Journal of Dental Sciences 19 (2024) 2090–2099

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Wnt/Ca^{2+} pathway inhibits neural differentiation of human dental pulp stem cells in vitro

Shi-Hua Wang [a,b], Shi-Rui Wang [b], Na-Na Luan [c], Xiao-Qian Sun [b,d], Yi-Ran Guo [b], Ying-Bin Yan [b,d**], Su-Xia Liang [a,b*]

[a] *Department of Operative Dentistry and Endodontics, Tianjin Stomatological Hospital, School of Medicine, Nankai University, Tianjin, China*
[b] *Tianjin Key Laboratory of Oral and Maxillofacial Function Reconstruction, Tianjin, China*
[c] *Department of Stomatology, Yancheng Third People's Hospital, Yancheng, China*
[d] *Department of Oromaxillofacial-Head and Neck Surgery, Tianjin Stomatological Hospital, School of Medicine, Nankai University, Tianjin, China*

Received 9 March 2024; Final revision received 11 April 2024
Available online 24 April 2024

KEYWORDS
Dental pulp stem cells;
Neural differentiation;
Wnt/β-catenin pathway;
Wnt/Ca^{2+} pathway;
WNT5A

Abstract *Background/purpose:* Dental pulp stem cells (DPSCs) have demonstrated significant potential for neuroregeneration. However, a full understanding of the specific mechanism underpinning the neural differentiation of DPSCs is still required. The Wnt signaling is crucial for the development of the embryonic neural system and the maintenance of adult neural homeostasis. This study aimed to investigate the role of the Wnt/Ca^{2+} pathway in the neural differentiation of human DPSCs (hDPSCs) and its modulation of the Wnt/β-catenin pathway.
Materials and methods: hDPSCs were cultured and divided into the control group and the neurogenic induction group (Neuro group). The mRNA and protein levels of neurogenic markers, Wnt/Ca^{2+}, and Wnt/β-catenin pathway indicators were determined using Quantitative real-time PCR and Western blotting. After inhibition of the Wnt/Ca^{2+} pathway using a WNT5A short hairpin RNA (shRNA) plasmid and subsequent neurogenic induction, neurogenic markers and Wnt/β-catenin pathway indicators in the NC-sh-Neuro group and WNT5A-sh-Neuro group were determined using Quantitative real-time PCR and Western blotting.
Results: Compared with the control group, the expression of the Wnt/Ca^{2+} pathway indicators (WNT5A, Frizzled 2, calmodulin-dependent protein kinase IIa, and nuclear factor of active T cells 1) decreased in the Neuro group. Conversely, the expression of WNT3A, total β-catenin

* Corresponding author. Department of Operative Dentistry and Endodontics, Tianjin Stomatological Hospital, School of Medicine, Nankai University, 75 Dagu Road, Heping District, Tianjin 300041, China.
** Corresponding author. Department of Oromaxillofacial-Head and Neck Surgery, Tianjin Stomatological Hospital, School of Medicine, Nankai University, 75 Dagu Road, Heping District, Tianjin 300041, China.
E-mail addresses: yingbinyan@qq.com (Y.-B. Yan), lliangyuer@163.com (S.-X. Liang).

https://doi.org/10.1016/j.jds.2024.04.011
1991-7902/© 2024 Association for Dental Sciences of the Republic of China. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

and active β-catenin in the Wnt/β-catenin pathway increased. Moreover, compared with the NC-sh-Neuro group, the WNT5A-sh-Neuro group exhibited a greater level of mature neural differentiation alongside elevated expression of the Wnt/β-catenin pathway indicators.
Conclusion: The Wnt/Ca^{2+} pathway inhibited neural differentiation of hDPSCs and has a negative effect on the Wnt/β-catenin pathway in vitro.
© 2024 Association for Dental Sciences of the Republic of China. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Dental pulp stem cells (DPSCs) have demonstrated significant potential for neural differentiation. Derived from the neural crest, DPSCs were found to express obvious neurogenic markers such as Nestin and βIII-tubulin without any induction.[1] Furthermore, when transplanted into rats with sensorineural hearing loss[2] and rodents with cerebral ischemic injury,[3] DPSCs exhibited remarkable capabilities of neuroprotection and regeneration in vivo. When subjected to neurogenic induction, DPSCs exhibited characteristic neurogenic marker expression, neuron-like morphology, and electrophysiological currents resembling those of neurons.[1] These findings have sparked a growing interest in DPSCs as a promising candidate for nerve regeneration in both medical and dental fields.

Wnt signaling is crucial for the maintenance of neural homeostasis, which can be categorized into canonical Wnt/β-catenin pathway and non-canonical pathways. Researchers have demonstrated that the canonical pathway positively regulated the DPSCs neural differentiation.[4,5]

The non-canonical Wnt/Ca^{2+} pathway is crucial for cell polarity, cell migration, and cell fate determination.[6] Research on it was limited, with most focusing on cancer, brain injuries, and inflammation, while its role in neural differentiation remains to be further established. It was found that the WNT5A/Frizzled 2 (FZD2)/calmodulin-dependent protein kinase II (CaMKII) signaling was abnormally activated in cases of hippocampal brain damage and worsening tissue damage.[7] In addition, when the calcium/calmodulin (Ca^{2+}/CaM) signaling was inhibited, directed differentiation of bone marrow mesenchymal stem cells (BMSCs) to neural cells was achieved.[8] However, some researchers have demonstrated the protective role of this pathway in neuronal differentiation and axon guidance.[9,10] The differences in these findings may be attributed to tissue origin, degree of differentiation, and the overall health condition of the research subjects.

The role of the Wnt/Ca^{2+} pathway in the differentiation of hDPSCs through the neuronal lineage remains unknown. Furthermore, there was intricate crosstalk between the Wnt signalings within biological processes.[11,12] Therefore, this study investigated the effect of the Wnt/Ca^{2+} pathway on hDPSCs neural differentiation and its modulation of the canonical pathway in vitro, to elucidate the mechanism involved in the hDPSCs neurogenesis and provided a theoretical foundation for future application in neural regeneration and tissue engineering.

Materials and methods

Ethics statement

Ethics approval was obtained from the Ethics Committee of Tianjin Stomatological Hospital (PH2022-B-008, 20220418). Informed consent was written by each donor.

Cell culture

Using the tissue block method, hDPSCs were isolated from the intact and healthy wisdom teeth extracted from donors aged 18—25 years. In brief, pulp tissue blocks (1 × 1 × 1 mm) were cultured in flasks containing alpha-minimum essential medium supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco), and then incubated in 5% CO_2 at 37 °C. The human dental pulp cells (hDPCs) were purified via limited dilution. To identify the cells, CD73, CD90, CD34, and CD45 were detected via flow cytometry, antibodies were purchased from BD Biosciences (San Jose, CA, USA). After osteogenic and adipogenic differentiation induction, alizarin red and oil red O staining were carried out.

Neural differentiation of hDPSCs

Cells were divided into the control group and the neurogenic induction group (Neuro group) induced as reported by Osathanon with some modifications.[13] Specifically, cells were seeded (1 × 10^5 cells/mL) into a low adhesion 60 mm-Petri dish (JET Biofil, Guangzhou, China) and induced with Neurobasal-A medium containing 2% B27 (Gibco), 20 ng/mL basic fibroblast growth factor (bFGF; Proteintech, Wuhan, China), 20 ng/mL epidermal growth factor (EGF; Proteintech) and 1% penicillin-streptomycin for 7 days in stage I. In stage II, spheres were mechanically pipetted into single cells and seeded (1 × 10^5 cells/mL) into 35 mm Petri dishes for another 7 days. The medium was replaced every two days.

Cell transfection

A control vector (NC-shRNA) or a WNT5A short hairpin RNA (WNT5A-shRNA) plasmid (Ribio Biotech, Guangzhou, China) was transfected into the hDPSCs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was detected by Western blotting (WB). An effective sequence

was selected for subsequent experiments and labeled the WNT5A-shRNA group. The shRNA sequences were as follows: WNT5A-shRNA-1: CGCCCAGGTTGTAATTGAA, WNT5A-shRNA-2: GCAAGTTGGTACAGGTCAA, WNT5A-shRNA-3: CTTCCAAGTTCTTCCTAGT. Subsequently, the NC-shRNA group and the WNT5A-shRNA group were simultaneously subjected to neurogenic induction and were then designated as the NC-sh-Neuro group and the WNT5A-sh-Neuro group, respectively.

Quantitative real-time PCR

Total cellular RNA was extracted using an RNA extraction kit and then reverse-transcribed into cDNA using Evo M-MLV RT Premix (Accurate Biology, Hunan, China). Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq II kit (Takara, Tokyo, Japan). GAPDH was used to normalize the target gene expression. The primer sequences are presented in Table 1. Primers of WNT3A were purchased from GeneCopoeia (Rockville, MD, USA) and others were from Sangon Biotech (Shanghai, China).

Western blotting

Cells were lysed using the Western and RIPA lysis buffer supplemented with protease and phosphatase inhibitors. A BCA protein assay kit was used to calculate the protein concentration. Following SDS-PAGE, samples were transferred to PVDF membranes. After being blocked, these membranes were incubated overnight at 4 °C with primary antibodies, and then incubated with HRP-conjugated secondary antibodies the following day. An ECL-chemiluminescent kit was used to visualize the membranes and the results were analyzed by Image Lab software (BioRad, Hercules, CA, USA). The primary antibodies were as follows: anti-Nestin (1:1000, Proteintech); anti-βIII-tubulin (1:2000, Proteintech); anti-MAP2 (1:5000, Proteintech); anti-Synaptophysin (SYP, 1:10000, Proteintech); anti-WNT3A (1:1000, Proteintech); anti-β-catenin (1:1000, Proteintech); anti-active β-catenin (1:1000, ABclonal, Wuhan, China); anti-WNT5A (1:1000, ABclonal); anti-CaMKIIa (1:1000, ABclonal); anti-p-CaMKIIa (1:2000, ABclonal); anti-NFATc1 (1:1000, ABclonal) and anti-GAPDH (1:1000, Beyotime, Shanghai, China).

Immunofluorescence

Immunofluorescence of adherent cells was performed as before.[14] While fixation, permeation, blocking and incubation with primary and secondary antibodies of spheres were performed in the collection tubes, then the spheres were dropped on slides and sealed with a sealing solution containing DAPI for observation using an inverted fluorescence microscope (Nikon Ti-S, Tokyo, Japan). The primary antibodies were anti-Nestin (1:200, Proteintech) and anti-MAP2 (1:200, Proteintech).

Statistical analysis

Data were presented as the mean ± SD for three or more independent replicates. Independent sample T-test and one-way ANOVA were used to quantify differences using SPSS 26.0 (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant ($n = 3$).

Results

Morphological profiling and characterization of hDPSCs

On the 7th day, new adherent primary hDPCs emerged from the tissue blocks (Fig. 1Aa), and by Day 21, cells were growing in colonies arranged radially around the blocks (Fig. 1Ab). After purification, single-cell clonal colonies were observed (Fig. 1Ac). Upon cultivation until passage 3–5, the hDPSCs exhibited a vortex growth pattern and a spindle-shaped morphology like fibroblasts (Fig. 1Ad). Flow cytometry analysis revealed a high expression of CD73 (98%) and CD90 (98.7%); however, the expression of CD34 (0.38%) and CD45 (0.04%) were found to be negative (Fig. 1B). Following osteogenic and adipogenic induction, respectively, prominent calcified nodules and orange lipid droplets were observed (Fig. 1C and D).

Table 1 Primers sequences of related genes.

Gene	Sense (5′–3′)	Antisense (5′–3′)
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
Nestin	GCCCTGACCACTCCAGTTTA	CCCAGATTTGCCCTTCACCT
βIII-tubulin	AGGTTCTCTTACATCGACCGC	AGATGCACTCACGCATGGTT
MAP2	CTGGGTCTACTGCCATCACTC	CCCCTTTAGGCTGGTATTTGA
SYP	GGACATGGACGTGGTGAATC	GATGGCGAAGACCCATTGC
WNT5A	GGCTCCACTTGTTGCTCGG	GTTATTCATACCTAGCGACCACC
FZD2	GTGCACCAGTTCTATCCGCT	CTCCGTCCTCGGAGTGGTTC
CaMKIIa	ACCACTACCTGATCTTCGACC	CCGCCTCACTGTAATACTCCC
NFATc1	GCAGAGCACGGACAGCTATC	GGGCTTTCTCCACGAAAATGA
β-catenin	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT

Calmodulin-dependent protein kinase IIa (CaMKIIa), Frizzled 2 (FZD2), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), microtubule associated protein 2 (MAP2), nuclear factor of active T cells 1 (NFATc1), Synaptophysin (SYP).

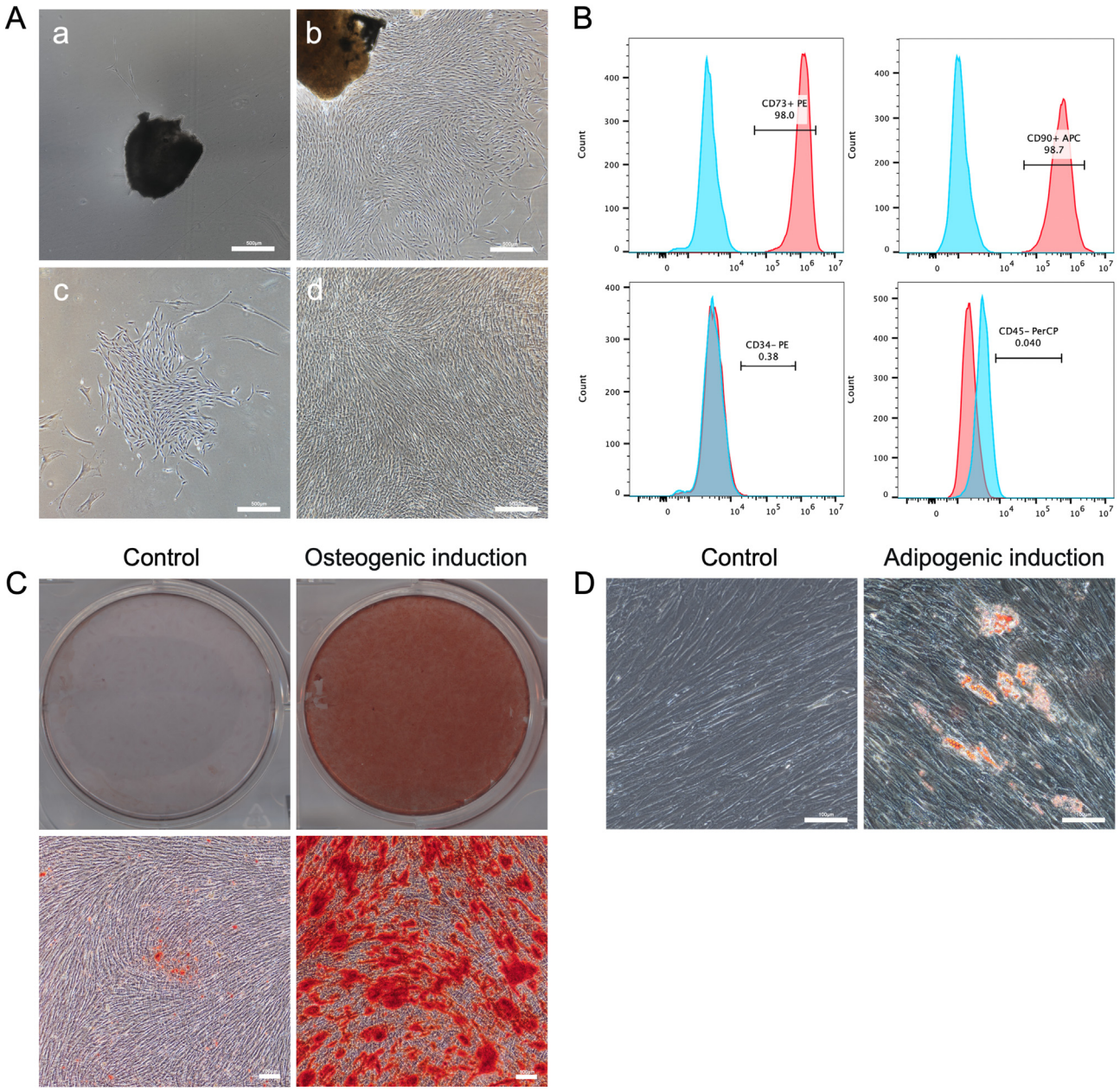

Figure 1 Characterization of human dental pulp stem cells. (A) Human dental pulp cells obtained from the tissue block (a, b) and purified by the limited dilution method (c, d) (scale bar 500 μm). (B) Flow cytometry analysis of human dental pulp stem cells (hDPSCs) surface markers including CD73 (98%), CD90 (98.7%), CD34 (0.38%) and CD45 (0.04%). (C) Alizarin red staining showed calcified nodules formed after osteogenic differentiation (scale bar 100 μm). (D) Oil red O staining showed orange lipid droplets formed after adipogenic differentiation (scale bar 100 μm). "Control": cells cultured in complete medium; "Osteogenic induction": cells subjected to the osteogenic induction; "Adipogenic induction": cells subjected to the adipogenic induction.

Neuron-like morphological characteristics and characteristic neurogenic marker expression in hDPSCs after induction

At the end of stage I, smooth-edged spheres with a diameter of 100–300 μm were observed; Immunofluorescence revealed a strong positive expression of Nestin in these spheres (Fig. 2Aa, b, 2Ba). At the end of stage II, the Neuro group exhibited a neuron-like alteration in morphology, primarily characterized by bipolar changes and the formation of distinct neurofilament-like structures. Moreover, microtubule associated protein 2 (MAP2) exhibited positive expression (Fig. 2Ac, d, 2Bc). Results of qRT-PCR and WB revealed that on the 7th day, expression levels of Nestin and βIII-tubulin increased after induction; however, there was no significant change in those of MAP2 and SYP (Fig. 2C and D). On the 14th day, expression levels of Nestin and βIII-tubulin were significantly downregulated; in contrast, those of MAP2 and SYP were upregulated (Fig. 2E and F).

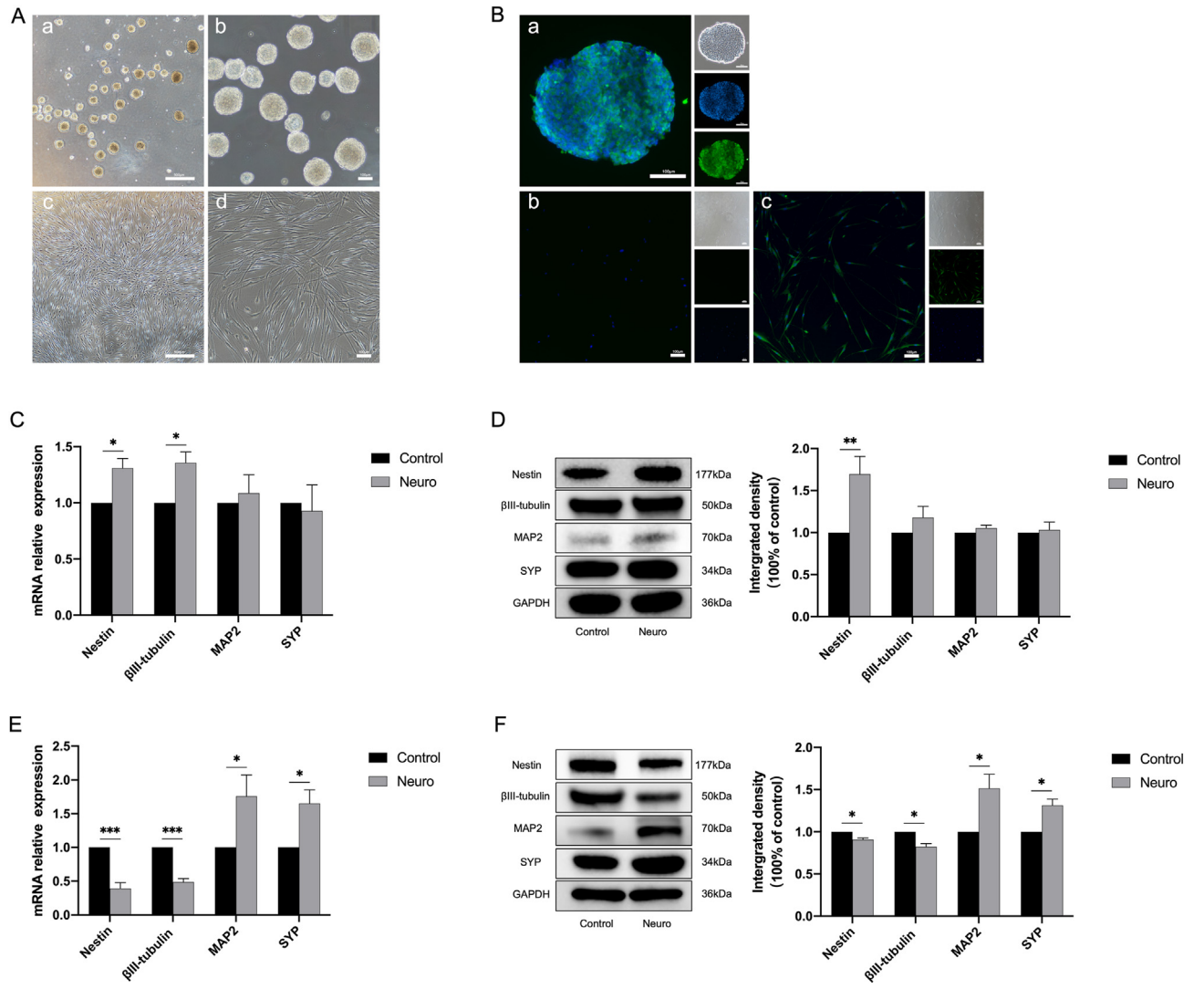

Figure 2 hDPSCs obtained neuron-like morphological characteristics and characteristic expression of neural markers after induction. (A) Neural differentiation was induced by a two-stage method. Neurosphere-like structures obtained after induction in stage I (a, b) and neurofilament-like structures formed after induction in stage II (c, d) (a, c: scale bar 500 μm, b, d: scale bar 100 μm). (B) Immunofluorescence revealed a strong positive expression of Nestin in the neurosphere-like structures at the end of stage I (a). The green fluorescence was Nestin, and the blue fluorescence was 4′,6-diamidino-2-phenylindole (DAPI, scale bar 100 μm). Immunofluorescence showed positive expression of microtubule associated protein 2 (MAP2) in the Neuro group (c) while negative in the control group (b) at the end of stage II. The green fluorescence was MAP2, and the blue fluorescence was DAPI (scale bar 100 μm). (C, D) mRNA and protein levels of Nestin and βIII-tubulin increased in the Neuro group on the 7th day. (E, F) mRNA and protein levels of Nestin and βIII-tubulin were downregulated; in contrast, those of MAP2 and Synaptophysin (SYP) were upregulated on the 14th day. "Control": cells cultured in complete medium; "Neuro": cells subjected to the neurogenic differentiation. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, n = 3.

Downregulation of the Wnt/Ca^{2+} pathway and upregulation of the canonical pathway were observed during the neural differentiation of the hDPSCs

A qRT-PCR assay revealed that on the 7th day, the Wnt/Ca^{2+} pathway indicators (WNT5A, CaMKIIa, NFATc1, and FZD2) in the Neuro group were downregulated. In contrast, the canonical pathway indicators (WNT3A, and β-catenin) were upregulated (Fig. 3A). On the 14th day, the trend was more pronounced (Fig. 3C). Similarly, WB analysis showed decreased expression of WNT5A, p-CaMKIIa and NFATc1 and increased expression of WNT3A, total β-catenin and active β-catenin on the 7th day (Fig. 3B) and 14th day (Fig. 3D). Protein level of CaMKIIa remained unchanged.

Wnt/Ca^{2+} pathway inhibition promoted neural differentiation of hDPSCs and activated the canonical pathway

Among the three sequences tested, WNT5A-shRNA-1 was the most effective in knockdown the WNT5A (Fig. 4A). A

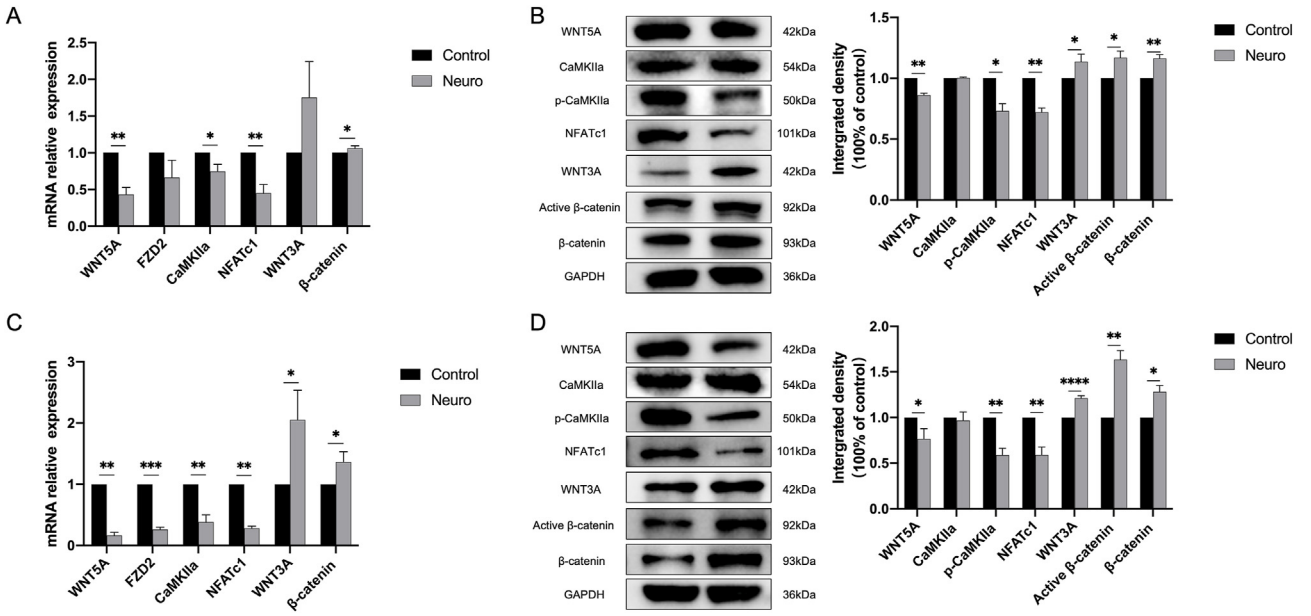

Figure 3 Downregulation of the Wnt/Ca^{2+} pathway and upregulation of the canonical pathway were observed during the neural differentiation of the hDPSCs. (A, B) mRNA and protein levels of the Wnt/Ca^{2+} pathway indicators, WNT5A, calmodulin-dependent protein kinase IIa (CaMKIIa), p-CaMKIIa, nuclear factor of active T cells 1 (NFATc1), and Frizzled2 (FZD2), were downregulated in the Neuro group, while the canonical pathway indicators, WNT3A, total β-catenin and active β-catenin, were upregulated on the 7th day. (C, D) The downregulation of indicators related to the Wnt/Ca^{2+} pathway was more pronounced, while the expression of WNT3A, total β-catenin and active β-catenin increased. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, n = 3.

substantial decrease in the protein level of the Wnt/Ca^{2+} pathway-related indicators was observed in the WNT5A-shRNA group by 24 h post-transfection (Fig. 4B). A qRT-PCR assay revealed that on the 7th day, compared to the NC-sh-Neuro group, expression levels of Nestin and βIII-tubulin in the WNT5A-sh-Neuro group decreased, while those of MAP2, SYP, WNT3A, and β-catenin elevated (Fig. 4C). On the 14th day, the trend was more pronounced (Fig. 4E). WB results were consistent with the qRT-PCR findings (Fig. 4D, F).

Discussion

Since DPSCs were first confirmed to possess the ability to differentiate towards functional neurons under neuronal inductive stimuli,[15] various methods have been devised to induce DPSCs to differentiate towards neural lineages in vitro. They varied in mode of cultivation, division of cultivation stage, and medium.[16] To induce hDPSCs into neural lineages in vitro, we followed a two-stage protocol using a serum-free induction medium. Previous research has demonstrated that a three-dimensional culture suitably mimics the in vivo microenvironment, facilitating material exchange and signal transmission between cells and the extracellular matrix.[17] A serum-free medium could effectively reduce safety risks such as host allergies or immune rejection.[18] In addition, the mild and prolonged induction achieved by adding biological factors such as bFGF and EGF proved effective in promoting DPSCs neural differentiation.[19]

At the end of stage I, the neurosphere-like structures formed, and the expression of immature markers (Nestin and βIII-tubulin) was upregulated. Nestin was highly expressed in neural progenitors but decreased with differentiation.[20] βIII-tubulin, a tubulin involved in microtubule formation, neuronal differentiation, and neurite formation, was significantly upregulated in immature neurons and decreased with maturation.[21] At the end of stage II, some cells displayed neuron-like changes in morphology. The expression levels of mature markers (MAP2 and SYP) were upregulated while those of immature markers were reduced. MAP2 was tightly related to the maturation of neurons, maintenance of synaptic plasticity, and post-mitotic neuronal dendrite stability and was often used as a mature neurogenic marker.[22] SYP, a synaptic vesicle membrane protein, was expressed extensively in brain tissue and was closely linked to the maintenance of synaptic stability.[23] Therefore, these results suggested that at least a portion of DPSCs have differentiated into neural cells after induction, and other growth factors may be needed for more efficient induction.

Subsequently, we investigated the effect of the two pathways on the neural differentiation of hDPSCs. β-catenin functions as the core regulator in the canonical pathway. When ligands are absent, cytoplasmic β-catenin is continuously degraded by the APGC complex composed of adenomatous polyposis, Axin, GSK-3β, and casein kinase 1 (CK1). When the canonical pathway is activated, the disorganized protein (Dvl) bound to the Fzd receptor is hyperphosphorylated by CK1 and subsequently hydrolyses

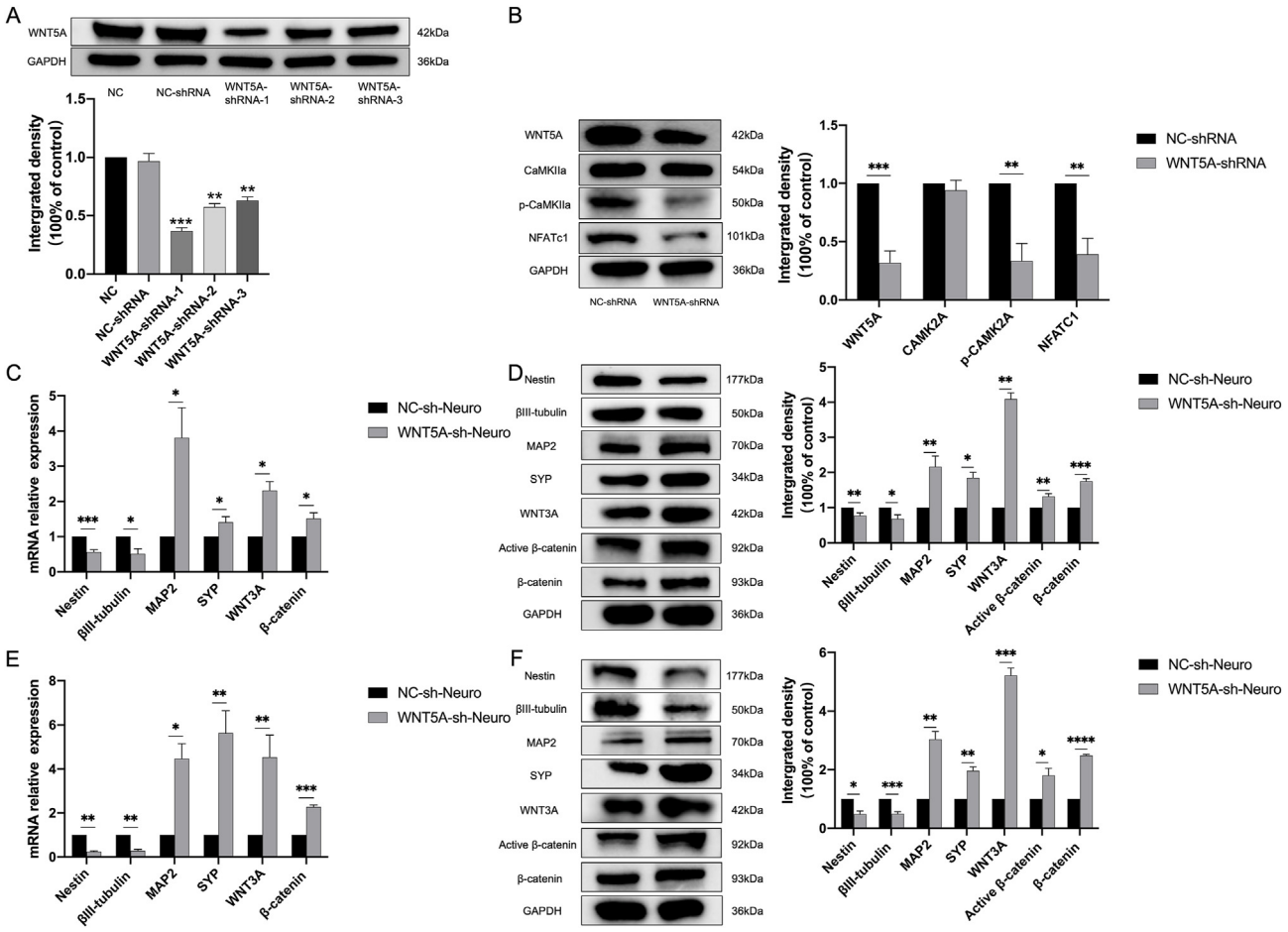

Figure 4 Inhibiting the Wnt/Ca^{2+} pathway promoted neural differentiation of hDPSCs and activated the canonical pathway. (A) WNT5A-shRNA-1 was shown to be the most effective sequence for the knockdown of WNT5A. (B) Protein level of Wnt/Ca^{2+} pathway-related indicators in the "WNT5A-shRNA" group decreased. (C, D) mRNA and protein levels of Nestin and βIII-tubulin in the WNT5A-sh-Neuro group decreased, while those of MAP2, SYP, WNT3A, total β-catenin and active β-catenin elevated on the 7th day. (E, F) mRNA and protein levels of Nestin and βIII-tubulin were further reduced, while those of MAP2, SYP, WNT3A, total β-catenin and active β-catenin remained elevated on the 14th day. "NC": Cells maintained in the complete medium; "NC-shRNA": cells transfected with control vector; "WNT5A-shRNA-1": cells transfected with WNT5A-shRNA-1 plasmid; "WNT5A-shRNA-2": cells transfected with WNT5A-shRNA-2 plasmid; "WNT5A-shRNA-3": cells transfected with WNT5A-shRNA-3 plasmid. WNT5A-shRNA-1 was selected for subsequent experiments and labeled the "WNT5A-shRNA" group. The NC-shRNA group and the WNT5A-shRNA group were simultaneously subjected to neural induction and were then designated as the "NC-sh-Neuro" group and "WNT5A-sh-Neuro" group, respectively. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$, n = 3.

Axin. Thus, the APGC complex disintegrated, and cytoplasmic β-catenin accumulated, which then entered the nuclear to bind the transcription factors such as the T cell factor (TCF) and the lymphoid enhancer factor (LEF) to regulate target gene.[24]

The canonical pathway is crucial for the neuronal differentiation of mesenchymal stem cells. It was shown that BMSCs differentiation into neural lineage cells was improved by activating this signaling.[25] In addition, this signaling was proved to be related to the age-dependent neuronal differentiation of DPSCs, and the addition of Wnt proteins could significantly enhance their differentiation capacity.[5] Moreover, DPSCs cultured on chitosan scaffolds were demonstrated to secrete more neurotrophic factors by activating this pathway.[4] In our study, we observed a gradual upregulation of WNT3A, total and active β-catenin expression in the "Neuro" group, indicating that the canonical pathway positively regulated the neural differentiation of hDPSCs, consistently with other researchers.

The Wnt/Ca^{2+} pathway is currently not well understood. As the representative ligand, when WNT5A binds to FZD2, G proteins and DVL are activated, which in turn activates phospholipase C. Then phosphatidylinositol bisphosphate (PIP2) is hydrolyzed into inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 stimulates the opening of calcium ion channels on the cell membrane, which facilitates the entrance of Ca^{2+} into the cell. It can also trigger the release of Ca^{2+} from the endoplasmic reticulum, thus intracellular Ca^{2+} concentration increased. Ca^{2+} combining with CaM then leads to the phosphorylation of CaMKII, ultimately impacting NFATc, cAMP-response element binding proteins and other transcription factors.[26]

During the neural differentiation of the DPSCs, the expression of the Wnt/Ca^{2+} pathway-related indicators decreased. Additionally, when the pathway was inhibited, DPSCs differentiated towards more mature cells that resembled neurons.

CaMKII is a multifunctional serine/threonine protein kinase located within the organelles of peripheral and central neurons. There are four isoforms of CaMKII: α, β, γ, and δ, with the α isoform being widely distributed in neural tissues. A review of the literature indicated that CaMKII had a bidirectional regulatory effect on neurite formation. For instance, It was found that transient elevation of Ca^{2+} under the cell membrane and activation of CaMKII could promote neurite branching and filopodia formation in neurons.[27] Similarly, WNT5A, a microhabitat factor found in the hippocampus, was confirmed to enhance neuronal differentiation while regulating the morphology of dendrites via the WNT5A/CaMKII pathway.[9] However, it has also been reported that activation of this signaling could lead to calcium overload in neurons, exacerbating brain damage.[7] Furthermore, CaMKII activation has been confirmed to contribute to the oxidative stress after hypoxia-ischemia brain injury leading to death of neural cells.[28] Although these research focused on nerve tissue, research on BMSCs also demonstrated that inhibition of CaMKII could rescue neural differentiating cells from death.[29] Similarly, salidroside was confirmed to facilitate BMSCs differentiation into neuron-like cells by blocking the Ca^{2+}/CaM/CaMKII signaling pathway.[8]

These findings indicated that CaMKII played varying biological roles in different tissues and under different physiological or pathological conditions. Studies have also confirmed that CaMKII/NFAT pathway was highly expressed during stem cell senescence,[6] abnormal activation of this signaling could induce cell apoptosis.[30] Consequently, inhibiting this signaling may help to maintain cellular stability during the process of DPSCs neural differentiation.

We observed opposing regulatory effects of the two pathways on the neural differentiation of DPSCs. By blocking the Wnt/Ca^{2+} pathway, the canonical pathway was triggered, allowing DPSCs to differentiate into mature cells that resembled neurons (Fig. 5). This finding validated the Wnt/Ca^{2+} pathway's inhibitory impact on the canonical pathway. The mechanism underpinning this interaction

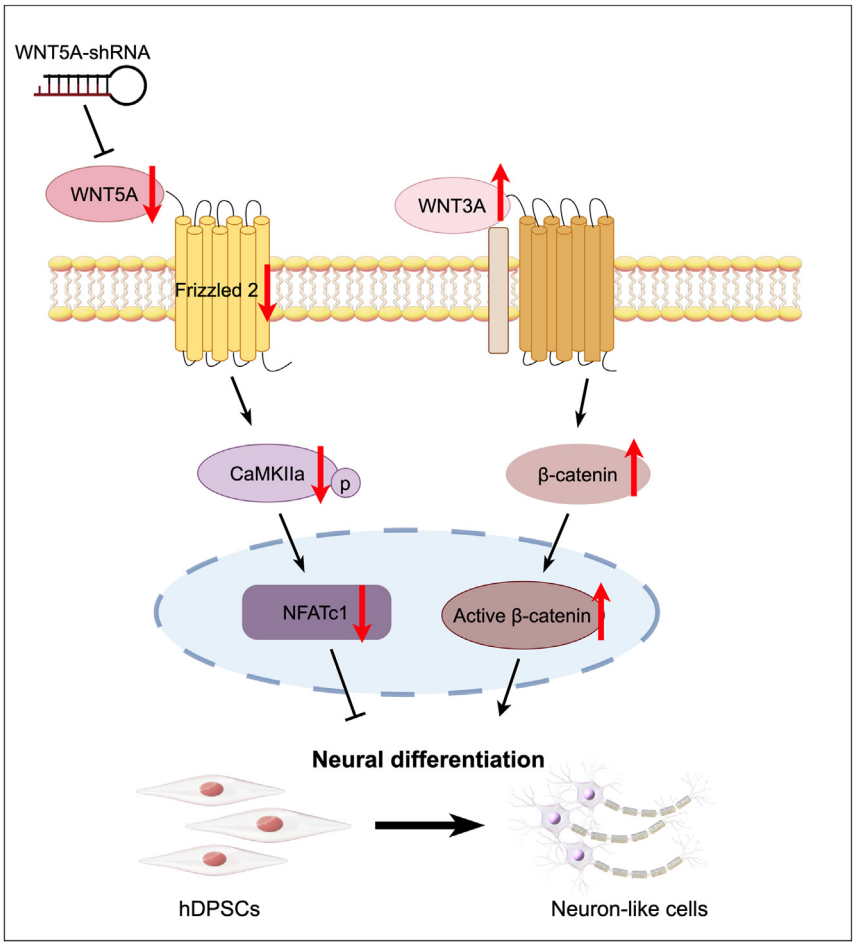

Figure 5 Schematic of the mechanisms proposed in this paper (by Figdraw). The Wnt/Ca^{2+} pathway exerted an inhibitory effect on the neural differentiation of the human dental pulp stem cells (hDPSCs), on the contrary, the canonical pathway played a promoting role. When the Wnt/Ca^{2+} pathway was inhibited by knocking down the WNT5A using the WNT5A-shRNA expression plasmid, the expression of the canonical pathway was more upregulated, which promoted the neural differentiation of the hDPSCs. Calmodulin-dependent protein kinase IIa (CaMKIIa), nuclear factor of active T cells 1 (NFATc1).

possibly involves the activation of TGF-β activated kinase 1 and nemo-like kinase by CaMKII. These kinases phosphorylate TCF and inhibit the transcription of the canonical pathway.[11] In addition, NFATc could competitively bind to Dvl with β-catenin, thereby disrupting the canonical pathway's control over the proliferation and differentiation of neural progenitor cells.[31]

In conclusion, the Wnt/Ca^{2+} pathway inhibited neural differentiation of DPSCs and exerted an inhibitory impact on the Wnt/β-catenin pathway in vitro. However, our research did not elucidate the specific site where the Wnt/Ca^{2+} pathway exerted an inhibitory effect on the canonical pathway. Therefore, further research is required to explore these aspects.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This study was supported by Tianjin Key Medical Discipline (Specialty) Construction Project (TJYXZDXK-078D), the Project of Graduate Supervisor of Tianjin Key Laboratory of Oral and Maxillofacial Function Reconstruction (2021KLDS01), Tianjin Key Medical Discipline (Specialty) Construction Project (TJYXZDXK-048A), and the Medical Talent Project of Tianjin City (TJSJMYXYC-D2-032).

References

1. Al-Maswary AA, O'Reilly M, Holmes AP, Walmsley AD, Cooper PR, Scheven BA. Exploring the neurogenic differentiation of human dental pulp stem cells. *PLoS One* 2022;17:e0277134.
2. Rawiwet V, Vijitruth R, Thonabulsombat C, Vongsavan K, Sritanaudomchai H. Evaluation of the efficacy of human dental pulp stem cell transplantation in Sprague-Dawley rats with sensorial neural hearing loss. *Eur J Dermatol* 2023;17:1207—14.
3. Wu T, Xu W, Chen H, et al. Comparison of the differentiation of dental pulp stem cells and periodontal ligament stem cells into neuron-like cells and their effects on focal cerebral ischemia. *Acta Biochim Biophys Sin (Shanghai)* 2020;52:1016—29.
4. Zhang J, Lu X, Feng G, et al. Chitosan scaffolds induce human dental pulp stem cells to neural differentiation: potential roles for spinal cord injury therapy. *Cell Tissue Res* 2016;366:129—42.
5. Yi Q, Liu O, Yan F, et al. Analysis of senescence-related differentiation potentials and gene expression profiles in human dental pulp stem cells. *Cells Tissues Organs* 2017;203:1—11.
6. Sarabia-Sánchez MA, Robles-Flores M. WNT signaling in stem cells: a look into the non-canonical pathway. *Stem Cell Rev Rep* 2024;20:52—66.
7. Niu LJ, Xu RX, Zhang P, Du MX, Jiang XD. Suppression of Frizzled-2-mediated Wnt/Ca^{2+} signaling significantly attenuates intracellular calcium accumulation in vitro and in a rat model of traumatic brain injury. *Neuroscience* 2012;213:19—28.
8. Zhao L, Zhao H-b, Pan Q, Li G, Wang J-n, Tang J-j. Salidroside induces the differentiation of mouse bone marrow mesenchymal stem cells into neuron-like cells mediated by calcium/calmodulin signaling pathway. *Chin J Tissue Eng Res* 2014;18:6019.
9. Arredondo SB, Guerrero FG, Herrera-Soto A, et al. Wnt5a promotes differentiation and development of adult-born neurons in the hippocampus by noncanonical Wnt signaling. *Stem Cell* 2020;38:422—36.
10. Shu Y, Xiang M, Zhang P, et al. Wnt-5a promotes neural development and differentiation by regulating CDK5 via Ca2+/calpain pathway. *Cell Physiol Biochem* 2018;51:2604—15.
11. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. *Mol Cell Biol* 2003;23:131—9.
12. Flores-Hernández E, Velázquez DM, Castañeda-Patlán MC, et al. Canonical and non-canonical Wnt signaling are simultaneously activated by Wnts in colon cancer cells. *Cell Signal* 2020;72:109636.
13. Osathanon T, Sawangmake C, Nowwarote N, Pavasant P. Neurogenic differentiation of human dental pulp stem cells using different induction protocols. *Oral Dis* 2014;20:352—8.
14. Zhang PP, Liang SX, Wang HL, et al. Differences in the biological properties of mesenchymal stromal cells from traumatic temporomandibular joint fibrous and bony ankylosis: a comparative study. *Anim Cells Syst (Seoul)* 2021;25:296—311.
15. Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cell* 2008;26:1787—95.
16. Heng BC, Lim LW, Wu W, Zhang C. An overview of protocols for the neural induction of dental and oral stem cells in vitro. *Tissue Eng Part B Rev* 2016;22:220—50.
17. Wu X, Su J, Wei J, Jiang N, Ge X. Recent advances in three-dimensional stem cell culture systems and applications. *Stem Cell Int* 2021;2021:9477332.
18. Solis-Castro OO, Boissonade FM, Rivolta MN. Establishment and neural differentiation of neural crest-derived stem cells from human dental pulp in serum-free conditions. *Stem Cells Transl Med* 2020;9:1462—76.
19. Rafiee F, Pourteymourfard-Tabrizi Z, Mahmoudian-Sani MR, et al. Differentiation of dental pulp stem cells into neuron-like cells. *Int J Neurosci* 2020;130:107—16.
20. Gonmanee T, Arayapisit T, Vongsavan K, Phruksaniyom C, Sritanaudomchai H. Optimal culture conditions for neurosphere formation and neuronal differentiation from human dental pulp stem cells. *J Appl Oral Sci* 2021;29:e20210296.
21. Katsetos CD, Legido A, Perentes E, Mörk SJ. Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. *J Child Neurol* 2003;18:851—67.
22. Yang L, Jurczak KM, Ge L, van Rijn P. High-throughput screening and hierarchical topography-mediated neural differentiation of mesenchymal stem cells. *Adv Healthcare Mater* 2020;9:e2000117.
23. Zhu X, Wang P, Liu H, et al. Changes and significance of SYP and GAP-43 expression in the hippocampus of CIH rats. *Int J Med Sci* 2019;16:394—402.
24. Hayat R, Manzoor M, Hussain A. Wnt signaling pathway: a comprehensive review. *Cell Biol Int* 2022;46:863—77.
25. Chen H, Li S, Xu W, et al. Interleukin-17a promotes the differentiation of bone marrow mesenchymal stem cells into neuronal cells. *Tissue Cell* 2021;69:101482.
26. Lu Y, Liu M, Guo X, et al. miR-26a-5p alleviates CFA-induced chronic inflammatory hyperalgesia through Wnt5a/-CaMKII/NFAT signaling in mice. *CNS Neurosci Ther* 2023;29:1254—71.

27. Sheng L, Leshchyns'ka I, Sytnyk V. Neural cell adhesion molecule 2 promotes the formation of filopodia and neurite branching by inducing submembrane increases in Ca2+ levels. *J Neurosci* 2015;35:1739—52.
28. Lu Q, Harris VA, Sun X, Hou Y, Black SM. Ca2+/calmodulin-dependent protein kinase II contributes to hypoxic ischemic cell death in neonatal hippocampal slice cultures. *PLoS One* 2013;8:e70750.
29. Jori FP, Napolitano MA, Melone MA, et al. Molecular pathways involved in neural in vitro differentiation of marrow stromal stem cells. *J Cell Biochem* 2005;94:645—55.
30. Huang Z, Peng Y, Ke G, Xiao Y, Chen Y. CaMKII may regulate renal tubular epithelial cell apoptosis through YAP/NFAT2 in acute kidney injury mice. *Ren Fail* 2023;45:2172961.
31. Huang T, Xie Z, Wang J, Li M, Jing N, Li L. Nuclear factor of activated T cells (NFAT) proteins repress canonical Wnt signaling via its interaction with dishevelled (DVL) protein and participate in regulating neural progenitor cell proliferation and differentiation. *J Biol Chem* 2011;286:37399—405.