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The Role of Autonomous Wntless in Odontoblastic Differentiation of Mouse Dental Pulp Cells

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Purpose: Wnt signaling plays an essential role in the dental epithelium and mesenchyme during tooth morphogenesis. Deletion of the *Wntless* (*Wls*) gene in odontoblasts appears to reduce canonical Wnt activity, leading to inhibition of odontoblast maturation. However, it remains unclear if autonomous Wnt ligands are necessary for differentiation of dental pulp cells into odontoblast-like cells to induce reparative dentinogenesis, one of well-known feature of pulp repair to form tertiary dentin.

Materials and Methods: To analyze the autonomous role of Wls for differentiation of dental pulp cells into odontoblast-like cells, we used primary dental pulp cells from unerupted molars of *Wls*-floxed allele mouse after infection with adenovirus for Cre recombinase expression to knockout the floxed *Wls* gene or control GFP expression. The differentiation of dental pulp cells into odontoblast-like cells was analyzed by quantitative real-time polymerase chain reaction.

Result: Proliferation rate was significantly decreased in dental pulp cells with Cre expression for *Wls* knockout. The expression levels of Osterix (*Osx*), runt-related transcription factor 2 (*Runx2*), and nuclear factor I-C (*Nfic*) were all significantly decreased by 0.3-fold, 0.2-fold, and 0.3-fold respectively in dental pulp cells with *Wls* knockout. In addition, the expression levels of *Bsp*, *Col1a1*, *Opn*, and *Alpl* were significantly decreased by 0.7-fold, 0.3-fold, 0.8-fold, and 0.6-fold respectively in dental pulp cells with *Wls* knockout.

Conclusion: Wnt ligands produced autonomously are necessary for proper proliferation and odontoblastic differentiation of mouse dental pulp cells toward further tertiary dentinogenesis.

Key Words: Dental pulp; Reparative dentin; Wntless

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Introduction

Dentinogenesis is the formation of dentin, a substance that forms the majority of teeth, and this process is performed by odontoblasts. Three types of dentinogenesis are commonly described, primary, secondary, and tertiary, although tertiary dentinogenesis only occurs under pathological conditions. The formation of tertiary dentin is distinguished by reactionary and reparative dentin[1]. Dental pulp tissue responds to dentin injury by laying down reactionary dentin secreted by existing odontoblasts or reparative dentin elaborated by odontoblast-like cells that differentiated from precursor cells in the absence of inner dental epithelium and basement membrane[2]. Under pulp exposure resulting in the destruction of the underlying odontoblast layer, the dentin-pulp complex regenerates through progenitor cell recruitment and differentiation into secreting cells and the stimulation of reparative dentinogenesis. The main objective of this healing process is to form a barrier of mineralized tissue to protect the underlying pulp from bacterial or toxin leakage. The dental pulp of the unerupted molars is enriched in osteo-dentinogenic progenitors engaged in the formation of coronal and radicular odontoblasts[3]. Dental pulp cells, as the progenitor cells of odontoblasts, undergo a cascade of events involving migration, proliferation and differentiation into odontoblast-like cells by multiple cytokines and paracrine signal molecules[3-5]. Clinically, the objectives of treatments such as direct pulp capping, partial pulpotomy, or the stepwise technique are to seal the pulp wound, induce odontoblast-like cell differentiation, and stimulate dentin secretion and mineralization in order to build a dentin bridge[6]. To improve the clinical outcome, knowledge of the molecular mechanisms involved in pulp healing must be better understood, and new biomaterials should be developed based on these results. Several aspects of the molecular mechanisms, including the progenitor cells and the odontoblast differentiation pathways, are still unclear.

Wnt/β-catenin signaling plays an essential role in the morphogenesis and cellular differentiation of many tissues including bone and tooth[7]. Wntless (Wls) is required for the normal secretion of Wnt ligands from cells[8,9]. Wls is highly specific for Wnt secretion, and, to date, Wnt-independent functions have not been reported[9]. Although most Wnt family members are expressed in the dental epithelium during early tooth morphogenesis, some Wnts, such as *Wnt5a* and *Wnt10a*, as well as Wnt signaling mediators, *Axin2* and *Lef-1*, are expressed in developing odontoblasts[10-13]. Zhu et al.[14] reported that tooth morphogenesis is arrested in tissue-specific ablation of *Wls* in the dental epithelium. We recently found that tissue-specific inactivation or constitutive stabilization of β-catenin (β-Cat) leads to disrupted odontoblast differentiation in roots or excessive dentin formation, respectively[15,16]. In addition, deletion of the *Wls* gene in odontoblasts appears to reduce canonical Wnt activity, leading to inhibition of odontoblast maturation and root elongation[17]. Although much evidence supports the possible roles of Wnt signaling in odontoblast differentiation and dentin formation, it remains unclear whether Wnt ligands secreted from pulp cells are involved in their odontoblastic differentiation and matrix formation. In this study, we analyzed the role of autonomous Wls in proliferation and odontoblastic differentiation of mouse dental pulp cells.

Materials and Methods

1. Primary Dental Pulp Cell Culture

All procedures were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animal. Experimental protocols and animal care methods were approved by the Animal Welfare Committee of Chonbuk National University. Primary dental pulp cells were

prepared from the coronal portions of pulps from unerupted first and second molars of 5- to 7-day-old *Wls*-floxed allele mouse as described previously[17,18]. Mouse dental pulp cells were cultured in α-MEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. Cells at passage 2 were treated with osteogenic medium (OM) consisting of growth media supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM β-glycerophosphates (Sigma-Aldrich) to induce their differentiation/mineralization for additional days as indicated.

2. Adenoviral Infection for Cre Expression

To knockout the floxed gene with Cre expression, cells were infected with either adenovirus for Cre recombinase combined with GFP (Ad-Cre) or GFP expression (Ad-GFP) (Vector Biolabs, Philadelphia, PA, USA) at a multiplicity of infection of 500 for 24 hours. The expression of GFP and Cre fused with GFP were viewed under a model LSM510 confocal laser scanning microscope (Carl Zeiss, Ostalbkreis, Germany).

3. Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 10 minutes. They were permeabilized by incubation in 0.2% Triton X-100 for 10 minutes at room temperature and then quenched in 3% H_2O_2 in the dark to block endogenous peroxidase activity. After rinsing with PBS, cells were blocked with 5% BSA in PBS for 30 minutes at room temperature. Cells were then treated with primary antibodies for Wls for 16 hours at 4°C. Normal rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for primary antibody was used as control. Alexa Fluor® dyes-conjugated second antibodies (Invitrogen, Carlsbad, CA, USA) were used for detection. DAPI (4',6-diamidino-2-phenylindol) was used for counterstaining. Cell staining was evaluated using a fluorescence microscope (Carl Zeiss).

4. Proliferation Assay

Proliferation rates of dental pulp cells were measured using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells were cultured in 24-multiwell plates and treated with

Table 1. Primer sequences for real-time quantitative polymerase chain reaction

Gene	Sense	Antisense
Acpt	CTCCTACCCCACAGATCCAC	TCAGAAATCGGCCCAGTTCT
Runx2	CCTCTGACTTCTGCCTCTGG	TAAAGGTGGCTGGGTAGTGC
Alpl	AGGGCAATGAGG TCACATCC	GCATCTCGTTATCCGAGTACCAG
Bsp	AAAGTGAAGGAAAGCGACGA	GTTCCTTCTGCACCTGCTTC
Oc	ACCCTGGCTGCGCTCTGTCTCT	GATGCGTTTGTAGGCGGTCTTCA
Osx	TCTCCATCTGCCTGACTCCT	AGCGTATGGCTTCTTTGTGC
Dspp	AACACATCCAGGAACTGCAGCACA	TGACTCGGAGCCATTCCCATCTCT
Wls	ACCGTGATGATATGTTTTCTG	TACCACACCATAATGATGAA
Axin2	AAGAAGGAGACCGGTCACAG	GGTCCTGGGTAAATGGGTGA
Nfic	GACCTGTACCTGGCCTACTTTG	CACACCTGACGTGACAAAGCTC
β-Cat	GCCATCTGTGCTCTTCGTC	ACACCCTTCTACTATCTCCTCC
Col1a1	CCGGAAGAATACGTATCACC	ACCAGGAGGACCAGGAAGTC
Dmp1	AGTGAGTCATCAGAAGAAAGTCAAGC	CTATACTGGCCTCTGTCGTAGCC
Opn	CCCGGTGAAAGTGACTGATTC	ATGGCTTTCATTGGAATTGC
Gapdh	TGCCCAGAACATCATCCCT	GGTCCTCAGTGTAGCCCAAG

10 μl/well of the kit solution. Absorbance was measured spectrophotometrically at 450 nm.

5. RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction

Total RNA preparation, cDNA synthesis and real-time (RT) quantitative polymerase chain reaction (qPCR) were performed as described previously[19]. The expression was normalized to that of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*). Specific primers sets used in the analysis are listed in Table 1.

6. Statistical Analysis

Data are presented as mean±standard errors from three separate experiments. Normal data with equal variance were analyzed using Student t-test. Significance was assigned for P-value ≤0.05 as indicated. All data were analyzed using SPSS statistical software (version 16.0; SPSS, Chicago, IL, USA).

Result

1. Odontogenic Gene Expression of Primary Dental Pulp Cells from Mouse Molars

Dental pulp cells are considered to be odontoblast progenitor cells that are capable of proliferation and

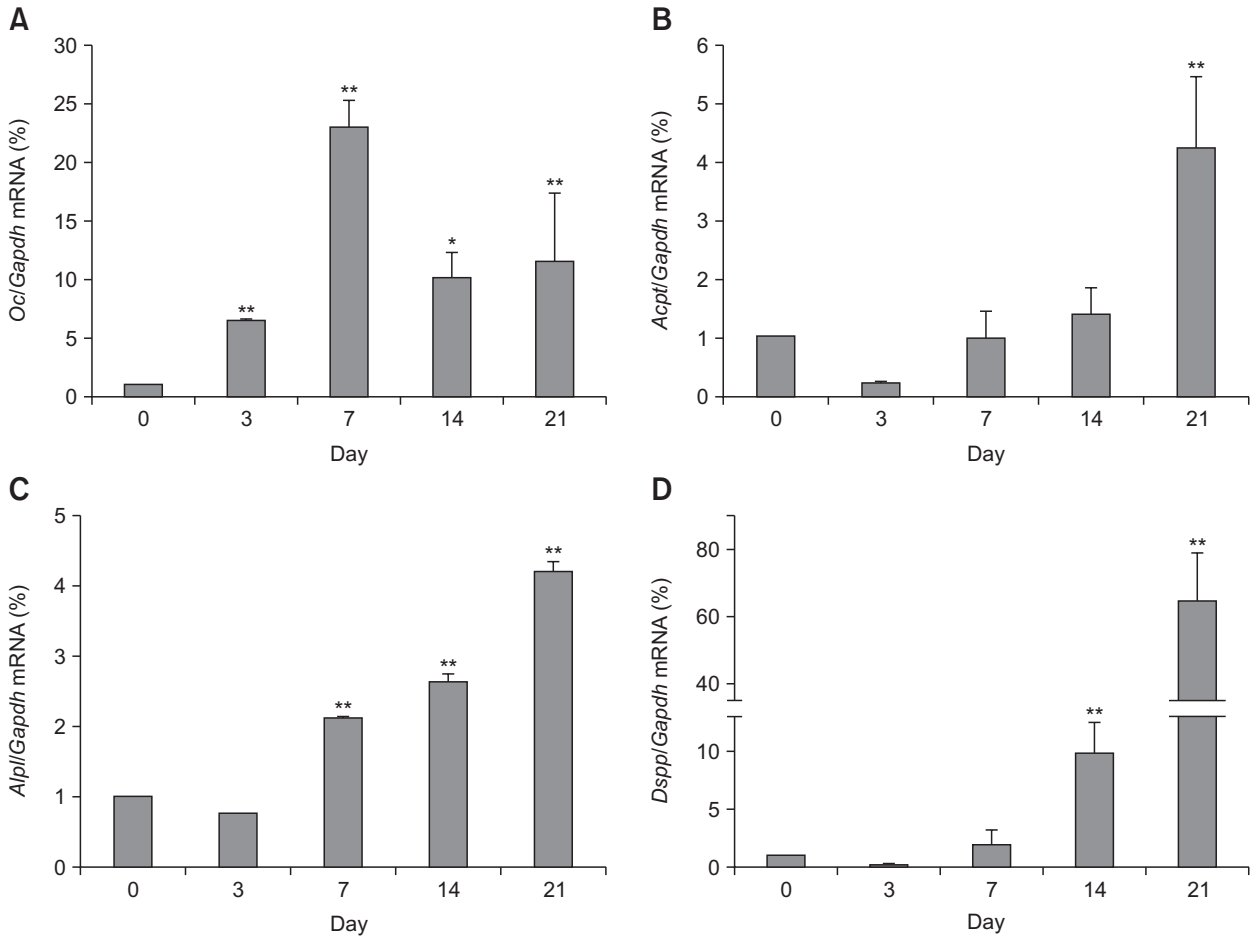

Fig. 1. Odontogenic gene expression of mouse dental pulp cells. Dental pulp cells were prepared from the coronal portions of pulps from unerupted first and second molars of 5- to 7-day-old mice and 90% confluent cells at passage 2 were treated with osteogenic medium containing 50 μg/ml of ascorbic acid and 10 mM β-glycerophosphate up to 21 days. The patterns of expression of *Oc* (A), *Acpt* (B), *Alpl* (C), and *Dspp* (D) for odontoblast differentiation were examined in these cultures at various time points by quantitative real-time polymerase chain reaction analyses. *P<0.05, **P<0.01 vs. day 0.

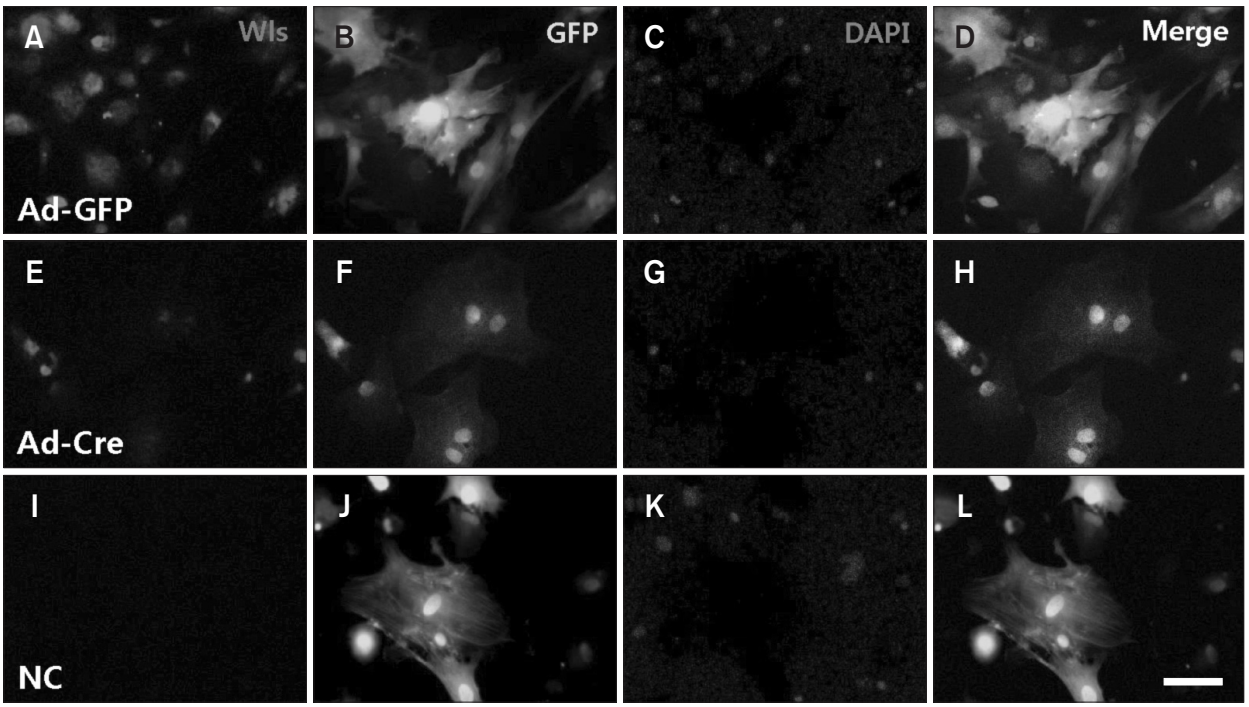

Fig. 2. Knockout of *Wls* gene in mouse dental pulp cells. To knockout *Wls* gene in primary dental pulp cells from *Wls*-floxed allele mouse, adenovirus for Cre recombinase (Ad-Cre) (E~H) was infected and adenovirus for GFP (Ad-GFP) was used as control (A~D). The expression of Cre recombinase was detected in the cells infected with Ad-Cre after 24 hours of infection as shown by GFP expressed with Cre recombinase (F) with reduced Wls expression (E). The control cells infected with Ad-GFP had GFP expression (B) with strong Wls expression (A). (C, G, K) DAPI (4',6-diamidino-2-phenylindol) was used for nuclear staining. Non-specific control immunoglobulin G was used for negative control (NC) in the cells infected with Ad-GFP (I~L). Scale bar=50 μm.

differentiation into odontoblast-like cells to produce new dentin[20]. To verify odontogenic differentiation of dental pulp cells, we investigated odontogenic gene expression of dental pulp cells. Cells were prepared from the coronal portions of pulps from unerupted first and second molars of 5- to 7-day-old mice and 90% confluent cells at passage 2 were treated with OM containing 50 μg/ml of ascorbic acid and 10 mM β-glycerophosphate up to 21 days. The patterns of expression of selected known markers for odontoblast differentiation were examined in these cultures at various time points by quantitative RT-PCR analyses. In cultures derived from molar dental pulp, highest levels of *osteocalcin* (*Oc*) mRNA were detected at day 7 with slight decreases but significantly expressed thereafter (days 14 and 21) (Fig. 1A). The expressions of *testicular acid phosphatase* (*Acpt*), *tissue-nonspecific alkaline phosphatase* (*Alpl*), and *dentin sialoprotein* (*Dspp*) were reduced at day 3, but increased at day 7 and gradually increased thereafter (Fig. 2B~D). The qPCR showed 64-fold increases in the level of *Dspp* at day 21 as compared to day 0 ($P<0.01$). The expression of *Dspp* in these cultures indicated the presence of odontoblast-like cells secreting dentin mineralized matrix.

2. Knockout of Wls Gene by the Expression of Cre Recombinase in Mouse Dental Pulp Cells

To determine the genetic role of autonomous *Wls* in dental pulp cells for odontoblastic differentiation, we used adenoviral Cre infection to inactivate *Wls* in primary dental pulp cells. The expression of Cre recombinase was detected in the nucleus of dental pulp cells infected with adenovirus for Cre expression (Ad-Cre) after 24 hours of infection as shown by GFP expressed with Cre recombinase (Fig. 2F). The control cells infected with adenovirus for

GFP expression (Ad-GFP) had strong expression of GFP at cytoplasm as well as nuclear (Fig. 2B). Wls expression was largely decreased in dental pulp cells infected with Ad-Cre after 24 hours of infection (Fig. 2E) when compared to the control (Fig. 2A), as shown by immunocytochemical staining. We also verified the specific staining of Wls in this experiments using non-specific control IgG, which are not bound to Wls in the cells infected with Ad-GFP (Fig. 2I).

3. The Role of Autonomous Wntless in Proliferation of Mouse Pulp Cells

To determine the role of autonomous Wls in dental pulp cells for cell proliferation, cell proliferation rates of dental pulp cells were measured for 24 hours after infection of Ad-Cre and Ad-GFP. As shown in Fig. 3, the proliferation rate dental pulp cells infected with Ad-Cre was significantly decreased to 66.6%±5.0% when compared with control Ad-GFP infected cells (100%±2.5%; P<0.05).

4. The Role of Autonomous Wntless in Odontogenic Differentiation of Mouse Pulp Cells

To confirm the knockout of *Wls* gene by the

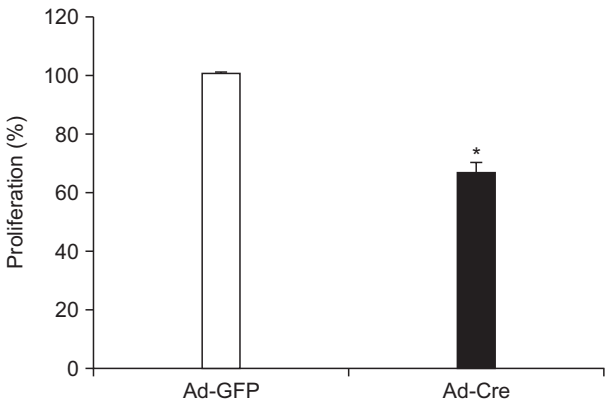

Fig. 3. The role of autonomous Wls in proliferation of mouse pulp cells. Cell proliferation rates of dental pulp cells were measured for 24 hours after infection of adenovirus for Cre recombinase (Ad-Cre) and adenovirus for GFP (Ad-GFP). *P<0.05 vs. control.

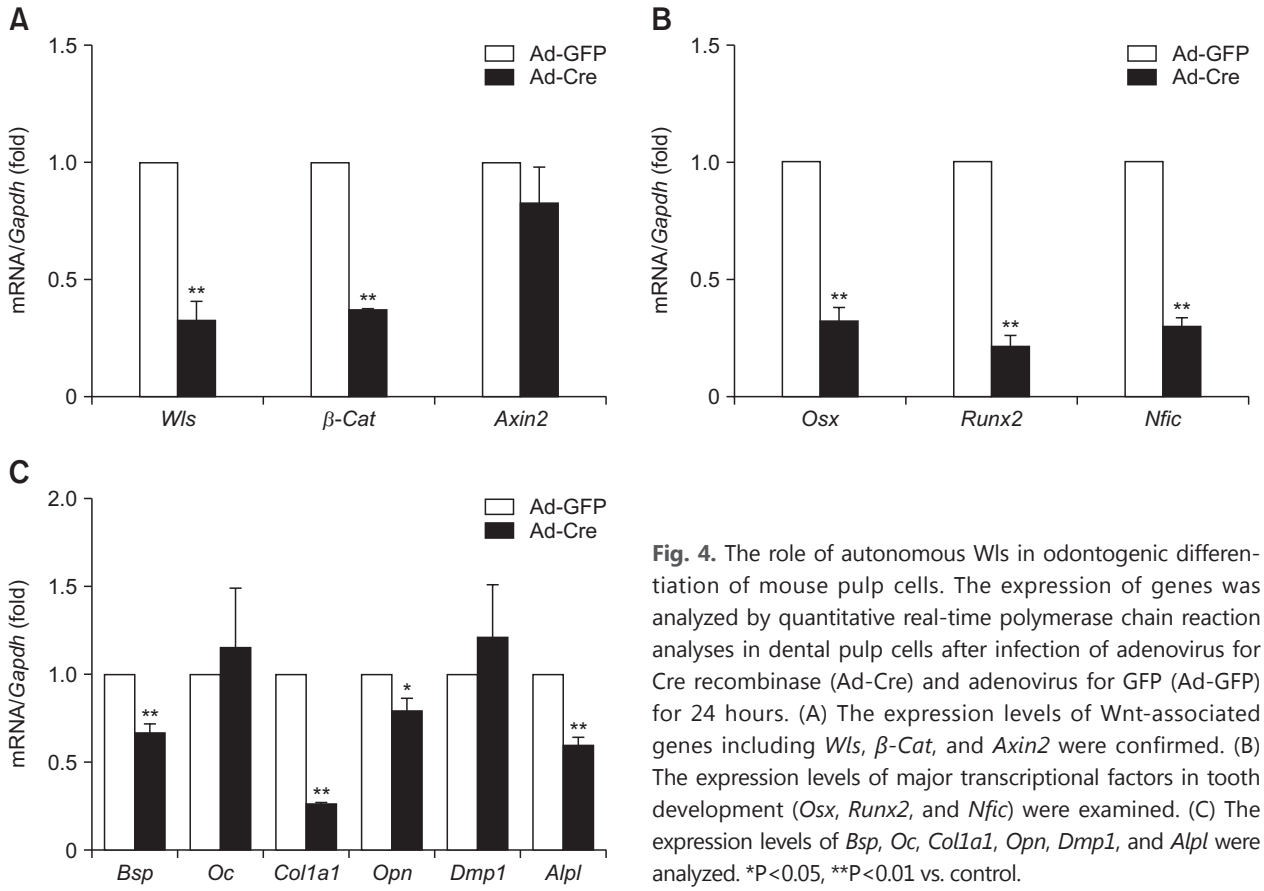

Fig. 4. The role of autonomous Wls in odontogenic differentiation of mouse pulp cells. The expression of genes was analyzed by quantitative real-time polymerase chain reaction analyses in dental pulp cells after infection of adenovirus for Cre recombinase (Ad-Cre) and adenovirus for GFP (Ad-GFP) for 24 hours. (A) The expression levels of Wnt-associated genes including *Wls*, *β-Cat*, and *Axin2* were confirmed. (B) The expression levels of major transcriptional factors in tooth development (*Osx*, *Runx2*, and *Nfic*) were examined. (C) The expression levels of *Bsp*, *Oc*, *Col1a1*, *Opn*, *Dmp1*, and *Alpl* were analyzed. *P<0.05, **P<0.01 vs. control.

expression of Cre recombinase in dental pulp cells, we examined the expression levels of Wnt-associated genes including *Wls*, *β-Cat*, and *Axin2* by RT qPCR. The expression levels of Wls and β-Cat were significantly decreased by 0.3-fold, and 0.4-fold respectively in dental pulp cells infected with adenovirus harboring *Cre* for 24 hours when compared to control (P<0.01) (Fig. 4A). However, the expression level of *Axin2* was not changed with *Wls* knockout. The expression levels of Osterix (*Osx*), runt-related transcription factor 2 (*Runx2*), and nuclear factor I-C (*Nfic*), which were transcriptional factors regulating tooth development[21], were all significantly decreased by 0.3-fold, 0.2-fold, and 0.3-fold respectively in dental pulp cells with *Wls* knockout (P<0.01) (Fig. 4B). Furthermore, we also examined the expression of matrix proteins and tissue nonspecific alkaline phosphatase (TNAP) as differentiation markers of odontoblasts by RT qPCR. The expression levels of *Bsp*, *Col1a1*, *Opn*, and *Alpl* were significantly decreased by 0.7-fold, 0.3-fold, 0.8-fold, and 0.6-fold respectively in dental pulp cells with *Wls* knockout (P<0.01, P<0.05 in *Opn* expression) (Fig. 4C). However, the expression level of *Oc* and *Dmp1* were not changed at this stage with *Wls* knockout.

Discussion

In this study, we analyzed the role of Wls for production of autonomous Wnt ligands in differentiation of mouse dental pulp cells into odontoblast-like cells for reparative dentinogenesis. To knockout the floxed *Wls* gene, we infected primary-cultured pulp cells from the molars of *Wls*-floxed allele mouse with adenovirus for Cre recombinase expression. Proliferation rate was significantly decreased in dental pulp cells with *Wls* knockout. The expression levels of major transcriptional factors in tooth development such as *Osx*, *Runx2*, and *Nfic* were all largely decreased in these *Wls*-knockout dental pulp cells. In addition, as differentiation markers of odontoblasts, the expression levels of the genes associated with matrix proteins (*Bsp*, *Col1a1*, *Opn*) and TNAP (*Alpl*) were significantly decreased in dental pulp cells with *Wls* knockout. These results suggest a critical role of autonomous Wls in odontoblastic differentiation of mouse dental pulp cells for reparative dentin formation.

The response mechanisms for carious and traumatic dental injury are critical for pulp survival and involve a series of highly conserved processes. The most common and well-known feature of repairing response is the formation of tertiary dentin, which is distinguished by reactionary and reparative dentin[1,2]. Reparative dentin is produced by odontoblast-like cells, which are differentiated from pulp cells and replace necrotic odontoblasts after more severe injury, whereas reactionary dentin is secreted by surviving odontoblasts in response to moderate stimuli leading to an increase in metabolic activity. The formation of reparative dentin needs the recruitment and proliferation of pulp cells, which might be re-activated after injury. At the injured site, pulp cells needs to proliferate and differentiate into odontoblast-like cells[22]. The adult pulp contains a heterogeneous cell population including odontoblast progenitor cells and consisting of fibroblast-like cells, macrophages and other nerve or capillary cells[20,23]. The reparative process requires the collaborative efforts of cells of different lineage in the injured pulp. The behavior of each of the contributing cell types during the phases of proliferation, migration, and matrix synthesis is still unclear. Due to cellular heterogeneity and the absence of data for specific genes exclusively expressed in dental pulp cells, it is limited to demonstrate the role of specific genes and signaling pathways in dental pulp by gene targeting technology *in vivo*. This study specifically validates the importance of autonomous Wls in proliferation and differentiation of dental pulp cells by gene knockout methods with Cre recombinase *in vitro*,

showing that *Wls*-deficient pulp cells had a defect in proliferation and differentiation with significant reductions in the expression of key odontoblast differentiation regulators and target molecules. In general, injury activates the endogenous Wnt pathway[24,25]. In a recent pulp cavity animal model, the response to pulpal injury was similar as shown that elevating Wnt signaling by either removing a negative Wnt regulator or by providing exogenous WNT3A protein was sufficient to significantly improve the pulp cavity's repair response[22]. In this model, pulp cells responded to the elevated Wnt stimulus by differentiating into secretory odontoblasts.

Little is known of the molecular mechanisms involved in dental repair and the recruitment and differentiation of pulp cells into odontoblast-like cells. A striking feature of the comparison between primary odontoblast and odontoblast-like cell differentiation is the absence of involvement of dental epithelium in the latter situation[26]. Despite of this critical importance, parallel molecular mechanisms may exist between dental repair and morphogenesis in the embryo, as suggested that some molecular mechanisms are common to dental development and repair[27]. The outcomes of these embryonic events show many similarities to the tissue-regenerative processes occurring during dental repair after injury. The exquisite regenerative capacity of the dentin-pulp complex offers many exciting challenges for the development of new biological approaches to dental tissue repair. Such approaches might lead to greater clinical emphasis on tissue regeneration in the tooth in the future.

Conclusion

Taken together, this study demonstrates that autonomously produced Wnt ligands are necessary for proper proliferation and odontoblastic differentiation of mouse dental pulp cells toward further tertiary dentinogenesis. These findings contribute to further understanding of the molecular mechanisms underlying odontoblastic differentiation of pulp cells and improving material science with it for a successive dental repair.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgement

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