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The WNT7B protein promotes the migration and differentiation of human dental pulp cells partly through WNT/beta-catenin and c-Jun N-terminal kinase signalling pathways

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ABSTRACT

Objective: The aim of this study is to investigate the role of the WNT7B protein in the migration and differentiation of human dental pulp cells (HDPCs).

Design: The effect of recombinant human WNT7B (rhWNT7B) on the proliferation and migration of HDPCs was evaluated by 5-ethynyl-2′-deoxyuridine (EdU), immunofluorescence staining of Ki67, flow cytometry and scratch assay; the differentiation of HDPCs was measured by alkaline phosphatase (ALP) staining, alizarin red staining, ALP activity, qPCR and western blot. The activation of the WNT/beta-catenin (WNT/β-catenin) and c-Jun N-terminal kinase (JNK) pathways was analysed by western blot, immunocytochemistry and dual luciferase assays. XAV939 and SP600125 , the inhibitors of the WNT/β-catenin and JNK pathways, were further applied to verify the mechanism.

Results: rhWNT7B repressed the proliferation but did not affect the apoptosis of HDPCs. In the presence of rhWNT7B, ALP and alizarin red staining were increased substantially in the HDPCs with osteogenic induction; the gene expression of *Runx2* and *Col1* in HDPCs was quite elevated compared with that induced in osteogenic medium without WNT7B measured by qPCR; The ALP activity was also increased with rhWNT7B stimulation in HDPCs after 7-day odontogenic culture; Western blot revealed that the expression of dentin sialophosphoprotein (DSPP) of HDPCs was up-regulated significantly with the addition of WNT7B as well. Further study showed that rhWNT7B activated the WNT/β-catenin and JNK signalling pathways in the differentiation of HDPCs. XAV939 and SP600125 can partly offset the effect of the WNT7B-induced differentiation of HDPCs.

Conclusion: WNT7B promoted the differentiation of HDPCs partly through the WNT/β-catenin and JNK signalling pathways.

1. Introduction

Naturally, dental pulp is surrounded directly by dentin and provides regenerative potential to form tertiary dentin postnatally (Arana-Chavez & Massa, 2004; Couve, Osorio, & Schmachtenberg, 2014). In the presence of mild stimulation such as early caries, the odontoblasts laying below dentin can survive by secreting reactionary dentin matrix to prevent the progress of the injury (Arana-Chavez & Massa, 2004; Couve et al., 2014). However, once the damage is intense enough like advanced caries, the pre-existing odontoblasts near the impaired site may die, and the precursor cells of odontoblasts, including dental pulp stem cells and mesenchymal stem cells in circulation, are recruited to the injured site to differentiated and synthesized mineralized tissue to preserve vital pulp (Ishizaka, Iohara, Murakami, Fukuta, & Nakashima, 2012; Kim, Lee, Kim, & Mao, 2010). This physiologically defence mechanism of dental pulp offers us the methods to treat many dental diseases, such as caries, abrasion, dental trauma and tooth sensitivity. However, in clinical practice, many patients with the dental diseases are still accepting root canal therapy, which ultimately produces discoloured and fragile teeth. Therefore, only relying on the self-repair of pulp is not enough to prevent dental diseases such as dental caries, and searching for the factors that promote reparative dentin formation is an effective way to prevent invasions and protect vital pulp tissue.

An increasing number of studies have shown that the precursor cells of odontoblasts reside in postnatal pulp, which can respond to multiple extracellular signals (Hunter et al., 2015; Li et al., 2014; Oh et al., 2012;

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Peng et al., 2010; Wang et al., 2010; Wang et al., 2013; Yang et al., 2015). Inflammatory stimulation initiates the network of various signalling pathways, which induces the mesenchymal stem cells in dental pulp to go through proliferation, migration and differentiation. More recently, studies on the WNT signalling pathway have enriched our understanding of its roles in the process of pulp repair (Li et al., 2014; Koizumi et al., 2013). The extracellular WNT ligands bind to their receptors on the progenitor cells of odontoblasts, activate intracellular signal transduction, and affect the cells' migration, proliferation and differentiation through beta-catenin(β-catenin)-dependent or −independent ways (Li et al., 2014; Peng et al., 2010; Wang et al., 2010; Wang et al., 2013). WNT5A has been reported to increase cell motility in human dental papilla cells (Wang et al., 2013). WNT6 has been demonstrated to promote the migration of human dental pulp cells (HDPCs) (Li et al., 2014). WNT3A and WNT6 have been indicated to increase the proliferation of the precursor cells of odontoblasts (Hunter et al., 2015; Li et al., 2014). WNT3A, WNT5A, WNT6, WNT10A and WNT11 have all been shown to promote odontoblasts differentiation (Hunter et al., 2015; Koizumi et al., 2013; Li et al., 2014; Lin et al., 2011; Peng et al., 2010; Wang et al., 2010; Wang et al., 2013; Yamashiro et al., 2007; Yang et al., 2015). It has been demonstrated that WNT7B was expressed in the process of tooth development by in situ hybridization (Sarkar & Sharpe, 1999). To further confirm the region of the secretion protein WNT7B that is active in tooth development, we employed immunohistochemistry and found that the WNT7B protein was expressed in the odontoblast layer at E18.5 (unpublished data), which suggests that WNT7B participates in the dentinogenesis in the embryonic stage. Due to the close relationship between dentinogenesis in the tooth development stage and in the process of reparative dentin formation, we speculate that WNT7B might play roles in dentin formation postnatally as well. However, the exact function of WNT7B in reparative dentin formation is still unclear.

WNT7B has been reported to be involved in cell proliferation and cell fate decisions (Joeng & Long, 2014; Lobov et al., 2005). Recently, Long et. al revealed that WNT7B dramatically elevated bone formation by increasing osteoblast number and function postnatally (Chen et al., 2014). We also evaluated the tooth phenotype of mice with WNT7B overexpression by the Dmp1 promoter, and found a thinner pre-dentin layer, more dentinal tubules, greater width of the inter-tubular dentin, higher micro-hardness values and the Ca/P ratio of dentin in the transgenic mice (data not shown). All the evidences suggest that WNT7B plays a role in dentinogenesis. Intracellularly, WNT7B has been demonstrated to activate the WNT/β-catenin signalling pathway and β-catenin-independent signalling pathways in different cell types (Arensman et al., 2014; Chen et al., 2014; Wang, Shu, Lu, & Morrisey, 2005). However, which intracellular pathways participate in the WNT7B-induced signals in HDPCs still needs to be elucidated.

The aim of this paper is to investigate the role of WNT7B in the migration and differentiation of HDPCs, which is the essential processes in dental pulp repair. We tested the proliferation, apoptosis, migration and differentiation of HDPCs with the stimulation of WNT7B. Furthermore, the WNT/β-catenin signalling pathway and intracellular signal c-Jun N-terminal kinase (JNK) were tested to understand the mechanism of WNT7B-induced dentinogenesis.

2. Materials and methods

2.1. Ethics and cell culture

This work was approved by the Ethics Committee of the State Key Laboratory of Oral Diseases of Sichuan University, and was performed in accordance with approved guidelines. Human pulp tissues were collected from the West China Hospital of Stomatology with patients' informed consent.

Primary HDPCs were cultured according to a previous study (Ye, Peng, Tan, & Zhou, 2006) and modifed as follows. We collected integrated non-carious human third molars of patients ages from 19 to 24 with the written consent. Freshly extracted third molar was split by a hammer to expose the pulp. The pulp tissue was isolated from the tooth and minced into small pieces by a surgical scissor. Then it is digested with 3 mg/ml collagenase (Sigma-Aldrich, CA, USA) and 0.5 mg/ml trypsin (Sigma-Aldrich) for 30 min at 37 °C. The tissue pieces were seeded into cell culture dishes (Corning Corp., Corning, NY, USA) with Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, NY, USA), 10% fetal bovine serum (FBS, Gibco, Life Technologies, NY, USA) and antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin, HyClone, UT, USA). The primary HDPCs were cultured in a humidified atmosphere at 37 °C with 5% CO2. HDPCs migrated from the tissue were collected and sub-cultured after confluence. HDPCs between the 3th and 5th passages were carried out in the experiment. For the following experiment, HDPCs were treated with 100 ng/ml recombinant human WNT7B (rhWNT7B) (Abnova, Walnut, CA, USA). To induce differentiation, we treated cells with odontogenic induction media (OM, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 0.1 μM dexamethasone) with 10% FBS. Media was changed every 2-3 days. For inhibition experiments, cells were pre-treated with specific inhibitors for 2 h, then cultured with new medium containing the inhibitor. For cell cycle and Annexin V staining, cells were treated with 100 ng/ml rhWNT7B for 2 days before testing.

2.2. Cell proliferation assay, cell cycle analysis and annexin V staining

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8, Dojindo, Japan) following the manufacturer's instructions. In addition, Edu Apollo 488 (RIBOBIO, Guangzhou, China) detection and Ki-67 ICC (1:1000, Abcam, Cambridge, MA, USA) were adopted to observe the influences of rhWNT7B on HDPCs. For cell cycle analysis, HDPCs were collected and fixed in pre-cooled 70% ethanol. After re-hydration, cells were treated with a cell cycle analysis kit (KeyGEN, Nanjing, China) following the manufacturer's instructions. For Annexin V staining, HDPCs cells were collected and stained with PI and Annexin V (KeyGEN, Nanjing, China) following the manufacturer's instructions. The proportion of different phases' cells and apoptotic cells was quantified by flow cytometry (Beckman, Miami, FL, USA), respectively.

2.3. Scratch assay

HDPCs were cultured and scratched as described in a previous study (Su et al., 2012). Then, HDPCs were incubated for 24 h in OM with or without WNT7B. The experiments were repeated three times. The quantification of migration is calculated by Image J (Iqbal, Szaraz, Librach, Gauthier-Fisher, & Librach, 2017).

2.4. Quantitative real-time PCR analysis

Total RNA was harvested by the RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA by using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The produced cDNA was used as templates for PCR reactions using SYBR Premix Ex Taq (Takara, Dalian, China) according to the manufacturer's instructions. *GAPDH* was used as a control. The specific primers were *GAPDH*, 5-GCACCGTCAAGGCTGAGAAC-3 (Forward), 5-ATGGTGGTGAAGACG CCAGT-3 (Reverse);

RUNX2, 5-GAACCACAAGTGCGGTGCAA-3 (Forward), 5-ACTGCTT GCAGCCTTAAATGACTCT-3 (Reverse).

Alkaline phosphatase (ALP), 5-AACATCAGGGACATTGACGTG-3 (Forward), 5-GTATCTCGGTTTGAAGCTCTTCC-3 (Reverse);

COL1, 5-TCTAGACATGTTCAGCTTTGTGGAC-3 (Forward), 5-TCTG TACGCAGGTGATTGGTG-3 (Reverse).