., 1996; Yu et al., 1996). Transient increase of intracellular Ca^{2+} can phosphorylate GSK3β at tyrosine-216 position (Hartigan and Johanson, 1999) through Pyk2 (Hartigan et al., 2001). Pyk2 activation induces multiple intracellular cascades, resulting in reorganization of the cytoskeleton (Ivankovic-Dikic et al., 2000), vesicular transport regulation (Andrev et al., 2001), regulating neurite outgrowth (Ivankovic-Dikic et al., 2000) and neurite retraction (Sayas et al., 2006). Inhibition of Pyk2 induces osteogenic differentiation, leading to a novel approach in the treatment of osteoporosis (Buckbinder et al., 2007). The molecule of PF-562271 is used as a competitive inhibitor for both FAK and Pyk2 and it produces an anti-tumor effect in xenograft tumor model after administration (Bagi et al., 2008). The molecule of PF-4618433 is used as specific inhibitor for Pyk2 (Han et al., 2009).

1.5 Notch Signaling Pathway

The Notch signaling pathway plays a key role during developmental embryos, especially in progenitor cell proliferation and specification (Cheng et al., 2010), angiogenesis, spermatogenesis, and neurogenesis (Hori et al., 2013). The Notch receptor is also recognized as an oncogenic protein, since more than 50% patients with T-cell lymphoblastic leukaemia have a mutant Notch1 and the Notch signaling is involved in carcinomas of the skin, the colon, the breast, and the lung as well as tumors in the CNS (Wilson A and Radtke F, 2006).

The Notch receptor, comprising of an extracellular (ECN), a transmembrane (TMN) and an intracellular (ICN) subunit, is a single-pass transmembrane protein and binds to its ligands of the DSL (Delta, Serrate, Lag-2) transmembrane proteins (Fig. 6). In mammals there are four Notch receptors, described as Notch1 (TAN1), Notch2, Notch3 and Notch4 (Int3), and five DSL ligands, indicated as Delta1, Delta3, Delta4, Jagged1 and Jagged2 (Bray, 2006).

The binding of DSL to the epidermal growth factor repeats (EGFR) in the ECN subunit of the Notch receptor leads to subsequent cleavages of it through alpha-secretase ADAM (a disintegrin and metalloprotease) protein and γ-secretase, resulting in the release of Notch intracellular domain (NICD), which translocates from the cytosol into the nucleus (Callahan and Egan, 2004) and forms a tri-protein complex with a DNA-bound protein CSL (suppressor of hairless lag-2, also known as RBPJ) and a transcriptional co-activator mastermind (Ong et al, 2006), activating the target genes. In the absence of NICD, this tri-protein complex is displaced by histone deacetylases (HDACs) and ubiquitous co-repressor proteins (Co-R) (Kopan et al., 2009; Imayoshi et al., 2013).

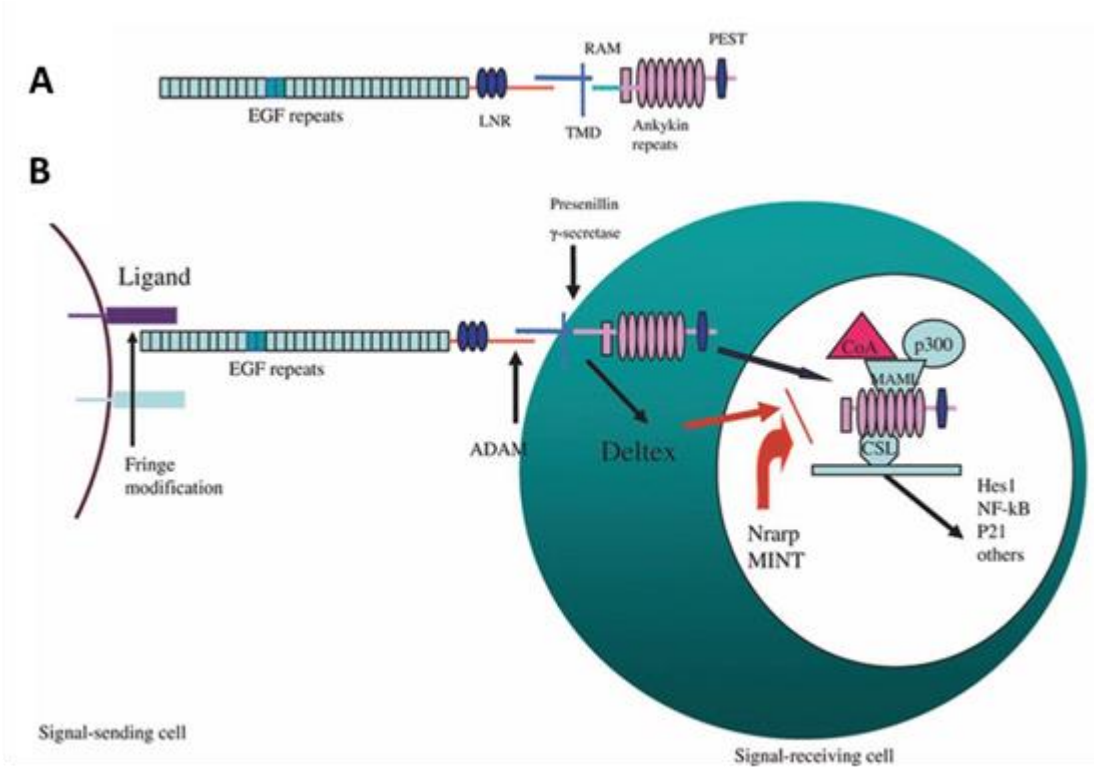

Figure 7. Schematic representation of Notch signaling pathway.
(A)*: The Notch receptor consists of an extracellular domain including EGF repeats, Lin12 notch repeats (LNR), a transmembrane domain (TMD) and an intracellular domain including a J-associated molecular domain (RAM), ankykin repeats and proline/serine/glutamic acid/threonine rich motifs.* ***(B)****: Notch signaling is initiated by a binding of Notch ligand expressed on an adjacent cell, followed by an enzymatic cleavage and release of intracellular Notch (NICD), which translocates into the nucleus and binds to the CSL transcription repressor and converts it into activator form, resulting in the transcription of the target genes (Diagram from Cheng et al., 2010).*

The well-known target genes in the Notch signaling pathway are the Hairy enhancer of split (HES) and its related repressor (HERP, also known as HEY) (Stylianou et al., 2006), which are encoded by a family of basic helix-loop-helix-(bHLH) transcriptional factors. During embryonic development, knockdown of the bHLH repressor genes results in an increase of *HES* genes, including *ES1*, *HES3* and *HES5*, and impairs NSC proliferation with premature neurogenesis and incomplete gliogenesis. In contrast, Math, Mash1 and neurogenin activate bHLH genes and drives neurogenesis. However, they also induce the expression of the Notch ligand Delta, which up-regulates HES1 and HES5 proteins to maintain cells in an undifferentiated state. In the developing CNS, a dynamic balance between these transcription factors is necessary to maintain cells in a differentiated or undifferentiated state.

1.6 ADAM Family

The members of the ADAM family are transmembrane-anchored protease, characterized by cleaving the extracellular domain of membrane-bound proteins and receptors. In developmental embryos, ADAMs plays a key role in cell-cell and cell-matrix interactions, cell adhesion as well as cell differentiation (Becherer and Blobel, 2003). So far 35 members of the ADAM family are identified in humans and vertebrates. In the Notch signaling, the ADAM can cleave the extracellular proteolysis site of the Notch receptor, resulting in activation of the Notch target genes. Dysregulation of ADAMs can induce diseases, for example, over-expression of ADAMs, such as ADAM9, ADAM12, and ADAM17, is associated with the breast cancer, and ADAM15 and ADAM10 with the prostate cancer. Abnormal ectodomain shedding by ADAMs can also lead to various disease, e.g., Alzheimer's disease (ADAM10), multiple-sclerosis (ADAM17), cardiovascular diseases (ADAM10 and ADAM17) (Weber and Saftig, 2012). Recently, a role of pharmaceutical inhibitors for ADAM17 in TNF-α mediated rheumatoid arthritis has been reported (kawaguchi and Hearing, 2011). In *in vitro* system, ADAM17 affects glial/neuronal cell fate decision during differentiation of NPCs (Romero Grimaldi et al., 2011).

1.7 Aim of the Study

The Wnt signaling pathway plays vital role in the developing CNS. Previous studies from our institute have shown that Wnt3a is involved in hNPC differentiation by regulating Wnt/β-catenin pathway (Mazemondet et al., 2011), spontaneous Ca^{2+} (Morgan et al., 2013) and HES5 amount (Hübner et al., 2010; Mußmann et al., 2014). Interestingly, Pyk2 activates STAT3 in HeLa cells (Shi and Kehrl, 2004) and HES5 regulates STAT3 activity in the differentiation of astrocytes in the CNS (Kamkura et al., 2004). Therefore, we hypothesized that Wnt3a may alternate the intracellular Ca^{2+} concentration, resulting in activation of Wnt/Ca^{2+}-pathway, which then activates the relative down-streams, e.g., CaMKII and Pyk2, and finally, affects neuronal differentiation (Fig. 8). Furthermore, since Pyk2 makes a co-immunoprecipitation together with GSK3-β, and regulates GSK3-β activity (Hartigan et al., 2001), we hypothesized that Pyk2 also interacts with Lrp6 and β-catenin, which contributes to the interaction between canonical and non-canonical Wnt signaling pathways (Fig. 8). Therefore, in the present study, 1) whether Wnt/Ca^{2+} pathway is involved in hNPC

differentiation; 2) whether the interaction between canonical and non-canonical Wnt signaling pathways in differentiating hNPCs is regulated by Wnt3a via Pyk2; and 3) whether the interaction between Wnt signaling and Notch signaling pathways is involved in hNPC differentiation, will be further investigated in order to understand how Wnt3a precisely regulates hNPC differentiation.

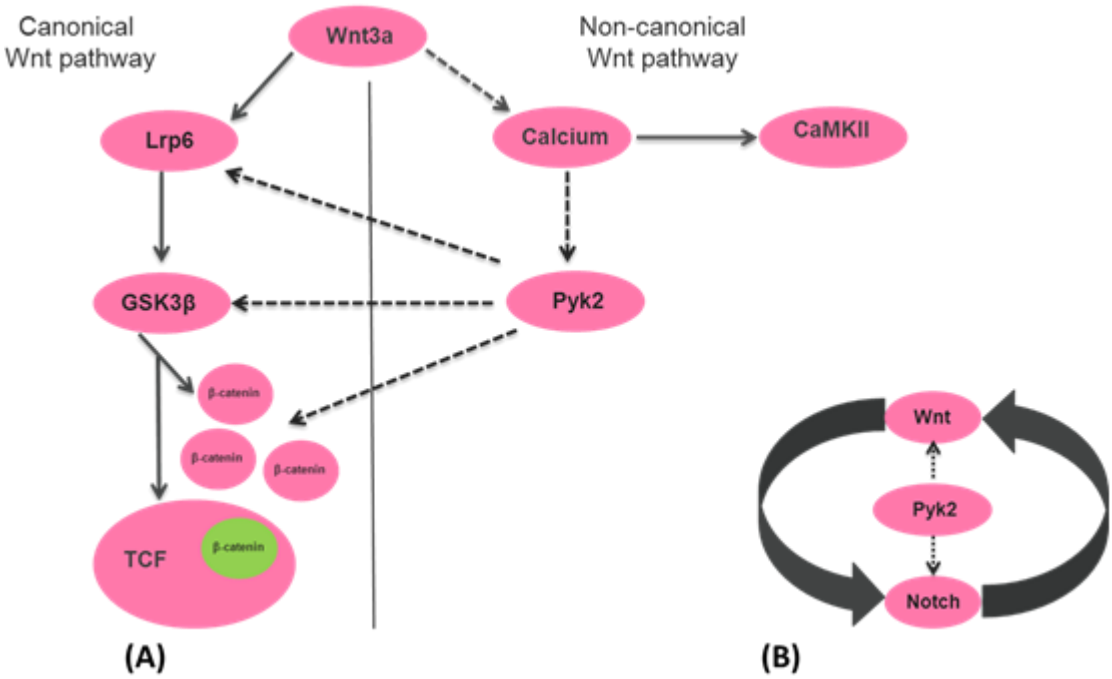

Figure 8: Hypothetical model to show interaction between canonical and non-canonical pathways via Pyk2 and Wnt-Pyk2-Notch signaling pathways induced by Wnt3a in the differentiating hNPCs.

(A): *Interaction between the canonical and non-canonical pathways via Pyk2. Wnt3a can induce both canonical and non-canonical pathways: in the canonical pathway, Wnt3a activates Lrp6, GSK3β and β-catenin. The stabilized β-catenin (shown in the green back ground) translocates into the nucleus to activate the target gene expression; in the non-canonical pathway, Wnt3a induces an increase of cytosolic* Ca^{2+} *concentration, which can activate CaMKII and Pyk2. Pyk2 may interact with other molecules of Lrp6, GSK3β and β-catenin.* ***(B):*** *Interaction between the Wnt pathway and Notch pathway through Pyk2. The dotted arrows indicate the proposed interactions and the solid arrows represent the known pathway.*

2 Materials and Methods

2.1 Materails

2.1.1 Cell Line

The ReNcell VM-197 cell line (hereafter as VM cell) supplied by the ReNeuron (Guildford, UK) is obtained from the midbrain of a ten-week old human fetus and immortalized by v-myc. VM cells are able to differentiate into neurons, astrocytes and oligodendrocytes (Donato et al., 2007, Hübner et al., 2010; Morgan et al., 2012) by in vitro system.

2.1.2 Cell Culture Media, Supplements, Buffers and Solutions

Cell culture	**Supplier (Composition)**
DMEM	Invitrogen
DMEM-F12	Invitrogen
Mouse laminin	Trevigen
B27	Invitrogen
EGF	Roche
bFGF	Roche
Heparin sodium salt	Sigma
Pencillin/Streptomycin	Sigma
Gentamycin	Invitrogen
L-Glutamine	Gibco
Poly-L-ornithine	Sigma
Poly-D-ornithine	Sigma
Trypsin/EDTA	Invitrogen
Trypsin/Benzonase	25 U/ml of Benzonase in Trypsin/EDTA
Trypsin-inhibitor	Sigma
Benzonase	Merck
Trypsin-inhibitor/Benzonase	1%HSA, 25U/ml Benzonase, 0.55mg/ml Trypsin inhibitor in DMEM/F12
Human Serum Albumin	Grifols
Normal Goat Serum (NGS)	Dako
DMSO	Sigma
Bovine Serum Albumin	Roth

Supplements	Supplier(Composition)
Recombinant Human Wnt3a	R & D Systems
PF-4618433	Snkinase

Buffers and Solutions	**Supplier (Composition)**
5X Lysis buffer	Promega
10x SDS Electrophoresis buffer	188mM Glycine+250mM Tris+3.5 mM SDS

SDS transfer buffer	39mM Glycine+48mM Tris+3.5mM SDS+20% ethanol
10x TBS	20mM Tris-HCl+150mM NaCl (pH 7.6)
TBST	TBS with 0.1% Tween-20
Blocking Solution	TBS with 5% BSA (Roth)

2.1.3 Antibodies

Antibodies used for Western blot (WB), immunocytochemistry (ICC) and FACS analysis were listed as following.

Primary Antibodies

Target	Type	Company	Application	Dilution
GAPDH	Mouse IgG_1	Abcam (ab8245)	WB	1:10000
GAPDH	Rabbit IgG_1	Santa Cruz (SC-335)	WB	1:1000
GSK3β	Mouse IgG_1	BD (610201)	WB	1: 3000
p-GSK3β (Ser9)	Rabbit polyclonal IgG	Cell Signaling (9336)	WB	1:2000
p-GSK3β (Tyr 216)	Rabbit polyclonal IgG	Santa Cruz (SC-9035)	WB	1:1500
β-catenin	Mouse IgG_1	Santa Cruz (SC-7963)	WB	1:1000
p-β-catenin (Ser33/37/Thr41)	Rabbit polyclonal IgG	Cell Signaling (9561)	WB	1:1000
LRP6	Rabbit polyclonal IgG	Cell Signaling (2560)	WB	1:1000
phospho-Lrp6 (Ser-1490)	Rabbit polyclonal IgG	Cell Signaling (2568)	WB	1:1000
CaMKII	Rabbit polyclonal IgG	Santa Cruz (SC-9035)	WB	1:1000
phospho-CaMKII (T216)	Mouse IgG1	Santa Cruz (SC-32289)	WB	1:250
Pyk2	Mouse monoclonal	Santa Cruz (SC-100379)	WB	1:200
phospho-Pyk2-(402)	Rabbit polyclonal IgG	Abcam (ab4800)	WB	1:250
ADAM10	Rabbit polyclonal IgG	Millipore (AB19026)	WB	1:1000
Tuji-1	Mouse IgG_1	Santa Cruz (SC-	FACS,	1:100

		51670)	ICC	1:500
Negative control	Normal mouse IgG	Santa Cruz (SC-2025)	FACS	1:100
Negative control	Normal rabbit IgG	Santa Cruz (SC-2027)	FACS	1:100

Secondary Antibodies

Target	Host	Conjugate	Company	Application	Dilution
rabbit IgG	goat	Alexa Flour 680	Invitrogen (A-21076)	WB	1:10000
rabbit IgG	goat	IRDye 800	Rockland (61-131-122)	WB	1:10000
mouse IgG	goat	Alexa Flour 680	Invitrogen (A-21057)	WB	1:10000
mouse IgG	goat	IRDye 800	Rockland (610-131-003)	WB	1:10000
rabbit IgG	goat	Alexa Flour 647	Invitrogen (A-21245)	FACS, ICC	1:1000
mouse IgG	goat	Alexa Flour 488	Invitrogen (A-11029)	FACS, ICC	1:1000

2.1.4 Technical Equipment and Kits

Technical Equipments

Name	Type	Supplier
Cell counter	CASY	Innovatis
Incubator	WTC	Binder
Sterile work bench	Antares	Sterile

Microscopy	Type	Supplier
Cell culture microscope	Eclipse TS100	Nikon
Fluorescence microscope	Biozero	Keyence
Fluorescence microscope	Eclipse TS102	Nikon

Electrophoresis	Type	Supplier
SDS-PAGE chamber	Criterion	Bio-Rad
Semi-dry-transfer chamber	Trans-Blot SD	Bio-Rad
Power-supplier	PowerPacHC	Bio-Rad

Centrifuges	Type	Supplier
Centriguge	Z383K	Hermle
Centrifuge	Z233MK-2	Hermle
Centrifuge	Universal 30 RF	Hettich

Centrifuge	Avanti J-25	Beckman Coulter

Miscellaneous	**Type**	**Supplier**
Vortexer	MS1	IKA
Spectrophotometer	Ultraspec3100pro	Amersham
Shaker	KM-2Akku	Edmund Bühler
Shaker	K2-50	Noctuna
Shaker	Titramax-100	Heidolph
Real-time PCR cycler	LightCyclernano1.0	Roche
Plate reader	Magellan	Tecan
Pipets	Reference	Eppendorf
pH meter		Mettler Toledo
Nucleofactor	NucleofectorII	Amaxa
FACS	FACSCalibur	Becton Dickenson
Balance	MCBA100	Sartorius
Heatingblock	Thermomixer	Eppendorf

Kits

Name	**Cat. Number**	**Supplier**
BCA Protein Assay Kit	23228	Pierce
FastLane Cell cDNA Kit	215011	Qiagen

2.1.5 Oligonucleotide Primers for Quantitative Real-Time PCR

Gene	**Annealing (°C)**	**Amplicon length (bp)/ Melting temp.(°C)**	**Primer sequence 5'-3' (forward and reverse)**	**Reference**
hG6PD	55°C	191/88	F: atcgaccactacctgggcaa R: ttctgcatcacgtcccgga	Hübner et al., 2010
hHES5	55°C	232/90	F: tcagccccaaagagaaaaac R: tagtcctggtgcaggctctt	Chen et al., 2006
hHES1	60°C	100/88	Property	Qiagen Cat no: QT00039648

2.2 Methods

2.2.1 Cultivation of Human Neuronal Progenitor Cells

For cell proliferation, VM cells were placed in a T-75 flask coated with 6 ml laminin (1:100 with ice-cold DMEM F12 for minimum 1 h) at 37°C under the condition with 5% CO_2 and 20% O_2. After 70-80% confluence in the proliferation media, cells were washed with 15 ml pre-warmed HBSS (37°C) and detached by adding 2.5 ml Trypsin/Benzonase, followed by 5 ml Trypsin-inhibitor/Benzonase solution for stopping reaction. Then cells were collected by centrifuged at room temperature for 5 min at 1500xg and resuspended with 5 ml warm medium for cell counting. For cell differentiation, 15 ml media without growth factors was added to the washed cells for incubation at 37°C under the condition with 5% CO2 and 20% O2 and cells were collected at different time points as wanted.

2.2.2 Cell Number Measurement and Seeding

CASY cell counter (Innovatis, Reutlingen, Germany) was used to count the cell number. After 25 µl of cell suspension was added to 10 ml CASY ton, the cell number was measured with an appropriate program and the numbers of the cells seeded in different types of plates were shown in the Table 1.

Table 1. The number of cells seeded in different types of plates.

Cell line		Type of plate	Surface area per well (cm^2)	Cell number/well	Proliferation time
VM cells		4-Well	2	50,000	48h
VM cells		6-Well	25	250,000	48h
VM cells		24-Well	2	50,000	48h

2.2.3 Protein Analyses

2.2.3.1 Preparation of Cell Lysates and Protein Measurement

Cells were washed with 1x PBS and were harvested on ice with 1x Lysis buffer (Promega, USA), containing phosphatase and protease inhibitors cocktails (Roche) for 15 min on shaking. Then cell solution was centrifuged at 4°C for 15 min at 15000x g and the supernatant was collected and stored at -80°C. Total protein

concentration was measured by bicinchoninic acid assay (BCA, Pierce, Rockford, IL, USA) according to manufacturer's guidelines using a plate reader (Tecan GENios, USA). Samples diluted with 5x sample buffer (Thermoscientific, Rockford, USA) were boiled for 5 min at 95 °C for Western blotting.

2.2.3.2 Western Blot Analysis

Equal amount of proteins were loaded in a vertical Tris-HCl gel with 4-15 % acrylamide concentration gradient (Criterion Precast, Bio-Rad) and run in a Criterion Cell (Bio-Rad) at 100-200 V in ice-cold water bath for about 2 h. PeqGOLD marker IV (PEQLAB, Erlangen, Germany) was used as the protein marker. Then proteins within the gel were transferred to nitrocellulose membranes (Hybond-ECL, Amersham) by a semi-dry blotting system (Trans-BlotSD, Bio-Rad) at 0.10 A per membrane for 2 h. The membranes were soaked in the blocking solution (5 % BSA or 5 % milk in TBST) for 2 h, followed by overnight incubation with primary antibodies at 4 °C on a shaker. Then membranes were washed for 5 min with TBST for 3 times and incubated in dark with suitable fluorescent secondary antibodies for 1.5 h. Thereafter, the membranes were washed 3 times for 5 min with TBST and air-dried in dark. Odyssey Infrared Imaging System (LI-COR Biosciences GmbH, Bad Homburg, Germany) was used to image and quantify the desired proteins. The membranes were scanned at a wavelength of 700 nm and 800nm for Alexa Fluor 680-labeled antibodies and IRDye 800CW-labeled antibodies, respectively. GAPDH was used as a loading control and the desired proteins were normalized to GAPDH for a relative expression level. Odyssey software version 1.4 was used to analyze the data.

2.2.3.3 FACS Analysis

Fluorescence-activated cell sorting (FACS) is used to analyze neurogenesis using neuronal marker β-tubulin in heterogeneous mixture of cell populations. After cultured in six-well plates were washed with HBSS, cells were subsequently treated with trypsin inhibitor/benzonase and trypsin/benzonase solutions. Then cells were fixed at room temperature in 1 % PFA in PBS for 20 min. After centrifugation at 1500x g at 4 °C for 10 min, the pellet was suspended in 500 µl of FACS wash buffer and stored at 4°C. For cell staining, the cells were centrifuged for 5 min at 4 °C at 2000x g. Wash buffer was removed and 25 µl of saponin buffer containing the

primary antibody were added to the pellet and incubated at room temperature on a shaker for 2 h. Cells added with normal mouse and normal rabbit IgG were used as negative controls. After 2 h cells were washed twice with 300 µl of saponin buffer and resuspended in 25 µl saponin buffer with appropriate secondary antibodies. Samples were incubated for 1 h at room temperature in dark with mild shaking. Finally cells were washed two times with 300 µl saponin buffer and centrifuged, followed by resuspended in 500 µl of FACS wash buffer and stored at 4°C in dark until the further analysis. Using FACS calibur (Becton Dickinson, San Jose, USA) in a combination with CellQuest Pro software, the samples (50,000 cells per sample) were counted under the same condition and the data were analyzed after aggregated cell and debris were filtered out.

2.2.3.4 Immunocytochemistry

Cells cultured on coverslips were fixed with 4 % PFA in PBS for 20 min at room temperature. After washed with PBS, the cells were blocked with 5% goat serum in PBS for 30 min and incubated overnight with primary antibody, containing 1% goat serum in PBS at 4 °C. On the next day, the cells were washed 3 times with PBS and incubated in dark with a secondary antibody for 1 h at room temperature. Finally, nuclear staining was performed with DAPI solution for 5 min. The coverslips were embedded carefully with mowiol, and stored at 4 °C in the dark until analysis. A control staining of the cells was performed with PBS solution instead of primary antibodies to check the background. Fluorescent images were generated using a fluorescent microscope (Bioxx-8000, Keyence, Germany).

Neurite length was measured in the images of immunocytochemistry using antibody against β-III tubulin - a specific neuronal marker - using the Keyence software according to the manufacture's guidance. The Images (each area size with 593 μm^2) were randomly chosen for quantification and at least 5 images were used for one independent experiment.

2.2.4 Intracellular Ca^{2+} Measurement

Intracellular Ca^{2+} was measured by using Ca^{2+} sensitive fluorescent dye Fura-2/AM (Invitrogen, Germany). The binding of Ca^{2+} to Fura-2/AM results in a change of the emission and excitation wave lengths from 340 nm to 380 nm and the ratio of this

shift was used to calculate the cytosolic Ca^{2+} concentration (Grynkiewicz et al. 1985). The exact determination of intracellular Ca^{2+} concentration depends on the K_d value of the dye, which is affected by temperature, pH, viscosity and the presence of other divalent ions especially Mg^{2+}. For Ca2+ measurement, cells growing on coverslips were incubated with Fura-2/AM with a final concentration of 5µM for 30 min at room temperature, and then washed three times with the extracellular solution, followed by a further incubation for 30 min to allow the binding of Ca^{2+} to Fura-2. The imaging was performed under the same perimeters using the image system of TillVision (v4.0, Till Photonics, Germany) with an illumination provider (Polychrome V, Till Photonics). The excitation wavelength can be shifted from Fura-2 with 340 nm to Ca^{2+}-Fura2 binding with 380 nm, and the exposure time was naturally 15 microseconds with adjusted depending on the signal intensity. Images were attained at 1Hz over a period of 1000 seconds. Recordings were made from the somas of the cells of interest by manual chosen. Ratio-metric movies were created off-line and the background subtraction was done per frame based on a specific region of the image containing no cells. This experiment was supported with the help of Dr. Peter Morgan.

2.2.5 Molecular Biological Methods

2.2.5.1 cDNA Synthesis

Fast Lane cDNA kit (Qiagen, USA) was used to generate cDNA, fallowing the manufacturer's protocol. In brief, cells were grown in plates with 4 or 24 wells and washed with 200µl of FCW buffer. After incubation with 80µl of FCP buffer for 10 min, genomic DNA was eliminated and cell lysates were collected for RNA synthesis. Template RNA was added to the reverse transcription master mix and cDNA synthesis was performed according to the manufacturer's guide lines. The synthesised cDNA sample was stored at -20°C for the further usage.

2.2.5.2 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed using the LightCycler Nano 1.0 (Roche) instrument with FastStart Essential DNA Green Master Mix (Roche) according to the guidelines of the manufacture. Primer information can be found in the section 2.1.5 and the efficiency

of the primers were calculated by plotting C_q values vs cDNA amounts of the serial diluted positive control as human total brain cDNA (Clontech). 1µl of cDNA, which was generated using Fast Lane cDNA kit (Qiagen) was used as the template. In the experiment, all the samples were run as triplicates and the cycling parameters can be found in the Table 2. Delta-delta C_t method (Pfaffl et al., 2000) was used to calculate the relative changes of the amount of mRNA. Desired, interested mRNA amount was normalized to the house keeping gene G6PD (Glucose-6-phosphate-dehydrogenase) and the relative changes were calculated in compared to the control.

Table 2. Cycling parameters used in quantitative real-time PCR

Step	Temparature (°C)	Time
Denaturation	95	10 min
Denaturation	95	20 sec
Annealing	See 2.1.5	20 sec
Extension	72	23 sec
Melting curve	60-95	

3 Results

3.1 Wnt3a Stabilize β-Catenin Only at Early Time Points

After cultured VM cells reached 80% confluence in the proliferation differentiation of VM cells was performed by the withdrawal of growth factors from the proliferation media and accompanied by adding Wnt3a (100ng/ml) to the medium.

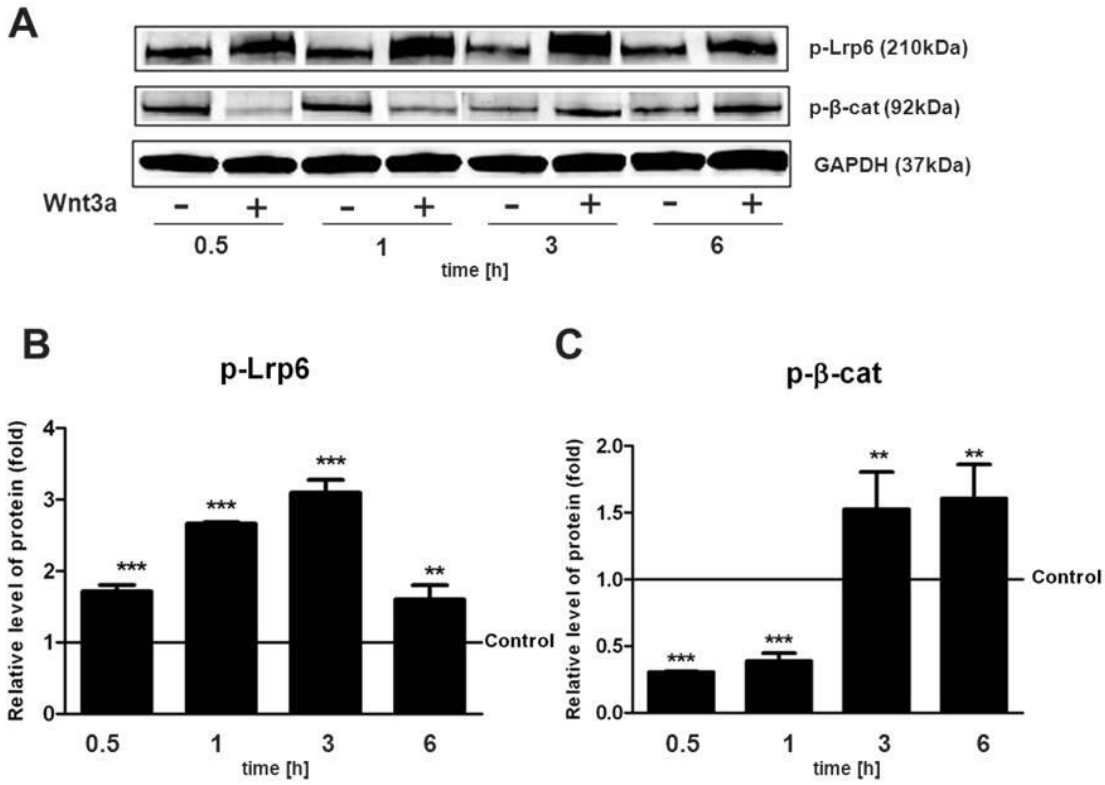

Figure 9: Wnt3a does not stabilize β-catenin at certain time points during VM cell differentiation.

Western blot analyses of phosphorylated Lrp6 at serine 1490 position (p-Lrp6) and phosphorylated β-catenin at serine 33/37/Thr47 position (p-β-cat) in differentiating VM cells at defined time points under stimulation with Wnt3a (100ng/ml). ***(A):*** *Western blot bands of p-Lrp6, p-β-catenin from cultured cell lysates treated with Wnt3a and HSA and harvested at 0.5h, 1h, 3h and 6h after differentiation. HSA serves as the control.* ***(B):*** *Quantified Western blot analyses by Odyssey Software for p-Lrp6 and p-β-catenin. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by black lines. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

Then the cells were harvested at the defined time points to investigate by Western blot analyses for target proteins, such as phosphorylated Lrp6 at Serine 1490 position (hereafter as p-Lrp6) and phosphorylated β-catenin at Ser-33/37/Thr 41 (hereafter as p-β-catenin). Results showed that under stimulation of Wnt3a, the

amount of p-Lrp6 in VM cells was increased in the defined time points from 0.5h onwards (Fig. 9A, B), confirming that Wnt3a can phosphorylate Lrp6 (Mazemondet et al., 2011).

However, stabilization of β-catenin, revealed by the decrease of p-β-catenin, was only observed at time points of 0.5h and 1h, but not at 3h and 6h (Fig. 9A, C). Therefore, it gave rise to the question why β-catenin was not stabilized at 3h and 6h points even under stimulation of Wnt3a with a higher amount of p-Lrp6.

3.2 Wnt3a Triggers Changes of Intracellular Ca^{2+}

Wnt3a can activate non-canonical pathways in some types of cells, e.g., articular chondrocytes (Nalesso et al., 2011), mesenchymal stem cells (Qu et al., 2013), and PC3 cells (Thrasivoulou et al., 2013). Previous study has shown that the spontaneous and transient Ca^{2+} signal was observed in VM cells (Morgan et al., 2013). Therefore, in the present study the effect of Wnt3a on intracellular Ca^{2+} concentration was investigated using Fura-2/AM-based method.

The results showed that in the condition with the extracellular medium containing Ca^{2+} ion (2 mM), Ca^{2+} transients were observed strongly in some cells treated with Wnt3a (Fig. 10B, D), but very weak in the cells treated with HSA, which serves as the control (Fig. 10A, C). Furthermore, when without Ca^{2+} ion in the medium, the Ca^{2+} transients were also observed under stimulation with Wnt3a, suggesting that Wnt3a can also trigger Ca^{2+} release from the intracellular organelles (Fig. 10E). This point was further identified by adding thapsigargin to medium, which discharges the Ca^{2+} from the intracellular organelles, especially endoplasmic reticulum (Thrasivoulou et al., 2013). When thapsigargin was added first into the medium and triggered Ca^{2+} release from the intracellular organelles, then the Ca^{2+} transients triggered by Ca^{2+} release from the intracellular pools under stimulation with Wnt3a was deleted (Fig. 10F). Taken together, these results showed that Wnt3a can increase the cytosolic Ca^{2+} concentration by influx of Ca^{2+} from extracellular area and release from intracellular organelles, suggesting that Wnt3a may induce Wnt/Ca^{2+}-pathway in the differentiating VM cells.

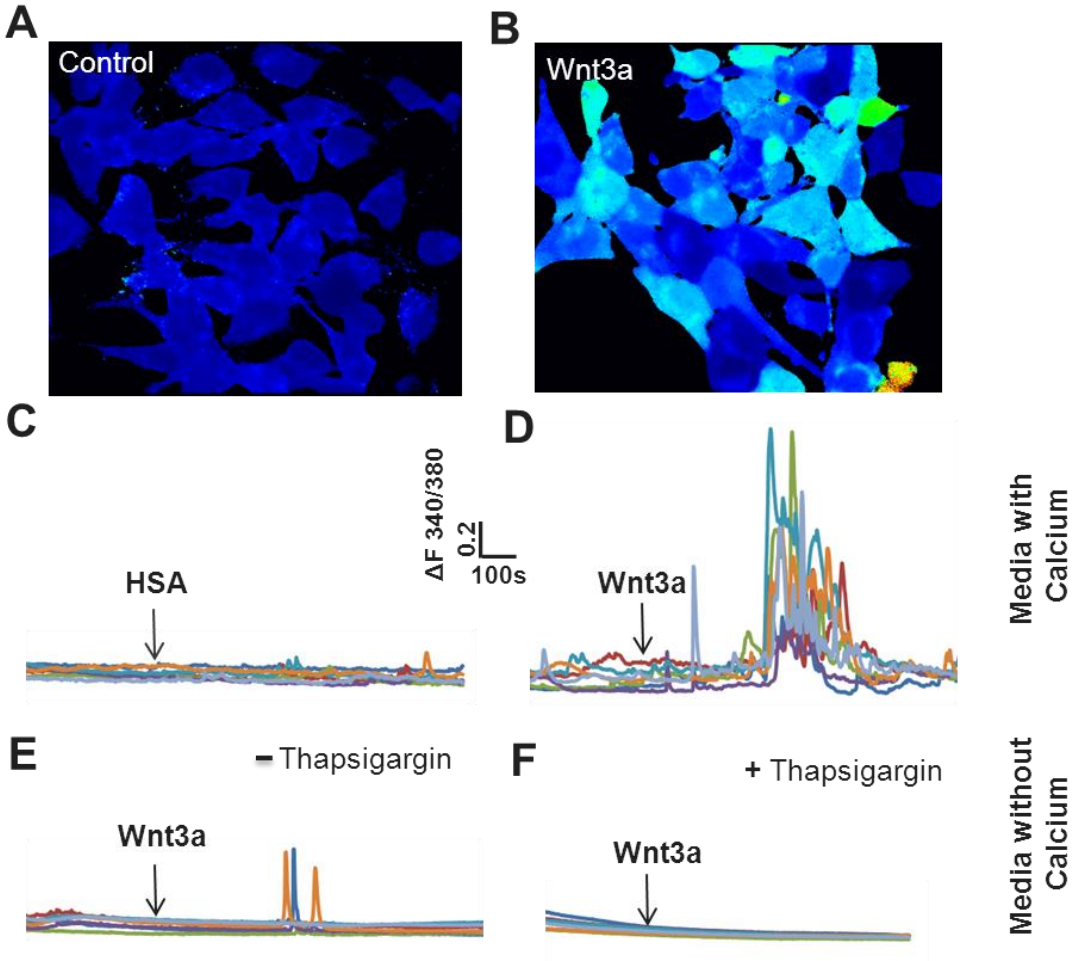

Figure 10: Wnt3a increases intracellular calcium concentration by calcium influx from medium and release from intracellular organelles.
(A-D): *Changes of Ca^{2+} transients are observed in VM cells by Fura-2/AM-based fluorescent method, where blue color indicates the low level of cytosolic calcium and yellow/green color the high level. When the extracellular medium contains calcium ion (2 mM), Wnt3a strongly induces the Ca^{2+} transients in cells indicated by yellow/green color (B) and peaks (D), but very weak in the control with HSA (A, C). The arrows indicate the time point to apply HSA and Wnt3a.* ***(E, F):*** *When the extracellular medium does not contain any calcium ion, the Ca^{2+} transients were observed in some Wnt3a treated cells in the absent with thapsigargin (E), but not in the first present with thapsigargin (F) in the medium.*

3.2.1 Wnt3a Regulates Calcium/Calmodulin Dependent Protein Kinase II

An increase of cytosolic Ca^{2+} concentration can induce a release of a Ca^{2+}-binding protein calmodulin (CaM), which subsequently binds to the calcium/calmodulin dependent protein kinase II (CaMKII), resulting in CaMKII activation via autophosphorylation at threonine-286 (T-286) position (Bossuyt and Bers, 2013). Therefore, CaMKII phosphorylation was further investigated under stimulation with

Wnt3a (100ng/ml) in differentiating VM cells. Western blot analyses showed that the amount of the total CaMKII were increased in all defined time points (Fig. 11A, B), while an increase of p-CaMKII only at 0.5h, but a decrease at 1h and 3h time points when compared to the control (Fig. 11A, B), suggesting that Wnt3a increased amount of CaMKII via the increase of cytosolic Ca^{2+} concentration.

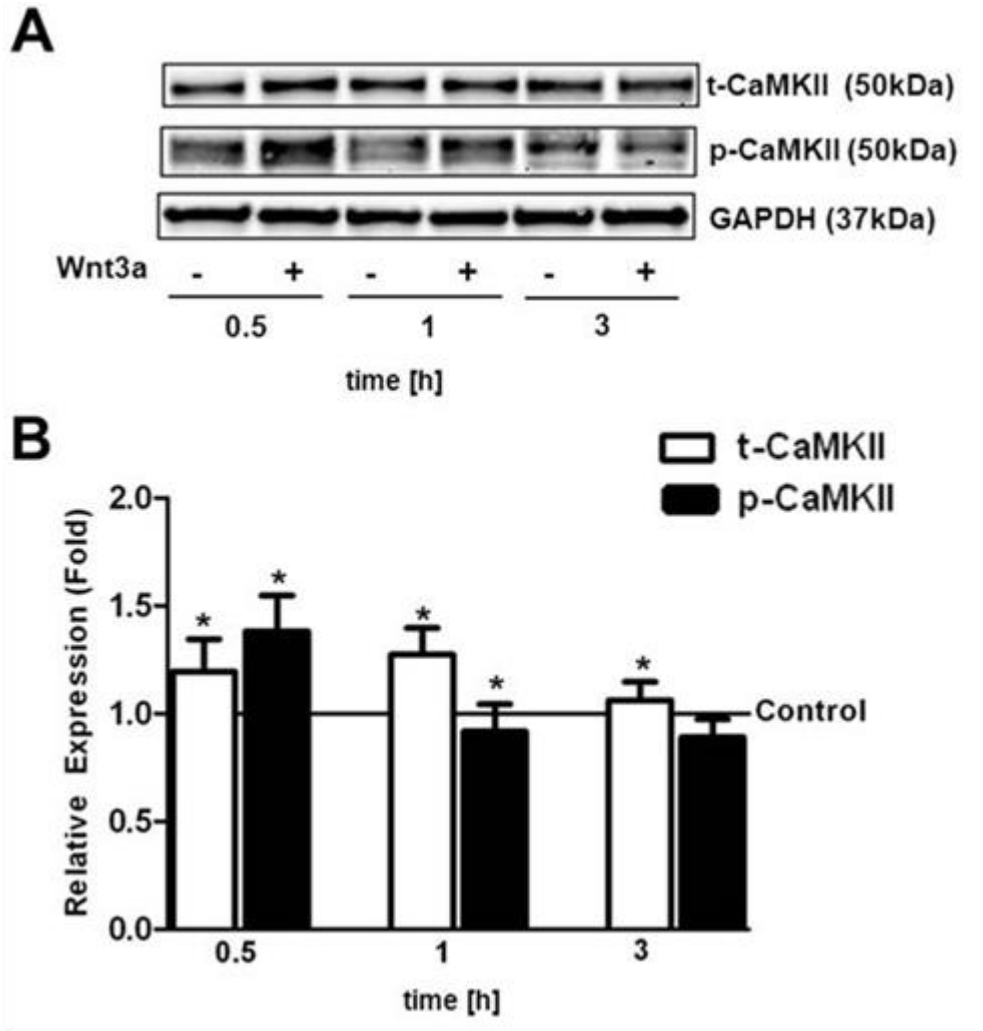

Figure 11: Wnt3a regulates calmodulin dependent protein kinase II in differentiating VM cells. *Western blot analyses of total CaMKII (t-CaMKII) and phosphorylated CaMKII at threonine 286 position (p-CaMKII) in differentiating VM cells at defined time points under stimulation with Wnt3a (100ng/ml).* ***(A):*** *Western blot bands of t-CaMKII and p-CaMKII from cultured cell lysates as well as the control using HSA at 0.5h, 1h, and 3h after differentiation.* ***(B)****: Quantified Western blot analyses by Odyssey Software for t-CaMKII and p-CaMKII. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

3.2.2 Wnt3a Triggers Phosphorylation of Proline-Rich Tyrosine Kinase 2

Phosphorylation of Pyk2 at Tyr-402 (p-Pyk2), which is the major phosphorylation site for the initiation of Pyk2 kinase activity, is regulated by Ca^{2+} concentration (Sharma and Kinsey, 2013). Therefore, we continued to investigate whether Wnt3a activates Pyk2 phosphorylation. After cultured under stimulation with Wnt3a (100ng/ml), VM cells were harvested at defined points and Western blot analyses was performed.

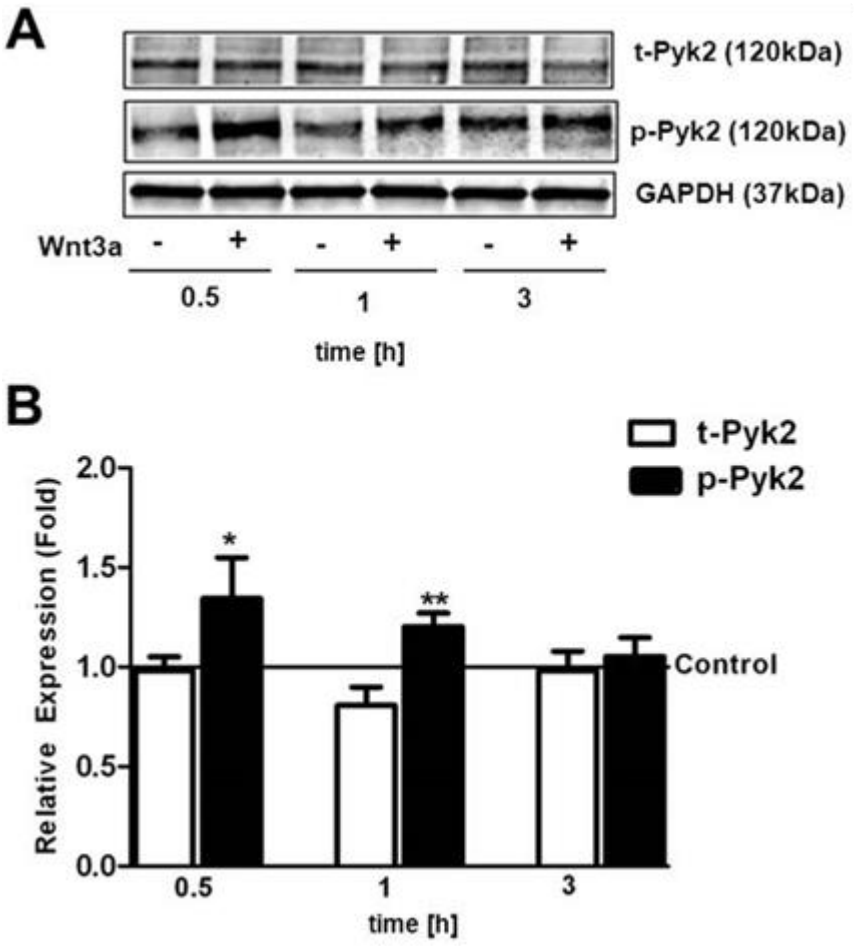

Figure 12: Wnt3a triggers Pyk2 phosphorylation during VM cell differentiation.
Western blot analyses of total Pyk2 (t-Pyk2) and phosphorylation of Pyk2 at tyrosine 402 position (p-Pyk2) during VM cell differentiation at defined time points under stimulation with Wnt3a (100ng/ml). ***(A):*** *Western blot bands of t-Pyk2 and p-Pyk2 from cultured cell lysates as well as the control using HSA at 0.5h, 1h, and 3h after differentiation.* ***(B):*** *Quantified Western blot analyses by Odyssey Software for t-Pyk2 and p-Pyk2. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

Results showed that the total amount of Pyk2 (t-Pyk2) was not changed at different time points, but the amount of p-Pyk2 significantly increased at 0.5h and 1h when compared to the control, suggesting that Wnt3a triggers Pyk2 phosphorylation in the differentiating VM cells.

3.3 Inhibition of Endogenous Pyk2 and Exogenous Overexpression of Pyk2

Next step, a novel Pyk2 specific inhibitor PF-4618433 (hereafter as PF-461; Han, et al., 2009, Lipinski et al., 2010) was chosen for inhibition of endogenous Pyk2 in the experiments. In order to find out at which concentration PF-461 works efficiently on Pyk2 function in VM cells, different concentration of PF-461 was screened in the cultured cell system. Cell survival results by CASY assay showed that the viability of cultured cells at 6h under the stimulation with PF-461 at concentration of 0.5 µM was the highest (Fig. 13A, B). Furthermore, Western blot analyses showed that both p-Pyk2 and t-Pyk2 were decreased at 0.5 and 3h under stimulation with PF-461 at concentration of 0.5 µM (Fig. 13C), suggesting that PF-461 specifically and efficiently inhibited Pyk2 in differentiating VM cells. Therefore, the concentration of 0.5 µM PF-461 was selected as an optimal dose for the experiments for the inhibition of endogenous Pyk2.

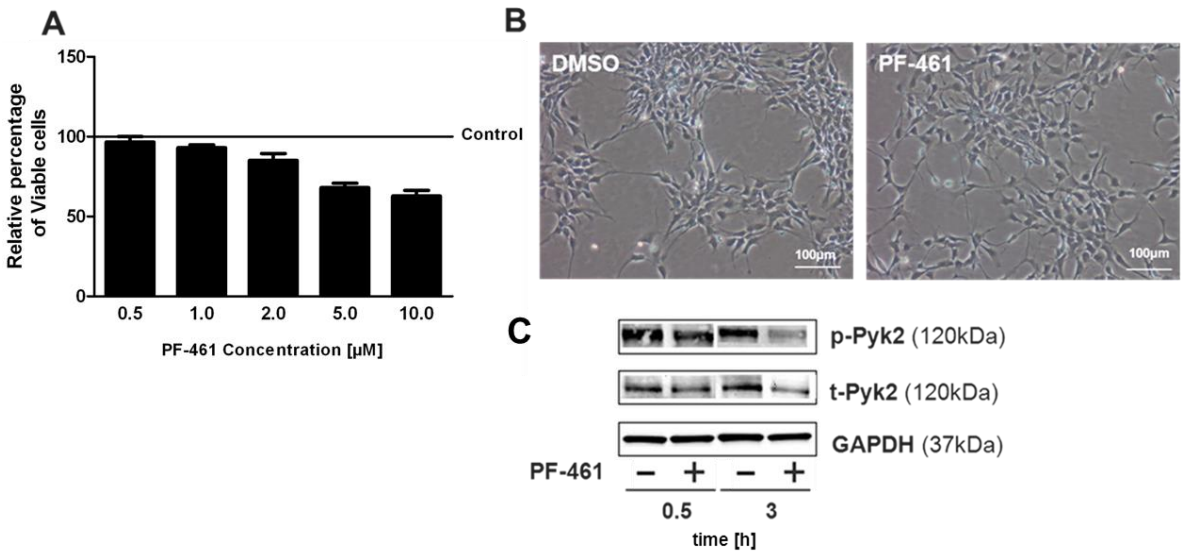

Figure 13: Effect of Pyk2 inhibitor PF-461 on Pyk2 in differentiating VM cells.
(A): *The cell viability was evaluated under stimulation with Pyk2 inhibitor PF-461 at different concentration as indicated in A as well as the control DMSO at 6h after cell differentiation using CASY assay. Relative percentages of the viability were calculated by normalizing the PF-461 treated cells to the control DMSO treated cells and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line.* ***(B):*** *Phase contrast images of differentiating cells treated with DMSO and PF-461 at 3h, respectively. Scale bar: 100 µm.* ***(C):*** *Western blot analyses of the decrease of p-Pyk2 and t-pPyk2 under stimulation with PF-461 (+), when compared to the control (-).*

In collaboration with the Institute for Experimental Gene Therapy und Tumour Research from University of Rostock, Pyk2 overexpression plasmid pWPXL+PTK2B was constructed (Fig. 14A) according the method described by others (Salmon and Trono, 2007; Alla et al., 2012) and stabilized Pyk2-overexpressing VM cells was obtained. By Western blot, the amount of Pyk2 was strongly increased in the Pyk2-overexpression cells (Pyk2-o/e; Fig. 14C) when compared to the control (Fig. 14C), suggesting that the constructed stabilized Pyk2-overexpressing cells can further used as exogenous overexpression of Pyk2 in the VM cells.

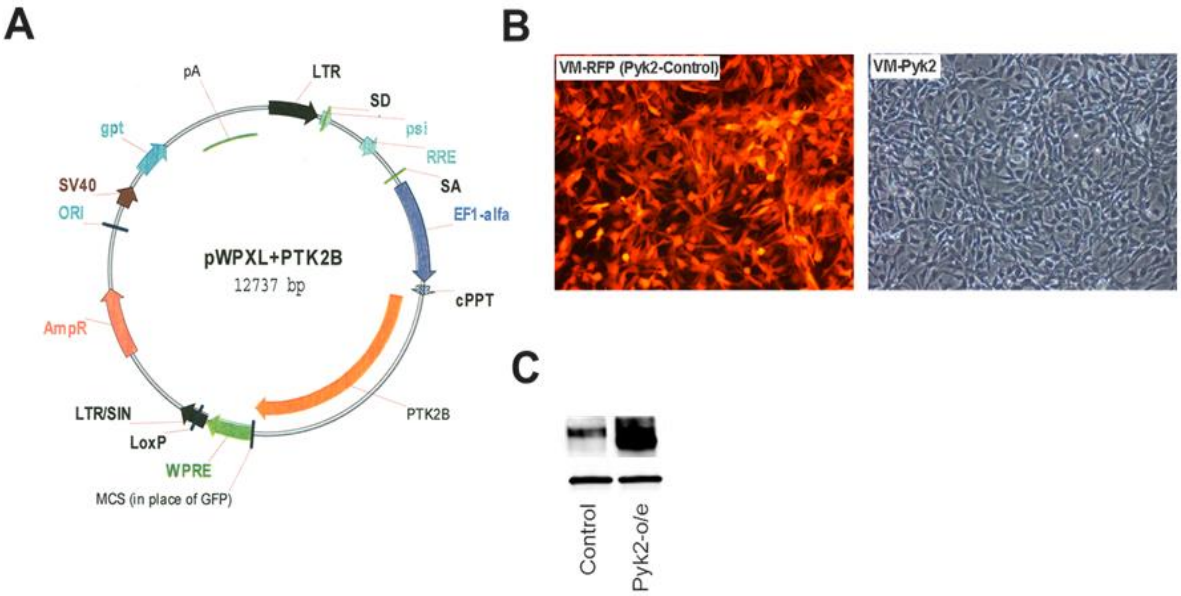

Figure 14: Pyk2-overexpressing plasmid map and Western blot analysis of Pyk2 amount in Pyk2-stabilized expression VM cells.
(A): *Plasmid map of pWPXL+PTK2B with an insertion of Pyk2 (PTK2B) in the lentiviral vector pWPXL.* ***(B):*** *The image under red-fluorescence (VM-RFP) of the control stabilized RFP expressing VM cells (red) and the phase contrast image of the stabilized Pyk2-overexpressing cells (VM-Pyk2) at 48 h in proliferating state.* ***(C):*** *Western blot analysis confirms that Pyk2 is strongly increased in the Pyk2-stabilized expression VM cells (Pyk2-o/e) when compared to the control.*

3.4 Effect of Pyk2 on Lrp6 Phosphorylation

Phosphorylated Lrp6 plays an important role in triggering Wnt/β-catenin dependent pathway. GSK3β regulates phosphorylation of Lrp6 and β-catenin (Zeng et al., 2005) and proline directed kinases regulates Lrp6 phosphorylation (Niehrs and Shen, 2010). Pyk2 is a member of the proline rich tyrosine kinase family, and therefore, it is interesting to investigate whether Pyk2 plays a role in the regulation of Wnt/β-catenin dependent pathway.

Therefore, in the present study, the effects of Pyk2 on Lrp6 phosphorylation were investigated using Pyk2-inhibitor PF-461 for Pyk2 down-regulation and stabilized Pyk2-overexpressing cells for Pyk2 exogenous overexpression (Pyk2 in Fig. 15C, D), respectively. After cells were treated in the different experimental groups with Wnt3a (100 ng/ml), PF-461 (0.5 µM), Pyk2-overexpression, and Wnt3a plus PF-461 or Pyk2-overexpression, the cultured VM cells were harvested at defined time points after differentiation. The results demonstrated that Wnt3a increased p-Lrp6 significantly at the defined time points (Fig. 15A-D). However, PF-461 and

overexpression of Pyk2 had no effect on Lrp6 (Fig. 15A-D), suggesting that Pyk2 has no effect on Lrp6 phosphorylation.

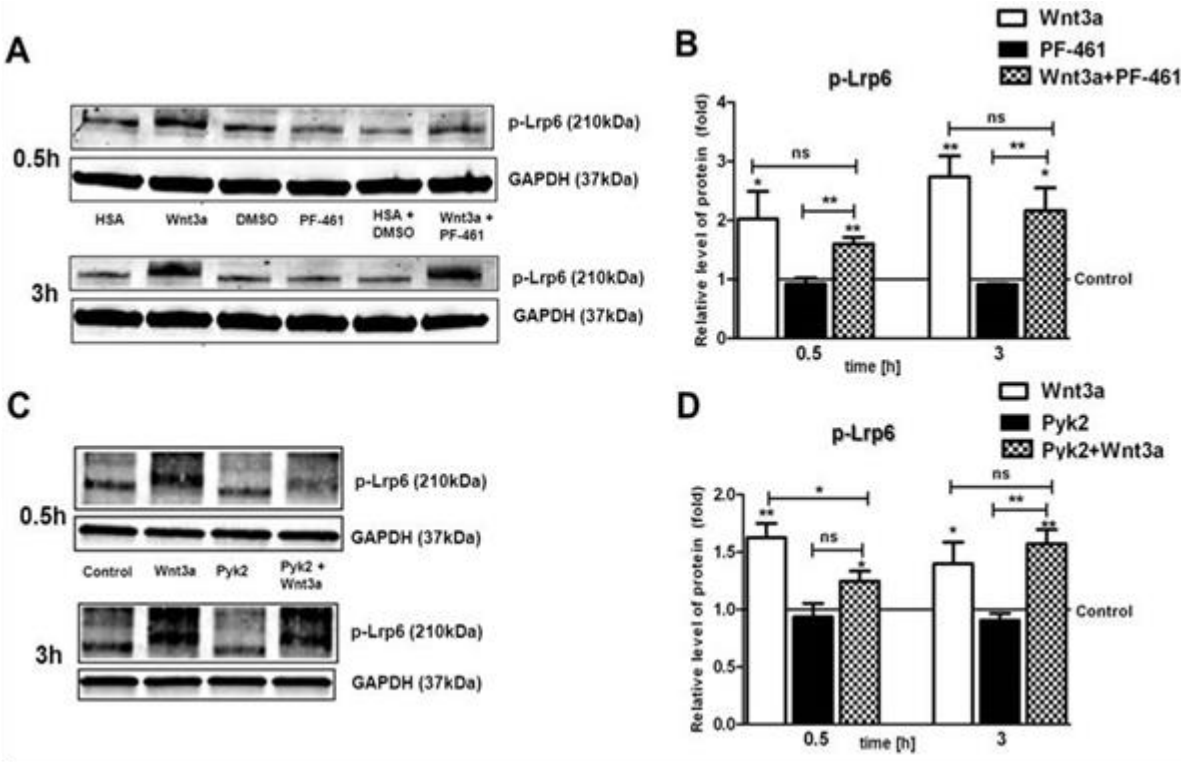

Figure 15: Effect of Pyk2 on Lrp6 phosphorylation in differentiating VM cells.
VM cells were treated with Wnt3a (100 ng/ml), PF-461 (0.5 µM), and Wnt3a plus PF-461 or in Pyk2-overexpression cells, respectively, and then harvested at 0.5h and 3h after differentiation. HSA and DMSO were used as controls for Wnt3a and PF-461, respectively. ***(A, B):*** *Western blot bands of p-Lrp6 (A) and quantified Western blot analyses by Odyssey Software for p-Lrp6 (B) in the control and treatment groups under stimulation with Wnt3a and/or PF-461 at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's-T-Test: *p< 0.05, **p< 0.01, ***p< 0.01.* ***(C, D):*** *Western blot bands of p-Lrp6 (C) and quantified Western blot analyses by Odyssey Software for p-Lrp6 (D) in distinct control and treatment groups with Wnt3a and Pyk2-overexpression cells (Pyk2) at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

3.5 Effect of Pyk2 on GSK3β Phosphorylation

GSK3β phosphorylation is a key step in the Wnt/β-catenin dependent pathway. However, the mechanism of regulating GSK3β phosphorylation is not totally clear (Taelman et al., 2010). Pyk2 can regulate GSK3β function (Sayas et al., 2006) and therefore, the effect of Pyk2 on GSK3β phosphorylation was investigated in differentiating VM cells. In the present study, the effects of Pyk2 on GSK3β phosphorylation were investigated using Pyk2-inhibitor PF-461 for Pyk2 down-

regulation and stabilized Pyk2-overexpressing cells for Pyk2 exogenous overexpression (Pyk2 in Fig. 16C-F), respectively. After the cells were treated in the different experimental groups with Wnt3a (100 ng/ml), PF-461 (0.5µM), Pyk2-overexpression, and Wnt3a plus PF-461 or Pyk2-overexpression, the cultured VM cells were harvested at defined time points after differentiation.

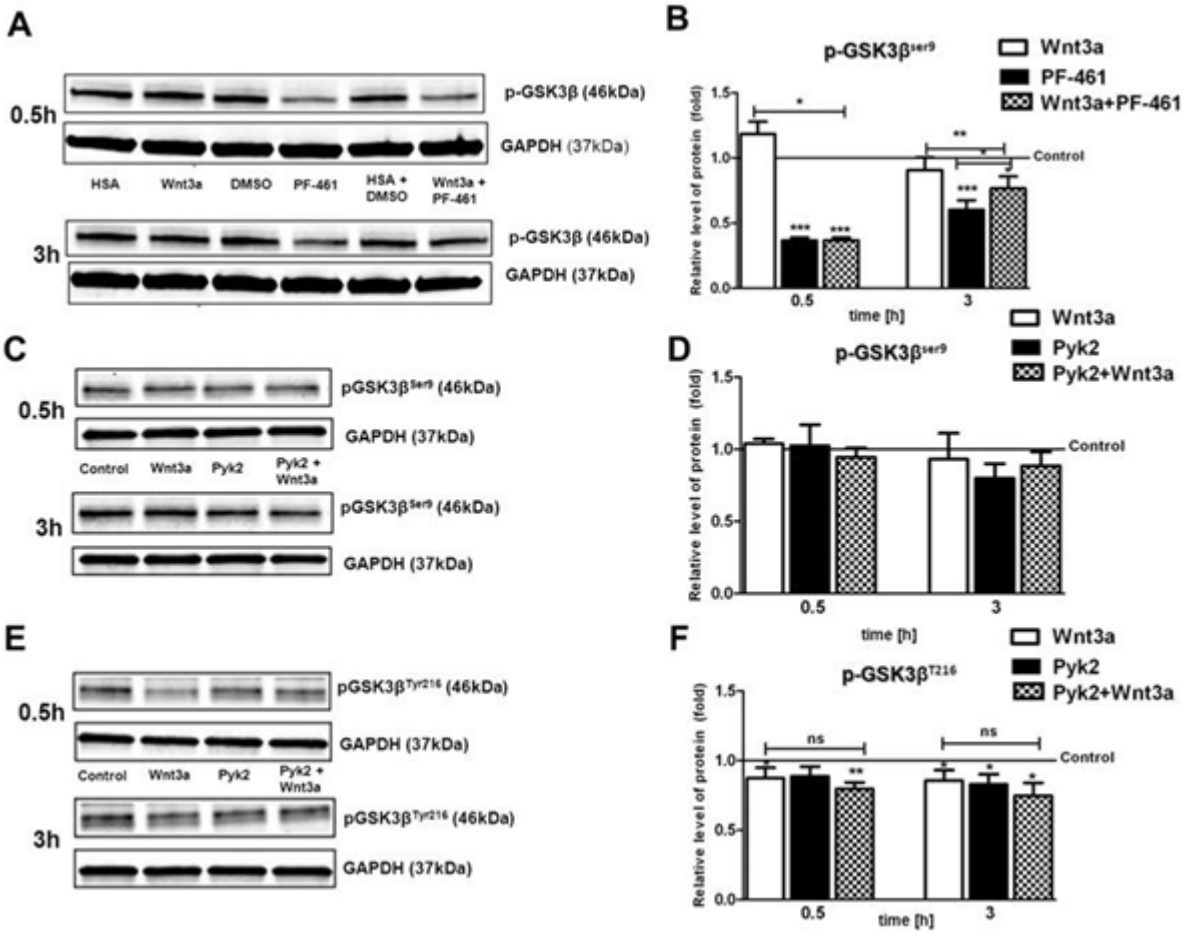

Figure 16: Figure 16: Effect of Pyk2 on GSK3β phosphorylation in differentiating VM cells.
VM cells were treated with Wnt3a (100 ng/ml), PF-461 (0.5 µM), Wnt3a plus PF-461 or in Pyk2-overexpression cells, respectively, and then harvested at 0.5h and 3h after differentiation. HSA and DMSO were used as controls for Wnt3a and PF-461, respectively. ***(A, B)****: Western blot bands of p-GSK3β^{Ser9} (A) and quantified Western blot analyses by Odyssey Software for p-GSK3β^{Ser9} (B) in the control and treatment groups under stimulation with Wnt3a and/or PF-461 at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's-T-Test: *p< 0.05, **p< 0.01, ***p< 0.01.* ***(C-F)****: Western blot bands of p-GSK3β^{Ser9} (C) and p-GSK3β^{T216} (E), quantified Western blot analyses by Odyssey Software for p-GSK3β^{Ser9} (D) and p-GSK3β^{T216} (F) in the control and treatment groups with Wnt3a and/or in Pyk2-overexpression cells at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

Experimental results demonstrated that at both 0.5h and 3h after cell differentiation, PF-641 alone significantly decreased the amount of phosphorylated GSK3β at serine 9 (p-GSK3β^{ser9}; Fig. 16A, B), indicating an increase of GSK3β activity (Cross et al., 1995). Furthermore, the amount of p-GSK3β^{ser9} in both Wnt3a and Wnt3a plus PF-461 groups was significant different (Fig. 16B), suggesting that PF-461 affected p-GSK3β^{ser9} even under stimulation with Wnt3a.

For exogenous overexpression of Pyk2, p-GSK3β^{ser9} amount was not changed in the defined time points after cell differentiation (Fig. 16C, D). However, the amount of phosphorylated GSK3β at tyrosine 216 (p-GSK3β^{T216}), was decreased at 0.5 h and 3 h after differentiation (Fig. 16E, F), suggesting that Pyk2 can decrease GSK3β activity, as indicated by a decrease of p-GSK3β^{T216} diminishes GSK3β activity (Hughes et al., 1993). Taken together, these data suggest that Pyk2 can decrease GSK3β activity, possibly playing a role in the interaction between the canonical and non-canonical pathways.

3.6 Effect of Pyk2 on β-Catenin Phosphorylation

The inhibition of Pyk2 can reduce β-catenin expression in bone-marrow stromal cells (BMSCs; Zhang et al., 2014). Therefore, the effect of Pyk2 on the expression of β-catenin was also evaluated after inhibition and overexpression of Pyk2 in differentiating VM cells.

In the present study, the effects of Pyk2 on β-catenin phosphorylation were investigated using Pyk2-inhibitor PF-461 for Pyk2 down-regulation and Pyk2-overexpressing cells for Pyk2 exogenous overexpression (Pyk2 in Fig. 17C, D), respectively. After the cells were treated in the different experimental groups with Wnt3a (100 ng/ml), PF-461 (0.5μM), Pyk2-overexpression, and Wnt3a plus PF-461 or Pyk2-overexpression, the cultured VM cells were harvested at defined 0.5 h and 3h time points after differentiation.

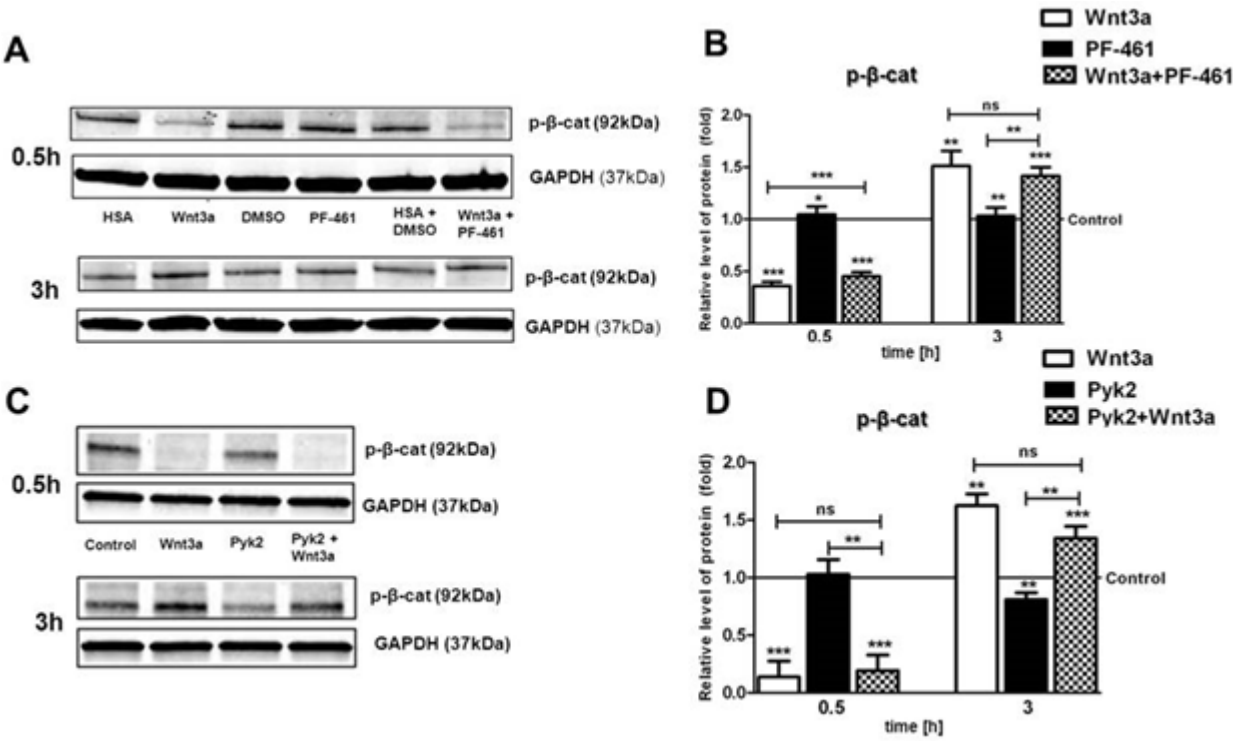

Figure 17: Effect of Pyk2 on β-catenin phosphorylation in differentiating VM cells.
VM cells were treated with Wnt3a (100 ng/ml), PF-461 (0.5 µM), Wnt3a plus PF-461 or in Pyk2-overexpression cells, respectively, and then harvested at 0.5h and 3h after differentiation. HSA and DMSO were used as controls for Wnt3a and PF-461, respectively. ***(A, B)****: Western blot bands of p-β-catenin (p-β-cat) and quantified Western blot analyses by Odyssey Software for p-β-cat (B) in the control and treatment groups under stimulation with Wnt3a and/or PF-461 at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's-T-Test: *p< 0.05, **p< 0.01, ***p< 0.01.* ***(C, D)****: Western blot bands of p-GSK3β (C) and quantified Western blot analyses by Odyssey Software for p-GSK3β (D) in the control and treatment groups with Wnt3a and/or in Pyk2-overexpression cells at 0.5h and 3h after differentiation. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

Results showed that Wnt3a decreased the phosphorylated β-catenin at Ser 33/37/Thr41 (p-β-catenin; unstabilization form) at 0.5h, but not at 3h (Fig. 17A, B). PF-461 alone increased p-β-catenin at 0.5 and 3h after differentiation, and PF-461 together Wnt3a also significantly increased p-β-catenin at 0.5h when compared to Wnt3a alone (Fig. 17A, B), suggesting that PF-461 may affect Wnt3a mediated p-β-catenin change. At 3h after differentiation, Wnt3a increased p-β-catenin, and PF-461 together with Wnt3a also increased p-β-catenin. However, the difference of p-β-catenin amount between the group of PF-461 together with Wnt3a and Wnt3a alone was not significant (Fig. 17A, B). Furthermore, the exogenous overexpression of

Pyk2 alone had no effect on p-β-catenin amount at 0.5h, but decreased it at 3h (Fig. 17C, D). However, the combination of Wnt3a and Pyk2 overexpression failed to stabilize β-catenin at 3h after differentiation (Fig. 17C, D). These data indicated that PF-461 may interfere β-catenin stabilization at defined time point, and exogenous overexpression of Pyk2 stabilized β-catenin at 3h, although the effect of Pyk2 on p-β-catenin may not involve in Wnt3a mediated p-β-catenin regulation in the differentiating VM cells.

3.7 Influence of Pyk2 on Notch Signaling Pathway

Previous report from our institute demonstrated that *HES5* gene is a key regulator in Wnt3a-induced VM cell differentiation (Mußmann et al., 2014) and ADAM10 also regulates Notch signalling pathway. As Wnt3a induces Pyk2 expression in VM cells, therefore, it is interesting to investigate whether Wnt3a can affect Notch signaling via Pyk2.

3.7.1 Effect of Pyk2 on *HES1* and *HES5*

In order to investigate whether the expression of Notch target factors - HES1 and HES5 - were affected by Pyk2, the effects of Pyk2 on Notch target factors, *HES1* and *HES5* gene were investigated using Pyk2-inhibitor PF-461 for Pyk2 down-regulation and Pyk2-overexpressing cells for Pyk2 exogenous overexpression (Pyk2 in Fig. 18B, D), respectively. After the VM cells were treated in different groups with Wnt3a (100 ng/ml), PF-461 (0.5μM), and Wnt3a plus PF-461 or in Pyk2-overexpression cells, respectively, the cultured VM cells were harvested at 3h and 6h time points after differentiation. Real-time PCR results showed that Wnt3a increased the mRNA amount of *HES1* (Fig. 18A), but decreased *HES5* (Fig. 18C) at 3h and 6h after differentiation, as demonstrated by previous published report; PF-461 increased the amount of *HES1* at 3h and 6h (Fig. 18A), and of *HES5* at 6h (Fig. 18C); A combination of PF-461 and Wnt3a also increased mRNA level of *HES1* (Fig. 18A), but decreased *HES5* (Fig. 18C). However, the differences of HES5 between the combination group and Wnt3a group were not significant (Fig. 18C). Furthermore, the exogenous overexpression of Pyk2 induced *HES1* mRNA expression at the defined time points (Fig. 18B), and *HES5* at 6h except of at 3h with a decrease (Fig. 18D). A combination of Wnt3a and Pyk2 transfection also increased mRNA level of

HES1 (Fig. 18B), but decreased *HES5* (Fig. 18D) at the defined time points except of 0.5h after differentiation. Moreover, exogenous overexpression of Pyk2 had no effects on *HES1* mRNA level, but Pyk2 significantly decreased *HES5*, when compared to the control (Fig. 18E), suggesting Pyk2 also affects *HES5* mRNA level at 3 days after differentiation.

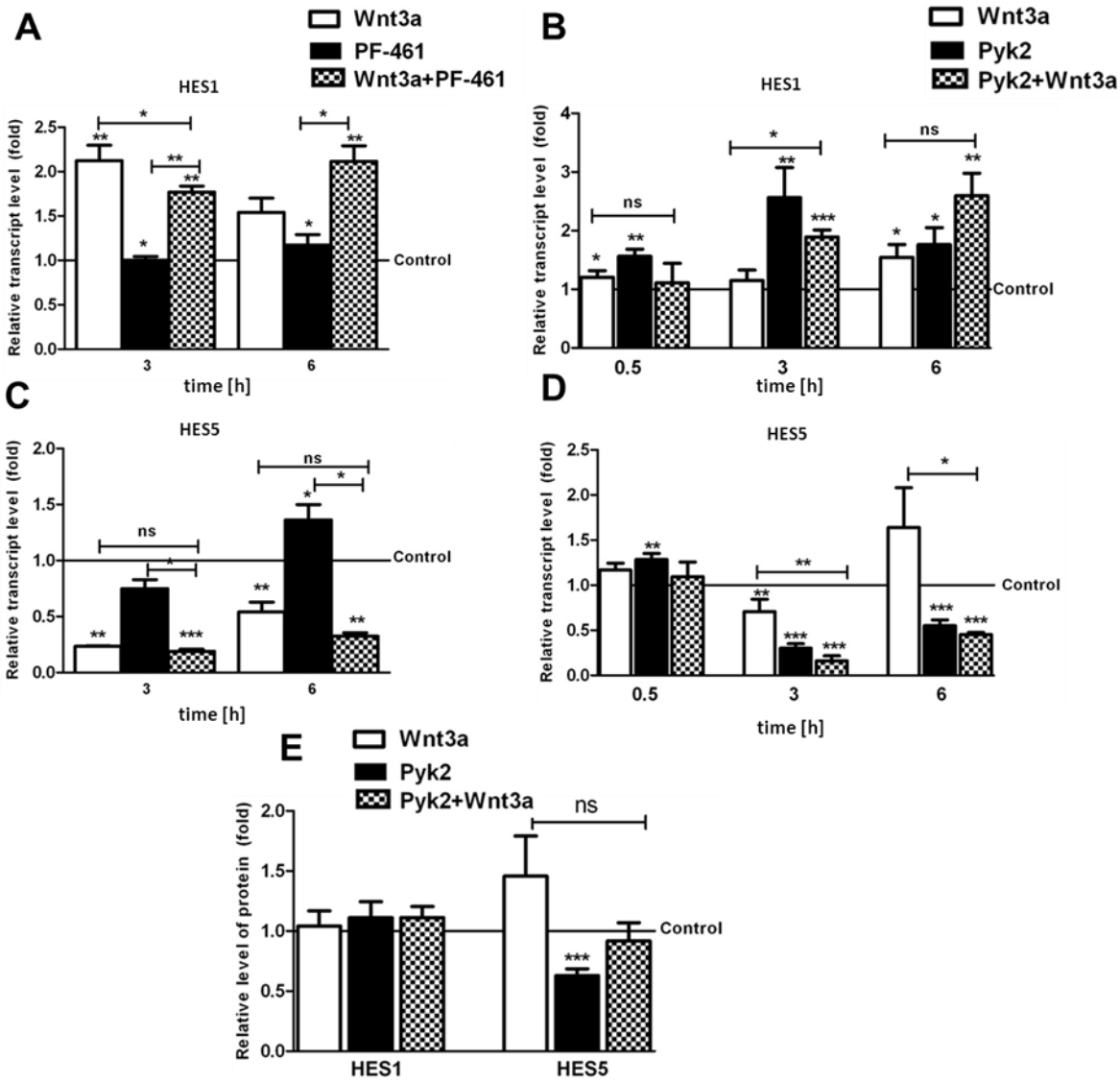

Figure 18: Effects of Pyk2 on *HES1* and *HES5* in VM cells revealed by real-time PCR analysis.
*VM cells were treated with Wnt3a (100 ng/ml), PF-461 (0.5 µM), Wnt3a plus PF-461 or in Pyk2-overexpression cells, respectively. Then the cells were harvested at 0.5h, 3h, 6h, and 3 days after differentiation. HSA and DMSO were used as controls for Wnt3a and PF-461, respectively. Quantified mRNA levels of HES1 (A, B, E) and HES5 (C, D, E) in the control and treatment groups under stimulation with PF-461 (A, C) and exogenous Pyk2 overexpression (Pyk2; B, D, E). Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black lines. Student's-T-Test: *p< 0.05, **p< 0.01, ***p< 0.01.*

3.7.2 Effect of Pyk2 on ADAM10

Previous report showed that Wnt3a regulates ADAM10 expression in mouse primary cortical neurons (Wan et al., 2012) and the down-regulation of ADAM10 affects HES5 protein level by in vivo and in vitro studies (Yan et al., 2014). Wnt3a can regulate Pyk2 expression and Pyk2 affects HES5, therefore, it is also interesting to investigate whether Pyk2 can regulate ADAM10. In the present, Pyk2-overexpressing cells were used for Pyk2 exogenous overexpression (Pyk2 in Fig. 19). After the cells were treated in the different experimental groups with Wnt3a (100 ng/ml) in the control and Pyk2-overexpression cells, the cultured VM cells were harvested at 0.5h and 3h time points after differentiation. Results showed that Wnt3a can reduce premature ADAM10 (110 kDa) at both 0.5h and 3h; the exogenous overexpression of Pyk2 also decreased the amount of ADAM10 at 0.5h and 3h (Fig. 19A, B), and a combination of Pyk2 transfection and Wnt3a also decreased ADAM10 protein expression at the defined time points (Fig. 19).

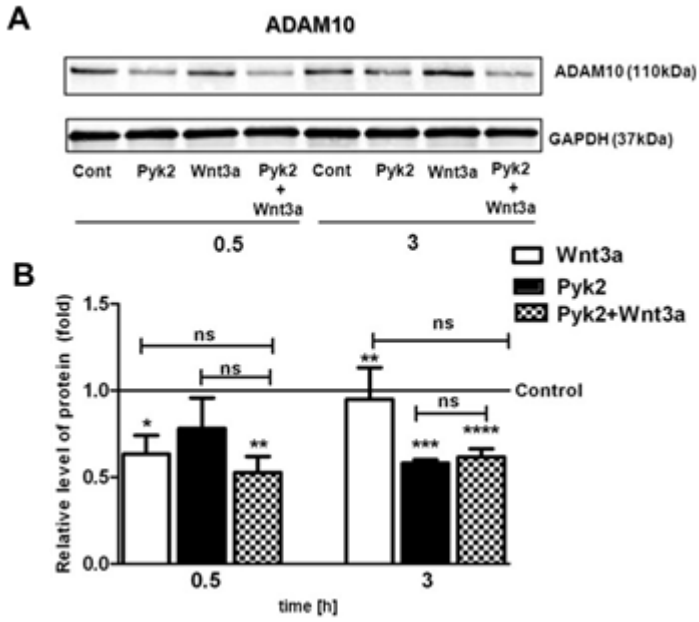

Figure 19: Effect of Pyk2 on ADAM10 expression.
*VM cells were treated with Wnt3a (100 ng/ml) in the control and Pyk2-overexpression cells, respectively, and then harvested at 0.5h and 3h after differentiation. HSA and pWPXL+RFP transfected cells were used as controls for Wnt3a and Pyk2-overexpression, respectively. Western blot bonds (A) and quantified protein level (B) of premature ADAM10 in the control and treatment groups as indicated. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

3.8 Effect of Pyk2 on Neurite Outgrowth

Previous studies have shown that Wnt3a induces neuronal genesis (Hübner et al., 2010) via HES5 (Mußmann et al., 2014) molecule in the differentiating VM cells. FAK and Pyk2 interact with integrin and growth factor receptors, increasing neurite outgrowth in PC 12 and SH-SY5Y cells (Ivankovic-Dikic et al., 2000). Therefore, it is interesting to study whether Pyk2 play a role in neurogenesis and neurite outgrowth in differentiation VM cells. After the cells were treated in the different experimental groups with Wnt3a (100 ng/ml) in the control and Pyk2-overexpression cells, the cultured VM cells were harvested for FACS and immunocytochemistry using antibody against β-III tubulin, a specific marker for neurons, at 3 days after differentiation. The results showed that as previous identified, Wnt3a induced neurogenesis in VM cells, but Pyk2 did not by FACS analyses (Fig. 20A). However, both Wnt3a and Pyk2 increased neurite length in the differentiating VM cells, as revealed by immunocytochemistry (Fig. 20B-F).

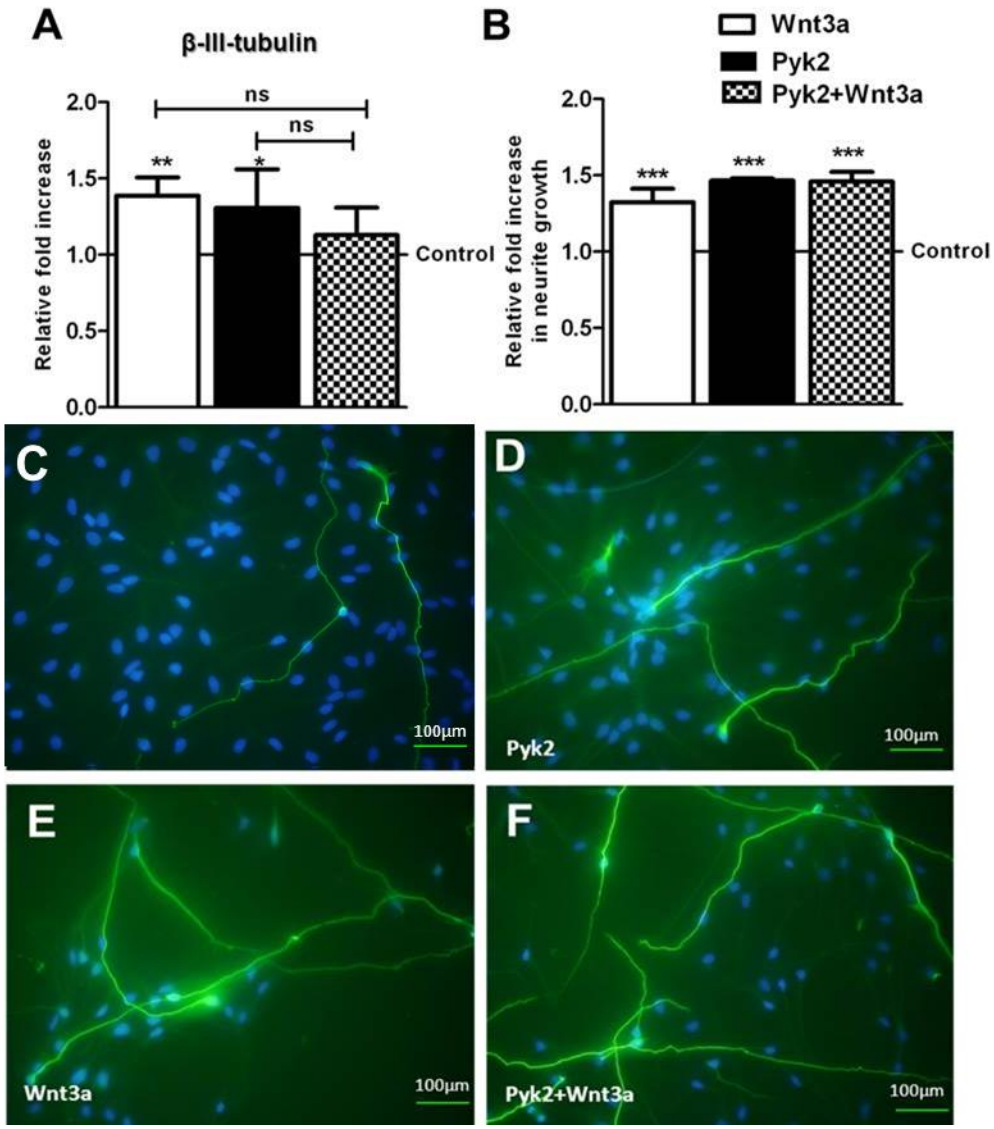

Figure 20:Effects of Pyk2 on neurogenesis and neurite length.

*VM cells were treated with Wnt3a (100 ng/ml) in the control and Pyk2-overexpression cells, respectively, and then harvested at 3 days after differentiation. HSA and pWPXL+RFP transfected cells were used as controls for Wnt3a and Pyk2-overexpression, respectively. FACS analysis and quantification of neurite length were measured in the control and treatment groups as indicated. Values were normalized to the control and presented as mean ± SEM from three individual experiments. The control was set to 1, as indicated by the black line. Student's T-Test was used: *p< 0.05, **p< 0.01, ***p< 0.01.*

4 Discussion

Wnt signaling plays an important role in tissue formation and neurogenesis during embryonic development. Previous studies from our laboratory have shown that Wnt signaling pathway is involved in the processes of hNPC differentiation (Hübner et al., 2010), including regulating spontaneous Ca^{2+} (Morgan et al., 2012) and *HES5* amount (Mußmann et al., 2014). However, mechanisms involving in the regulation of hNPC differentiation in VM cells are still not totally clear. Recently, Wnt3a has been found to induce both canonical and non-canonical pathways in some types of cells (Nalesso et al., 2011). Therefore, in the current research, I study whether Wnt3a induces a non-canonical pathway and how the interaction between Wnt canonical and non-canonical pathways affects expression of their target genes and proteins, and neuronal differentiation.

4.1 Activation of Lrp6 and Stabilization of β-catenin

It was shown that Wnt3a induces phosphorylation of Lrp6 at serine 1490 position and stabilization of β-catenin. Several studies have explained about how Wnt signalling affects β-catenin, e.g., 1) phosphorylated Lrp6 induced by Wnt3a directly inhibits GSk3β activity (Piao et al., 2008); 2) Wnt3a induces disruption of GSK3β-Axin interaction and causes a separation of GSK3β from β-catenin, resulting in β-catenin stabilization (Liu et al., 2005); 3) under Wnt3a stimulation, GSK3β triggers dephosphorylation of Axin at Ser497/500 and causes dissociation of Axin with Lrp6 and β-catenin, resulting in the inhibition of β-catenin phosphorylation (Kim et al., 2013); 4) an increase of cytosolic $Ca2^{+}$ concentration and activation of PKCα mediated by Wnt5a result in degradation of β-catenin (Gwak J et al., 2006).

The previous study in our institute has shown that Wnt3a increases Lrp6 phosphorylation during VM cell differentiation (Mazemondet et al., 2011), but the relationship between activation of Lrp6 and stabilization of β-catenin has not been focused. In the current study, we found that Wnt3a induces phosphorylation of Lrp6 at serine 1490 position and subsequently stabilizes β-catenin at 0.5h and 1h after VM cell differentiation, but not at 3h and 6h points (Fig. 9). This phenomenon is also revealed in RKO cells (Hernandez et al., 2012) and L-cells (Kim et al., 2013). Recently, several studies have further reported the novel mechanisms about Lrp6 phosphorylation and β-catenin stabilization. GSK3β bounds to Axin, interacts with

cytosolic region of Lrp6, which is phosphorylated at serine/threonine residues in PPPS/TP motifs (Wantae et al., 2013).

Wnt3a induces formation of phosphotidylinositol 4,5-biphosphate (PIP2), which in turn activates Lrp6 (Pan et al., 2008). Furthermore, phospholipase C (PLC) cleaves PIP2 into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and IP3 releasing from the membrane triggers an increase of Ca^{2+} in the cytosol (Kühl, 2004). Therefore, we asked whether Wnt3a also activates Wnt/Ca^{2+} pathway in the differentiating VM cells and how these processes are related to the degradation of β-catenin at the defined time points under a high level of phosphorylated Lrp6 after differentiation. Indeed, Wnt3a can induce an increase of cytosolic Ca^{2+} concentration by a Ca^{2+} influx from the extracellular medium and release from the intracellular organelles, resulting in an increase of activated phosphorylated CaMKII and Pyk2 (Fig. 10). Niehers and co-workers found that, an unknown proline directed kinase mediates the phosphorylation of Lrp6 at Serine 1490 and Threonine 1479 positions (Niehrs and Shen, 2010). Since Pyk2 is a proline-rich tyrosine kinase, we hypothesised that Pyk2 can induces Lrp6 phosphorylation and mediates the stabilization of β-catenin. However, overexpression of Pyk2 failed to induce phosphorylation of Lrp6 in VM cells (Fig. 15 C, D), but induces stabilization of β-catenin (Figs. 17, 21). Therefore, the reason why the β-catenin is degraded underlying the high level of phosphorylated Lrp6 is still unclear and should be further investigated.

4.2 Wnt3a Triggers Wnt/Ca^{2+} Pathway

Free cytosolic Ca^{2+} plays a role as the second messenger to trigger distinct pathways and normal regulation of free cytosolic Ca^{2+} concentration is important for physiological processes in cells. Wnt3a can elicit intracellular Ca^{2+} in rat embryonic hippocampal neurons (Avila et al., 2010) and in human articular chondrocytes (Nalesso et al., 2011). In the present study, the data showed that Wnt3a increases cytosolic Ca^{2+} concentration mediated by the influx of Ca^{2+} from the extracellular medium and also the release from the intracellular organelles, as revealed with the help of thapsigargin (Fig. 10), which can abolish a release of Ca^{2+} from endoplasmic reticulum under treatment with Wnt5a, Wnt9B, Wnt10B in PC3 cells (Thrasivoulou et al., 2013). Furthermore, Wnt3a can increase activated phosphorylated CaMKII in VM

cells (Fig. 11), possibly mediated by the increase of cytosolic Ca^{2+} concentration, suggesting that Wnt3a can trigger Wnt/Ca^{2+} -pathway in the differentiating VM cells.

An increase of cytosolic Ca^{2+} concentration activates CaMKII protein in a two-step process. The binding of Ca^{2+} to calmodulin enhances phosphorylation of the kinase domain of CaMKII at threonine 286 position, resulting in the formation of an active form of CaMKII with an autonomous activity, although the detailed mechanism is still not clear (Hoffman et al., 2011). Wnt3a activates Ca^{2+}/CaMKII non-canonical pathway together with β-catenin dependent canonical pathway, regulating distinct transcriptional targets in differentiation of human articular chondrocytes (Nalesso et al., 2011). Wnt5a can activate CaMKII to promote ventral cell fates in *Xenopus* embryos (Kühl et al., 2000) and increase intracellular Ca^{2+} levels in rat hippocampal neurons (Varella-Nallar et al., 2010), resulting in an increase of nitric oxide (NO) generation lasting for only 1h and modulating N-methyl-D-aspartate (NMDA) receptors (Munoz et al., 2014). Subsequently, NMDA receptors can promote Ca^{2+} influx and regulates p-CaMKII (T286) formation in some type of cells, e.g., rat cortical neurons (Zhou, et al., 2012). Whether Wnt3a affects NO and NMDA activation and results in activation of CAMKII in VM cells is still unknown.

4.3 Wnt3a Activates Pyk2

Activation of G-protein couple receptors and hydrolysis of PIP2 lead to phosphorylation of Pyk2 in PC12 cells (Lev et al., 1995) and PIP2 is also involved in phosphorylation of Lrp6 at Serine1490 position induced by Wnt3a (Pan et al., 2008). In the present study, Wnt3a increases the amount of p-Lrp6 protein (Fig.9) and cytosolic Ca^{2+} concentration in VM cells (Fig.10). Furthermore, Wnt3a also induces phosphorylation of Pyk2 at Tyrosine 402 position (p-Pyk2$^{(402)}$) in VM cells (Fig. 12). To the best of our knowledge, this is the first report describing that Wnt3a can induce phosphorylation and activation of Pyk2 in cells.

Regulation of Pyk2 phosphorylation in cells is complicated and has been described in some types of cells. For example, transient increase of intracellular Ca^{2+} concentration induces activation of Pyk2 in rat epithelial cells (Yu et al., 1996). In vitro experiment has shown that a few micro-molar Ca^{2+} concentration is enough to induce p-Pyk2$^{(402)}$ formation and cytosolic Ca^{2+} influx from extracellular medium is the main source of Ca^{2+} to activate Pyk2 (Kinsey, 2013). p-Pyk2$^{(402)}$ is the first and major phosphorylated form response to Ca^{2+} transient (Wu, et al., 2006). Reduction of

NMDA receptors mediated by decrease of Ca^{2+} influx leads to a decrease of Pyk2 activity in rat hippocampus after the happening of cerebral ischemia (Liu et al., 2003; Ma et al., 2004). EGF triggers IP3 generation, resulting in transient Ca^{2+}, which induces Pyk2 activation in fibroblasts (Xie et al., 2008). Furthermore, TRPM2 (Transient receptor potential cation channel member 2) channel mediated Ca^{2+} influx activates Pyk2 in human monocytes (Yamamoto et al., 2008).

Pyk2 is mainly distributed in the CNS and some other tissues and plays a very important role for keeping normal physiological function of cells. For example, activation of Pyk2 induced by transient Ca^{2+} is essential for fertilization of oocytes from the rat (Meng et al., 2006) and the zebrafish (Sharma et al., 2013). Pyk2 is essential for regulating osteoprogenitor cell development and bone formation, and Pyk2-knockout mice demonstrate an increased osteogenesis, causing high bone mass. Similar results have also been observed in rates treated with the Pyk2 inhibitor PF-431396 (Buckinder et al., 2007) and in human mesenchymal stem cells treated with PF-461 (Han et al., 2009), offering a basic strategy for osteoporosis treatment.

In the current study, PF-461 can successfully inhibit Pyk2 activity and decrease Pyk2 amount in VM cells (Fig. 13), suggesting that PF-461 is a potential candidate for inhibition of Pyk2. Furthermore, structural analyses revealed that PF-461 is more specific and selective to inhibit Pyk2, whereas PF-431396 inhibits both Pyk2 and FAK (Han, 2009). Taken together, in differentiating VM cells Wnt3a triggers Wnt/Ca^{2+}-pathway, increases cytosolic Ca^{2+} concentration (Fig. 10) and activates CaMKII (Fig. 11), and also induces phosphorylation and activation of Pyk2 (Fig. 12), which can be inhibited by its antagonist PF-461 (Fig. 13). Based on the function of Pyk2, it is important to study whether Pyk2 can regulate the components of Wnt/β-catenin pathway during VM cell differentiation (see below in detail).

4.4 Effect of Pyk2 on Components in Wnt/β-Catenin Singaling Pathway

Protein tyrosine kinases and the Wnt signalling pathway play very important roles that regulate various developmental processes and there is an inter-connection between both cell signals. For example, non-receptor tyrosine kinase Pyk2 has a cross-talk with the components of Wnt/β-catenin pathway - GSK3β and β-catenin. In the present study, Pyk2 had no effect on p-Lrp6 (Fig. 15), but decreased GSK3β

activity (Fig. 16) and stabilized β-catenin (Fig. 17). Taken together, these results suggest that Pyk2 is involved in Wnt/Ca^{2+}-pathway, and interacts with Wnt/β-catenin dependent pathway, in which Pyk2 decreases GSK3β activity and stabilizes β-catenin (Fig. 21).

In multiple myeloma, stabilization of β-catenin is achieved by Pyk2 overexpression, resulting in transcription of c-Myc and cyclin D1 (Zhang et al., 2014). In mammalian cells, intracellular release of Ca^{2+} causes trafficking of β-catenin into the nucleus (Thrasivoulou et al., 2013) and inhibition of Pyk2 increases the degradation of β-catenin (Zhang et al., 2014), suggesting that Pyk2 plays a role in the stabilization of β-catenin. Indeed our findings also revealed that inhibition of Pyk2 by PF-461 destabilizes β-catenin, but overexpression of Pyk2 stabilizes β-catenin (Fig. 17), suggesting the important role of Pyk2 in regulation of Wnt/β-catenin pathway in hNPCs.

The outcome of Wnt signal transduction in cells is decided by different factors, such as phosphorylation of Dvl (Sen, 2005; Gao and Chen, 2010) and the ability of Wnt ligand to form a complex with the upstream of Dvl - Fz receptors and co-receptor (Lrp6, Ror1/2; Grumolato et al., 2010). Growth factor receptor bound protein 2 (Grb2), a downstream molecule of Dvl2, can bind to Dvl2, as revealed by immunoprecipitation and Grb2-knockdown in HEK293 cells, in which it inhibits the Wnt3a-mediated β-catenin signalling (Crampton et al., 2009). On the other hand, p-$Pyk2^{(402)}$ facilitates the binding of Pyk2 to Grb2 (Blaukat et al., 1999), regulating Wnt/β-catenin pathway through Dvl2, Rac1, Jnk and c-jun (Crampton et al., 2009). These findings support our results that Pyk2 can interact with the components of Wnt/β-catenin dependent pathway (Figs. 16, 17).

Although it is still a preliminary step in integration of cell singling cascade, deep focus might answer a lot of unresolved questions about how Wnt signaling works. The Wnt ligand induces intracellular Ca^{2+} increase and leads to stabilization of β-catenin (Avila et al., 2010; Thrasivoulou et al., 2013). The trafficking of β-catenin into the nucleus mediated by Pyk2 destroys VE-cadherin function of the cell-cell adhesion in vascular endothelial cells (Van Buul et al., 2005). Transient increase of intracellular Ca^{2+} increases GSK3β activity, promoting tau-protein phosphorylation in SH-SY5Y cells (Hartigan J A et al., 1999). All these data strongly support the concept, which was recognized in the current study, that Pyk2 activation induces by

an increase of cytosolic Ca^{2+} via Wnt3a stimulation plays an important role in the regulation of β-catenin stabilization in hNPCs.

4.5 Effect of Pyk2 on Notch Singaling Pathway

In Notch signaling pathway, the interaction between a ligand in one cell to the receptor in another cell triggers proteolytic cleavages of Notch receptor subsequently by either ADAM10 or ADAM17 and then by γ-secretase, resulting in a release of the intracellular fragment, which interacts with the nuclear factors to regulate the target gene expression, including Hes1, Hes5, Hey1, and Hey2 (Takebe et al., 2015). Previous studies from our institute have shown that Wnt3a promotes hNPCs differentiation, possibly mediated by Hes5 molecule (Mußmann et al., 2014). Down-regulation of ADAM10 also decreases HES5 protein expression by both in vivo and in vitro systems (Yan et al., 2014). Furthermore, deletion of Wnt/β-catenin signaling inhibits ADAM10 upregulation in mouse cortical neurons (Wan et al., 2012). FAK protein also regulates activity of Notch signaling.

Interestingly, overexpression of Pyk2 decreases ADAM10 protein expression in the VM cells (Fig. 19). A strong reduction of Notch target genes including *HES5* has been found in ADAM10-knockout mice (Zhuang et al., 2015). Down-regulation of ADAM10 strongly decreases HES5 expression in chicken spinal cord, resulting in precociously neuronal differentiation in the spinal cord and hNPCs (Yan et al., 2014). Therefore, Pyk2 triggered by the increase of cytosolic Ca^{2+} concentration mediated by Wnt3a can also affect Notch signalling by affecting ADAM10 and HES5 expression, resulting in an increase of neurogenesis and neurite outgrowth (Figs. 18-21).

4.6 Effect of Pyk2 on Neurogenesis and Neurite Outgrowth

Over expression of Wnt3a induces neurogenesis in mice hippocampal stem and progenitor cells (Lie et al., 2005). Wnt3a can induce neurogenesis in hNPCs through β-catenin independent pathway (Hübner et al., 2010). Overexpression of Pyk2 decreases *HES5* expression (Fig. 18D, E) in VM cells and induces neurite outgrowth (Fig. 20). Increasing neurite outgrowth in PC12 and SH-SY5Y cells has been observed under the condition with autophosphorylation of Pyk2, induced by growth factors and integrin signaling and and the inhibition of Pyk2/FAK blocks neurite

outgrowth (Ivankovic-Dikic et al., 2000). Interestingly, overexpression of Pyk2 reduces the *HES5* expression (Fig. 18D, E) in differentiating VM cells, supporting the concept of the involvement of Pyk2 in neurogenesis. Pyk2 and GSK3β are co-immunoprecipitated in various cells (Hartigan et al., 2001). Inhibition of Pyk2 by tryphostin A9 and GSK3β by SB216763 partially inhibit neurite retraction in B103-LPA_1 cells (Sayas et al., 2006), suggesting that an involvement of Wnt-Pyk2 signaling in the differentiation of neurons. Although further studies are necessary to confirm the existence of Wnt-Pyk2 signaling in VM cells, the preliminary data from the present study support our hypothesis, i.e., Pyk2 induced by Wnt3a can mediate neurogenesis in VM cells.

4.7 Integration of Wnt singaling and Notch Singaling via Pyk2

Different signalling pathways, such as Wnt signaling, Notch signaling, and hedgehog signalling, cooperate with each other in embryogenesis and organism development. The cross-talk between the signaling pathways has been always paid attention by scientists to find out more key molecules for invention of new drugs, which are used in treatment for various diseases (Takebe et al., 2015).

Although β-catenin and Ca^{2+} play a key role in different canonical and non-canonical Wnt pathways, respectively, it is still not clearly understand how Wnt signalling is regulated. The current study identified that Pyk2 can decrease GSK3β and stabilize β-catenin and also decrease ADAM10 and HES5 expression, further extending a connection between Wnt signaling and Notch singaling via Pyk2 in differentiating hNPCs.

Furthermore, Pyk2 can bind to STAT3, activating STAT3 activity and forming a positive-feedback loop between them (Verma et al., 2015). The binding of HES protein to STAT3 induces a cross-talk between the Notch and STAT pathways (Kamakura et al., 2004). The current results together with our previous data (Mußmann et al., 2014; Yan et al., 2014) demonstrate that decreased ADAM10 and HES5 by Wnt3a and Pyk2 during hNPCs differentiation make a connection between Wnt and Notch signaling pathways through Pyk2.

Taken together, Pyk2 is involved in both Wnt signalling and Notch signalling and integrate both signalling pathways, suggesting an important role of Pyk2 in hNPC differentiation (Fig. 21).

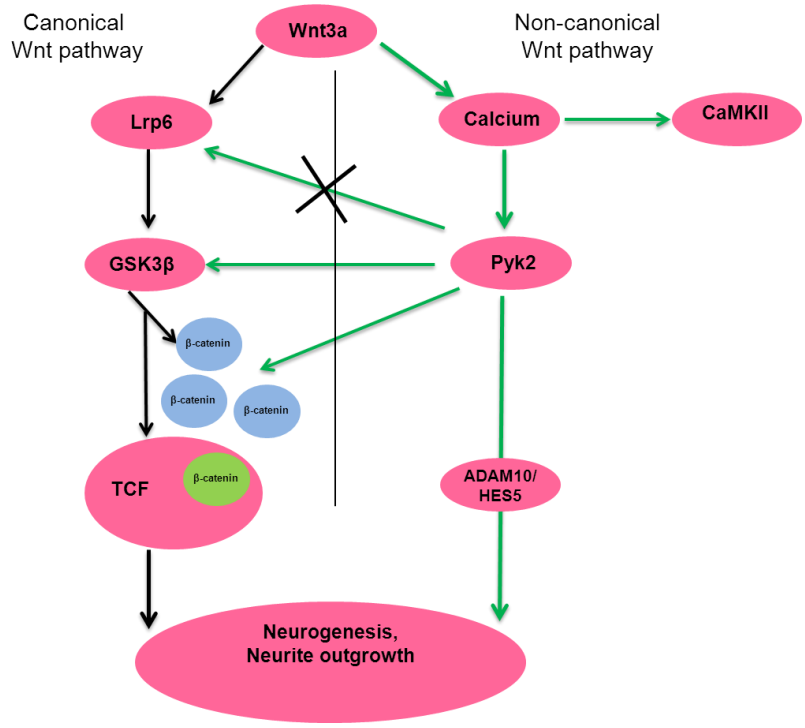

Figure 21. Simplified model to show interaction between canonical and non-canonical pathways via Pyk2 and Wnt-Pyk2-Notch signaling pathways induced by Wnt3a in the differentiating NPC. *Wnt3a increases cytosolic Ca^{2+} concentration, which can activate CaMKII and Pyk2. Subsequently, Pyk2 decreases GSK3β activity and stabilizes β-catenin, but not Lrp6, suggesting a crosstalk between Wnt/β-catenin and Wnt/Ca^{2+}-pathways. Furthermore, Pyk2 decreases ADAM10 and HES5, and promote neurite outgrowth, suggesting that Pyk2 also involves in Notch signaling.*

4.8 Outlook

The present data show that Wnt3a increases cytosolic calcium concentration by increasing calcium influx from extracellular space and calcium release from the intracellular organelles and induces activation of CaMKII and Pyk2, suggesting that Wnt3a can trigger Wnt/Ca^{2+} dependent pathway during hNPC differentiated. Activation of Pyk2 can decrease GSK3β activity and stabilizes β-catenin, suggesting an interaction between canonical and non-canonical Wnt signaling pathways during differentiation of hNPCs. Furthermore, overexpression of Pyk2 decreases the *HES5* mRNA amount and ADAM10 protein in the differentiating hNPCs, suggesting an involvement of Pyk2 in the Notch signaling pathway. Finally, Pyk2 overexpression

promotes neuronal differentiation and increases neurite length, suggesting a role of Wnt and Pyk2 in neurogenesis.

Although the data shown in this study is very interesting, still some questions should be further investigated. For example, why is β-catenin degraded underlying a high level of p-Lrp6 at 3h and 6h after differentiation of hNPC under Wnt3a stimulation? Answer this question may give more detail to discover how other factors are involved in β-catenin regulation, and how they work together with Wnt3a to regulate β-catenin function, and what is the key role of Lrp6 in Wnt/β-catenin dependent pathway. Furthermore, Wnt3a at a low concentration induces the Wnt/β-catenin dependent pathway, but at a high concentration triggers the Wnt/Ca^{2+} dependent pathway, modulating precisely articular chondrocyte phenotype (Nalesso et al., 2011). In this study, Wnt3a can trigger Wnt/Ca^{2+} dependent pathway, therefore, it is interested to find out whether during hNPC differentiation, Wnt3a induces Wnt/β-catenin and Wnt/Ca^{2+} dependent pathways is also in a dose-dependent manner, and which specific Wnt receptor mediates this different Wnt signaling pathways. Moreover, Pyk2 is involved in both Wnt and Notch signaling pathways, and it is also interested to study how Pyk2 regulates these two pathways and induces the interaction between them.

5 Summary

HumanNPCs (hNPCs) are the suitable cell source for stem cell-based replacement of lost neurons in translation medicine for neurodegenerative disease and the benefit of cellular therapy is dependent on cell survival and differentiation after transplantation.

In the present study, effects of Wnt3a on the differentiation of hNPC were studied using VM cell line as a cell model. The results showed that Wnt3a induces phosphorylation of Lrp6 and stabilization of β-catenin at early 0.5h and 1h, but not 3h and 6h after differentiation, suggesting other molecular mechanisms also regulate β-catenin stabilization. Furthermore, Wnt3a increases transient cytosolic Ca^{2+} concentration by triggering Ca^{2+} influx from the medium and Ca^{2+} release from the cellular organelles, as indicated by thapsigargin experiment. Furthermore, Wnt3a increases Ca^{2+} target genes CaMKII and Pyk2 in the differentiating VM cells. Pyk2 is a member of non-receptor PTKs and plays an important role in regulating cellular signaling pathways. Therefore, effects of Pyk2 were further investigated after endogenous Pyk2 inhibited by PF-461 or exogenous overexpression in stable Pyk2-overexpressing VM cells. The results demonstrated that Pyk2 has no effect on Lrp6 phosphorylation, but decreases GSK3β activity and stabilizes β-catenin, suggesting there is an intercross between Wnt/β-catenin and Wnt/Ca^{2+}-pathway via Pyk2. Moreover, Pyk2 increases Notch targeting gene HES1, but decreases HES5 as well as ADAM10, suggesting Pyk2 is involved in Notch signaling during VM cell differentiation. Finally, Pyk2 increases neurite outgrowth at 3 days after VM differentiation.

Wnt3a induces neurogenesis and neurite outgrowth via downregulation of HES5 mediated by Pyk2 was found out in the present study and Pyk2 is involved in not only Wnt signaling pathway, but also Notch signaling pathway, suggesting a crosstalk between Wnt signaling and Notch signaling via Pyk2.

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7 Abbreviations

AD	Alzheimer disease
ADAM	A disintegrin and metalloprotease
ADSC	Adipose derived stem cell
AKos	Albrecht Kossel Institute for Neuroregeneration
APC	Adenomatous poliposis coli
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
bFGF	Basic fibroblast growth factors
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
CaCN	Calcineurin
CADTK	Calcium dependent tyrosine kinase
CAKβ	Cell adhesion kinase β
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent kinase II
cDNA	Complementary DNA
mRNA	Messenger RNA
CK1	Casein kinase-1
CNS	Central nervous system
CSL	Suppressor of hairless lag-2, also named as CBF-1
Ct	Cycle threshold
DAG	Diacylglycerol
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid

DSL	Delta Serrate Lag-2
Dvl	Dishvelled
ECN	Extracellular
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor repeats
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinases
FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
Fz	Frizzled
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRB2	Growth factor receptor bound-2
GSK3β	Glycogen synthase kinase 3β
h	Hour
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase
HERP	HES related repressor
HES	Hairy enhancer of split
hESC	Human embryonic stem cell
hNPC	Human neural progenitor cell
HSA	Human serum albumin
ICC	Immunocytochemistry

ICN	Intracellular
iPS	Induced pluripotent stem cell
JNK	c-Jun terminal kinase
LEF	Lymphoid enhancer factor
LNR	Lin12 Notch repeats
Lrp5/6	Low density receptor related protein
MAML	Mastermind-like protein
MAPK	Mitogen activated protein kinase
MS	Multiple sclerosis
NFAT	Nuclear factor activated T-cell
NICD	Notch intracellular domain
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cell
PBS	Phosphate buffer saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PFA	Paraformaldihyde
PIP2	Phosphotidylinositol-4,5-biphosphate
PLC	Phospholipase C
PTK2β	Protein tyrosine kinase 2β
PTKs	Protein tyrosine kinases
Pyk2	Proline-rich tyrosine kinase 2 (also named as PTK2β)
q-RT-PCR	Quantitative real-time PCR
RAC1	Ras-related C3 botulinum toxin substrate-1
RAFTAK	Related adhesion focal tyrosine kinase

RBPJ	Recombining binding protein suppressor of hairless
RHOA	Ras homolog gene A
RNA	Ribonucleic Acid
ROCK	Rho associated kinase
STAT3	Signal transducer and activator of transcription 3
TCF	T-cell factor
THAP	Thapsigargin
TMD	Transmembrane domain
TMN	Transmembrane
WB	Western blot
β-Trcp	β-transducin repeat containing E3 ubiquitin protein ligase

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9 Declaration

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen habe ich als solche kenntlich gemacht

Rostock, 16. 11. 2015

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10 Curriculum Vitae

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2008-2009	Masters in Science (Molecularbiology); Skövde University, Sweden. Thesis: Role of NFAT3 in the differentiation and proliferation of embryonic stem cells.

Publications

1. **Talabattula VA**, Arndt Rolfs, Jiankai Luo. Pyk2 mediated Wnt signaling in the differentiation of human neural progenitor cells. (Manuscript under preparation).
2. Yan X, Lin J, **Talabattula VA**, Mußmann C, Yang F, Wree A, Rolfs A, Luo J. ADAM10 negatively regulates neuronal differentiation during spinal cord development. PLoS One. 2014. 9: e84617.
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July 14-18, 2012	8th FENS forum of Neuroscience, Barcilona, Spain
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