Biochemical and Biophysical Research Communications 478 (2016) 527–532

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

p38 MAP kinase is required for Wnt3a-mediated osterix expression independently of Wnt-LRP5/6-GSK3β signaling axis in dental follicle cells

Yukihiko Sakisaka [a], Sousuke Kanaya [a, b], Takashi Nakamura [c], Masato Tamura [d], Hidetoshi Shimauchi [a], Eiji Nemoto [a, *]

[a] Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan
[b] Liason Center for Innovative Dentistry, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan
[c] Department of Dental Pharmacology, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan
[d] Department of Biochemistry and Molecular Biology, Hokkaido University Graduate School of Dentistry, Sapporo 060-8586, Japan

ARTICLE INFO

Article history:
Received 13 July 2016
Accepted 18 July 2016
Available online 19 July 2016

Keywords:
Wnt3a
Osterix
p38 MAPK
β-catenin
Dental follicle cells

ABSTRACT

Wnt3a is a secreted glycoprotein that activates the glycogen synthase kinase-3β (GSK3β)/β-catenin signaling pathway through low-density-lipoprotein receptor-related protein (LRP)5/6 co-receptors. Wnt3a has been implicated in periodontal development and homeostasis, as well as in cementum formation. Recently, we have reported that Wnt3a increases alkaline phosphatase expression through the induction of osterix (Osx) expression in dental follicle cells, a precursor of cementoblasts. However, the molecular mechanism by which Wnt3a induces Osx expression is still unknown. In this study, we show that Wnt3a-induced Osx expression was inhibited in the presence of p38 mitogen-activated protein kinase (MAPK) inhibitors (SB203580 and SB202190) at gene and protein levels, as assessed by real-time PCR and immunocytohistochemistry, respectively. Pretreatment of cells with Dickkopf-1, a potent canonical Wnt antagonist binding to LRP5/6 co-receptors, did not influence Wnt3a-mediated p38 MAPK phosphorylation, suggesting that Wnt3a activates p38 MAPK through LRP5/6-independent signaling. On the other hand, pretreatment with p38 MAPK inhibitors had no effects on the phosphorylated status of GSK3β and β-catenin as well as β-catenin nuclear translocation, but inhibited Wnt3a-mediated β-catenin transcriptional activity. These findings suggest that p38 MAPK modulates canonical Wnt signaling at the β-catenin transcriptional level without any crosstalk with the Wnt3a-mediated LRP5/6-GSK3β signaling axis and subsequent β-catenin nuclear translocation. These findings expand our knowledge of the mechanisms controlling periodontal development and regeneration.

1. Introduction

The Wnt family consists of 19 secreted glycoproteins with lipid modification that mediate developmental and post-developmental cellular physiology including proliferation, differentiation, migration, and apoptosis [1]. In the canonical Wnt/β-catenin pathway, Wnt proteins bind to frizzled transmembrane receptors and low-density-lipoprotein receptor-related protein (LRP) 5/6 co-receptors located on the cell surface, resulting in the inhibition of glycogen synthase kinase-3β (GSK3β). In the absence of Wnt signaling, GSK3β phosphorylates β-catenin, leading to its ubiquitination and subsequent degradation in the proteasome. However, the binding of Wnt protein to its receptors results in the inhibition of GSK3β activity and the cytosolic accumulation of β-catenin, which is translocated to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors [1].

The dental follicle is an ectomesenchymal tissue surrounding the developing tooth germ and has an important role in regulating tooth eruption. Furthermore, existing data indicate that this tissue contains stem cells and progenitor cells for periodontal tissues, which are composed of alveolar bone, periodontal ligament, and cementum [2]. When triggered appropriately, dental follicle cells

* Corresponding author. Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi Aoba, Sendai 980-8575, Japan.
E-mail address: e-nemoto@dent.tohoku.ac.jp (E. Nemoto).

http://dx.doi.org/10.1016/j.bbrc.2016.07.076
0006-291X/

are considered to be able to differentiate toward a cementoblast/osteoblast phenotype [3–6]. Although the specific mechanisms controlling follicle cell differentiation toward a cementoblastic phenotype are not yet fully understood, it has been reported that constitutive stabilization of β-catenin in dental mesenchyme leads to excessive cementum formation in conditional β-catenin stabilized mice [7,8]. Conversely, disruption of Wnt/β-catenin signaling in dental mesenchyme of conditional knockout mouse arrests the differentiation of cementoblasts [9], suggesting that canonical Wnt/β-catenin signaling is required for cementum formation.

The p38 mitogen-activated protein kinase (MAPK) pathway is known to regulate osteoblast differentiation [10]. Recent studies have suggested the possibility of crosstalk between p38 MAPK and canonical Wnt signaling. Among the mechanisms, GSK3β plays a critical role in crosstalk between canonical Wnt and p38 MAPK signaling pathways [11–15]. p38 MAPK inactivates GSK3β by direct phosphorylation at its C terminus, leading to activation of canonical Wnt signaling [11,12]. Conversely, GSK3β inhibits p38 MAPK through the inhibition of the upstream MAPK kinase, MAKK4, which indicates that Wnt-mediated GSK3β inhibition may derepress p38 MAPK [13,14]. On the other hand, p38 MAPK phosphorylates myocyte enhancer factor 2, which promotes its association with β-catenin, leading to nuclear-translocation of β-catenin and enhancement of canonical Wnt signaling [15].

Previously, we have reported that Wnt3a increases alkaline phosphatase (ALP) expression through the induction of osterix (Osx) expression in dental follicle cells [5]. In this study, we examined the molecular mechanism by which Wnt3a induces Osx expression. Here, new evidence is provided showing that Wnt3a-induced Osx expression requires activation of p38 MAPK through LRP5/6-independent signaling, and that p38 MAPK modulates canonical Wnt signaling at the β-catenin transcriptional level without any crosstalk with Wnt3a-mediated GSK3β activity and subsequent β-catenin nuclear translocation.

2. Materials and methods

2.1. Reagents

Recombinant mouse Wnt3a (rWnt3a) was purchased from Peprotech (Rocky Hill, NJ, USA). Recombinant mouse Dickkopf-1 (DKK1) (5897-DK) was purchased from R&D Systems Inc. (Minneapolis, MN, USA). 4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-benzamide (IWR-1), ascorbic acid, Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PD98059, SB203580, SB202190, and SP600125 were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA).

2.2. Cell culture

SVF4, an immortalized murine dental follicle cell line [3], was kindly provided by Dr. MJ Somerman (National Institutes of Health, MD, USA), and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin G and 100 μg/ml streptomycin. All tissue culture reagents were purchased from Gibco®/Life Technologies (Carlsbad, CA, USA), unless otherwise indicated.

2.3. Cell stimulation

A confluent monolayer of cells was stimulated with rWnt3a in DMEM containing 5% (v/v) FBS and 50 μg/ml ascorbic acid (defined as complete DMEM) for the indicated time. For some experiments, cells were pretreated with inhibitors, followed by stimulation with rWnt3a in complete DMEM in the continuous presence of the inhibitors for the indicated time. When IWR-1 or MAPK inhibitors were used for pretreatment, samples, including the control, were adjusted to contain 0.1% (v/v) DMSO in the culture medium.

2.4. Reverse transcription and real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was extracted using Qiashredder and RNeasy® Kits (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions, and treated with DNase (DNA-free™, Ambion Inc., Austin, TX, USA). Total RNA transcription into cDNA was performed using a Transcriptor First Strand cDNA Synthesis Kit® (Roche Diagnostic Co., Indianapolis, IN, USA) according to the manufacturer's instructions. Primers were designed using LightCycler probe design software® (Roche Diagnostics GmbH), and primer sequences for each mouse gene encoding Osx and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows (forward/reverse): *Osx* (5′-CGGGTCAGGTACAGTG-3'/5′-ACCATGACGACAAGGG-3′); and *Gapdh* (5′-ACCACAGTCCATGCCATCAC-3′/5′-TCCACCACCCTGTTGCTGTA-3′). For real-time PCR, the amplification profile was 40 cycles at 94/10, 55/30, and 72/30 [temperature (°C)/time (sec)]. PCR was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBR Green Supermix® (Bio-Rad) with optimized levels of 3 mM $MgCl_2$ and 0.5 μM of each primer. After amplification, one cycle of a linear temperature gradient from 55 °C to 95 °C at a transition rate of 0.5 °C/2 s was performed to assess the specificity of the PCR products. For each run, water was used as a negative control. Relative expression levels of transcripts are shown after normalization to the corresponding sample expression level of *Gapdh*, as described previously [16].

2.5. Western blotting

Cells were harvested with Cell Lysis Buffer® (Cell Signaling Technology, Danvers, MA, USA) as per the manufacturer's instructions. Cell proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan) using a semidry transblot system (ATTO). The blot was blocked for 1 h with 0.5% (w/v) non-fat skim milk in phosphate buffered saline (PBS) with 0.1% (v/v) Tween 20, followed by incubation for 1 h with rabbit anti-phosphorylated β-catenin (Ser33/37/Thr41), anti-β-catenin, anti-phosphorylated GSK-3β (Ser9), anti-GSK-3β, anti-phosphorylated LRP-6 (Ser1490), anti-LRP6, anti-phosphorylated p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, or anti-β-actin antibodies at 1:1000. All antibodies were obtained from Cell Signaling Technology. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) at 1:2000 for 1 h at room temperature. Proteins were detected using ECL Prime Western blotting detection reagents (GE healthcare, Madison, WI, USA) according to the manufacturer's instructions and visualized by Molecular Imager® ChemiDoc™ XRS plus (Bio-Rad). The intensity of the expression was quantified with densitometry scanning using Image Lab software (Bio-Rad).

2.6. β-Catenin/TCF luciferase assay

The pTopflash construct (Upstate Biotechnology, Lake Placid, NY, USA) contains a firefly luciferase reporter under the control of two repeats, each containing three copies of the TCF binding site upstream of the thymidine kinase minimal promoter. pFopflash constructs (Upstate Biotechnology) containing mutated TCF binding sites were used as a negative control. pRL-CMV-*Renilla* luciferase